LOCAL IMMUNE RESPONSES IN EOSINOPHILIC ESOPHAGITIS

Implications for disease pathophysiology and dietary management

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Local immune responses in eosinophilic esophagitis

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Lokale immuunresponsen in eosinofiele oesofagitis

Implicaties voor de pathofysiologie en dieetmanagement van de aandoening
(met een samenvatting in het Nederlands)

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CONTENTS

CHAPTER 1

General introduction and thesis outline

1.1 EOSINOPHILIC ESOPHAGITIS - AN EMERGING DISEASE

Once considered a rare condition, eosinophilic esophagitis (EoE) – a chronic, allergen-driven disorder of the esophagus – is now one of the most common conditions diagnosed during the evaluation of food impaction in adults.[1,2] Since the first case description back in 1978,[3] current prevalence has been estimated at 34.2 cases per 100,000 inhabitants in the USA and Europe, and is higher for adults (42.2) than for children (34.4).[4] Multiple population-based studies from Europe[5-7] and the USA[8,9] have shown that this is a true increase rather than the effect of raised awareness or improved diagnostic methods. EoE has been described in all age groups, but predominantly affects men (3:1 male-to-female ratio) with an onset from school age to midlife and a personal or family history of atopic disorders.[10,11] EoE substantially impacts patient's quality of life because of symptoms, dietary restrictions, and the social and psychological implications of living with food-related illness.[12,13] Current treatment options can be effective but may not provide long-term disease control for all patients due to differences in disease endotype.[13,14] It is therefore crucial that we continue to unravel the complex etiology and pathophysiology of EoE to enable future therapies to be more effective.

1.2 MAKING THE DIAGNOSIS OF EOE

EoE is defined as "an esophageal disease characterized clinically by symptoms related to esophageal dysfunction and histologically by an eosinophil-predominant inflammation".[15] The diagnosis of EoE is complex and based on a combination of esophageal symptoms, and endoscopic and histologic findings.[16] Age-related differences in clinical presentation of EoE have been identified in children and adults. EoE in children presents with non-specific symptoms, including vomiting, nausea, food refusal, abdominal pain, and failure to thrive. Adults, on the other hand, typically exhibit symptoms related to esophageal narrowing, such as solid food dysphagia and food impaction (Figure 1).[17] Endoscopic signs of EoE are detected in ~95% of the symptomatic patients.[18] Like symptoms, endoscopic findings vary among children and adults, and change with level of inflammation. Linear furrows, exudates, and edema are the most common endoscopic features of EoE in children, while in adults often a combination of inflammatory and fibrotic signs is detected, including strictures and rings.[19,20]

The healthy esophagus is devoid of eosinophils. To date, the peak count of 15 or more eosinophils per high power field (standard size of ~0.3 mm^2) assessed within the esophageal epithelium – while other causes of esophageal eosinophilia are ruled out – is the gold standard for the diagnosis of EoE.[21] Since eosinophilic inflammation in EoE is patchy, six biopsies sampled from multiple sites of the esophagus are needed to identify EoE with a high degree of accuracy.[22] Other histologic features of EoE include basal zone hyperplasia, dilated intercellular spaces (spongiosis), eosinophil abscesses (clusters of intraepithelial eosinophils), superficial layering of eosinophils, surface epithelial alteration, dyskeratotic epithelial cells, and lamina propria fibrosis.[23] Currently,

upper endoscopy with biopsy is needed for the definitive diagnosis of EoE and for monitoring disease activity,[24] as the search for noninvasive biomarkers (e.g. in serum, saliva, and stool) has thus far been largely unsuccessful.[25]

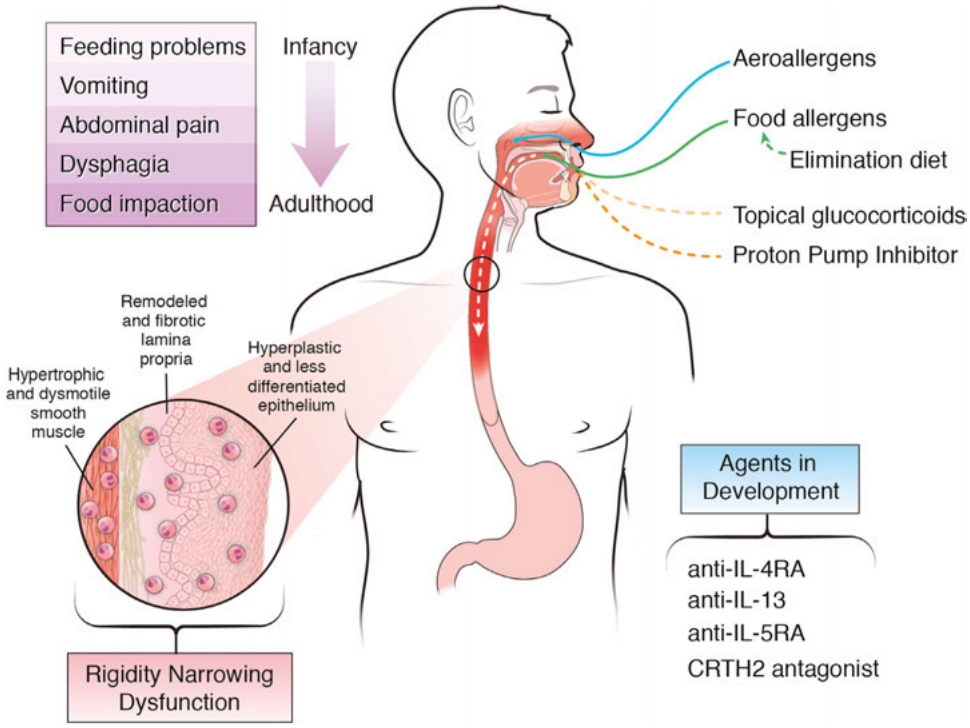

Figure 1. Clinical and pathologic manifestations of EoE. Both food and aeroallergens have been implicated in EoE. Clinical and histologic disease remission can be achieved by current (dietary elimination, glucocorticoids, PPIs) and future (e.g. biologics) interventions. The presenting symptoms are age-dependent. Figure obtained from O'Shea et al.[66] with permission.

1.3 THERAPEUTIC OPTIONS FOR PATIENTS WITH EOE

EoE can be controllable with appropriate treatment. The goals of treatment are to improve symptoms, and to prevent disease progression and ensuing complications such as fibrosis, which can be accomplished by both dietary and medical treatment. EoE requires life-long therapy, with relapse occurring rapidly after therapy cessation.[26] The effectiveness of therapy is currently limited by side effects, lack of long-term disease control, and adherence.[13] Current treatment options for EoE patients include swallowed topical glucocorticoids, proton pump inhibitors (PPIs), dietary elimination, and esophageal dilation (Figure 1). In addition, knowledge of EoE pathophysiology has led to the development of biologics targeting key players in EoE.

1.3.1 Medical therapy

To date, only two medications – budesonide orodispersible tablets (a swallowed topical glucocorticoid) and dupilumab (a biologic that targets IL-4Rα to inhibit IL-4 and IL-13 signaling) – have been approved for EoE by European regulatory authorities.[27,28] Swallowed topical glucocorticoids have been used as a first-line treatment because of their safety and proven efficacy, inducing clinical and histologic remission in up to 90% of patients depending on the formulation.[29] The main drawback of topical glucocorticoid treatment is that almost all patients relapse rapidly after discontinuation of the therapy.[30,31] In addition, the main side effect of swallowed topical glucocorticoids is oral *Candida albicans* infection, which occurs in 10-15% of the patients.[15] PPIs are used off-label and yield partial resolution of symptoms in EoE patients with PPI-responsive esophageal eosinophilia.[32,33] A secondary therapy for EoE is esophageal dilation, a mechanical procedure that successfully addresses strictures and luminal narrowing in the majority of patients, particularly older teenagers and adults.[34,35] Although esophageal dilation is highly effective in achieving long-lasting symptom relief, it does not affect the underlying inflammatory process.[35]

Recent insights in the pathophysiology of EoE have encouraged the investigation of disease-modifying biologic agents directed at blocking the molecular pathways that lead to inflammation in EoE. The biologics investigated in clinical trials thus far include monoclonal antibodies against IL-5 (mepolizumab and reslizumab), IL-5Rα (benralizumab), IL-13 (cendakimab), IL-4Rα (dupilumab), IgE (omalizumab), TNF-α (infliximab), Siglec-8 (lirentelimab), and TSLP (tezepelumab). Their clinical efficacy has been extensively reviewed elsewhere.[36-38] Notably, of the aforementioned monoclonal antibodies, only dupilumab (anti–IL-4Rα) was found to improve both histological and clinical symptoms.[27] Dupilumab has recently been approved by the European Commission and the Food and Drug Administration, making it the first and only targeted medicine specifically indicated to treat EoE in both Europe and the USA.

1.3.2 Empiric and targeted elimination diets

Considering the fact that EoE is mainly driven by food allergens, avoiding contact between food allergens and the local esophageal immune system could open the door towards non-medical treatment. The interest in dietary therapy has recently emerged as a result of the limitations associated with medical therapies, and its potential to achieve sustained drug-free remission if the causative foods are avoided.[39] Currently, the challenge is to identify these causative foods, as conventional allergy tests – including skin prick tests, atopy patch tests, and serum IgE – are not reliable in the search for the foods that should be eliminated from the diet to achieve remission.[40] Consequently, the only option up to now comprises of empiric elimination diets, while there are usually only a few causative foods. These diets, such as the four- or six-food elimination diet, are effective for many,[41] but are very impactful in daily life, difficult to adhere to, and patients may be at risk of developing nutritional deficiencies.[42-44] It is beyond doubt that the identification and subsequent elimination of only the causative food (groups) is the preferred treatment option for EoE. More promising testing approaches should incorporate the current understanding of the pathophysiology of EoE, including that this disease may be a form of local mucosal allergy. Therefore, food challenge tests focusing on the true location of the immune response – that is the

esophageal mucosa – and not the skin or serum may be the way to go to develop reliable tests for the identification of causative foods for targeted elimination diets.[45]

1.3.3 Nutraceuticals to target esophageal inflammation

Nutraceuticals have received considerable interest lately due to their potential therapeutic effects and safety. Nutraceuticals are foods or food components that other than nutrition also provide medical or health benefits, including the prevention and treatment of a disease. Examples include but are not limited to specific vitamins, amino acids, and fatty acids (e.g. short-chain fatty acids, omega-3 polyunsaturated fatty acids). Nutraceuticals can exert their immunomodulatory effects through the interaction with the (gut) microbiota, receptors on cell membranes, and modification of epigenetics.[46] Over 25 years ago, the amino-acid based elemental diet – in which all proteins are eliminated and the nitrogen source is provided exclusively by single amino acids – paved the way for dietary interventions in EoE patients.[47] Although the amino-acid based elemental diet is highly effective (85-95% disease remission rates) in EoE patients of all ages,[15] it is not feasible for permanent use because of its poor palatability and impaired socialization. Elemental diets are thus usually combined with elimination diets.[48] Interestingly, besides their hypoallergenic properties, amino acids may have immune-modulating effects itself.[49] Indeed, De Rooij *et al*.[50,51] demonstrated that adult EoE patients given four-food elimination diet plus an amino-acid based formula improved the expression of several genes normally dysregulated in EoE compared with patients who were only given the elimination diet. Furthermore, Brusilovsky *et al*.[52] recently showed that vitamin D has important IL-13 antagonistic properties with the capacity to regulate esophageal epithelial barrier function, suggesting that vitamin D supplementation in EoE, either alone or in combination with other therapies, could be effective. Dietary supplementation of specific nutraceuticals may offer a potential effective, safe, inexpensive and acceptable solution to EoE patients.

1.4 PATHOPHYSIOLOGY OF EOE

The pathophysiology of EoE is multifactorial and results from the complex interplay between genetic, environmental and immunologic factors. It is postulated that a dysfunctional esophageal epithelial barrier, either acquired or genetically inherited, allows food antigens to pass the esophageal mucosa, facilitating contact between antigens and the immune system to elicit a local type 2 immune response in an environmentally or genetically predisposed individual, leading to tissue eosinophilia, inflammation, and ultimately esophageal remodeling (Figure 2).

1.4.1 Environmental and genetic factors conferring a predisposition to EoE

Multiple studies have reported a strong familial component to EoE, with an increased risk of developing EoE in first degree relatives or siblings of patients affected by the disease.[53,54] Comparing monozygotic (i.e., identical) and dizygotic twins is an excellent way of studying the contribution of genetic factors to a disease. Alexander *et al*.[53] demonstrated that monozygotic twins had a 41% disease concordance, while non-twin siblings had a 2.4% concordance, and 0.05% for non-

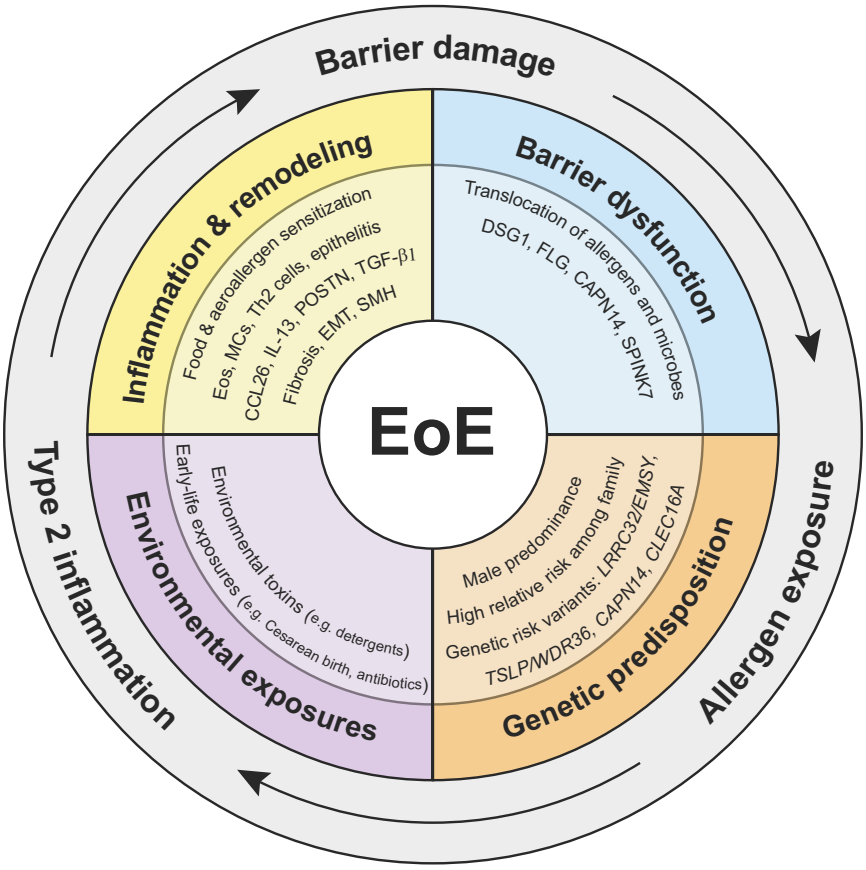

Figure 2. The pathogenetic mosaic and vicious circle of EoE. Local type 2 inflammation and esophageal barrier dysfunction are hallmark pathologic features of EoE. Continuous inflammation leads to esophageal remodeling. These pathologic features may be induced by genetic predisposition or specific environmental influences. A disrupted barrier enhances allergen exposure and type 2 inflammation, leading to the accumulation of immune cells (e.g. eosinophils, mast cells, and Th2 cells) in the esophageal mucosa and production of type 2 cytokines (e.g. IL-13) that further damage the barrier, providing a vicious circle of leaky barriers and chronic inflammation. Abbreviations: EMT, epithelial-to-mesenchymal transition; Eos, eosinophils; MCs, mast cells; SMH, smooth muscle hypertrophy.

related individuals. Interestingly, dizygotic twins have a disease concordance of 22%, which is 2-fold lower than monozygotic twins, but nearly 10-fold higher than non-twin siblings. Non-twin siblings and dizygotic twins are expected to have similar shared genetic influences, suggesting that the increased rate of EoE in dizygotic twins compared with non-twin siblings can be attributed to early-life environmental factors.[53,54] Indeed, maternal fever, cesarean delivery, preterm birth, and antibiotic or acid suppressant use in infancy have been identified as factors that may increase the risk of pediatric-onset EoE, while having a furry pet at home was protective.[55] Furthermore,

Akdis[56] has suggested that the alarming increase in prevalence of allergic diseases like EoE could be attributed to the increased exposure to noxious environmental triggers, such as detergents, and emulsifiers, surfactants and enzymes present in processed foods, which may damage epithelial barrier function and thereby allowing transit of food allergens locally into the esophagus.

Genetic variations in specific regions of the genome can increase the risk of developing EoE. Among the reported genome-wide association studies, genetic variants at four loci have been consistently found in EoE: 5q22.1 (*TSLP/WDR36*), 2p23.1 (*CAPN14*), 11q13.5 (*LRRC32/EMSY*), and 16p13.13 (*CLEC16A*).[57-62] A recent large genome-wide association study by Chang *et al.*[60] identified 11 additional EoE risk loci: 2q12.1 (*TMEM182*), 5q31.1 (*RAD50*), 6p22.3 (*SOX4*), 8q22.1 (*MATN2*), 10q21.1 (*PRKG1*), 11p15.4 (*RHOC*), 11p13.4 (*SHANK2*), 13q12.13 (*GPR12*), 15q22.2 (*RORA*), 15q23 (*SMAD3*), and 18q12.2 (*GALNT1*). The associated variants at 5q31.1 (*RAD50*), 15q22.2 (*RORA*), and 15q23 (*SMAD3*) have previously been linked to other allergic diseases,[63] indicating that the susceptibility to EoE is mediated by both EoE-specific and general atopic disease loci, which may act together to increase risk.[64] Interestingly, five sex-specific EoE risk loci were identified, providing evidence of distinct genetic mechanisms for female and male patients that may explain the observed sex difference in prevalence rate.[60] The identified genetic variants to date most often affect either epithelial barrier function or type 2-mediated immune responses.[65,66] The current data support a model in which genetic risk variants affect gene expression, leading to changes in immune and epithelial cell function. These changes, together with environmental disease risk-modifying factors, are hypothesized to increase the risk of EoE.[67]

1.4.2 Impaired esophageal barrier function

The primary function of the epithelial barrier is to protect the deeper mucosal and submucosal layers from infections, environmental toxins, and allergens.[56] The human esophageal epithelium is non-keratinized and is made up of three distinct layers (from bottom to top): the basal cell layer, prickle cell layer, and squamous cell layer (superficial layer of long flat cells). The maintenance of the intact esophageal epithelial barrier depends on the coordinated expression of epidermal differentiation complex (EDC) proteins, tight junctions, adherens junctions, and desmosomes.[68] However, in EoE, the esophageal epithelial barrier is frequently disrupted, as demonstrated by reduced transepithelial electrical resistance and mucosal impedance,[69-72] most likely caused by a profound loss of epithelial differentiation.[73] Dilated intercellular spaces (spongiosis) and basal zone hyperplasia are prominent histologic features of defective epithelial differentiation in EoE,[74] which is further demonstrated by lost expression of EDC proteins filaggrin (FLG), involucrin (IVL) and several small proline-rich protein (SPRR) family members (SPRR4, SPRR1A, SPRR3).[73,75,76] In addition, loss of desmoglein-1 (DSG1) expression as seen in EoE is sufficient to induce esophageal barrier dysfunction.[77] These histologic and molecular changes are reproduced in differentiated esophageal epithelial cells *in vitro* in the presence of the type 2 cytokine interleukin (IL) 13 and are accompanied by impaired barrier function.[78]

Esophageal epithelial cells are a rich source of proteases and antiproteases that are part of a normal homeostatic surveillance mechanism.[73,79-81] However, in EoE, there is a defect in the regulation of protease/antiprotease responses. More specifically, loss of the antiprotease serine

peptidase inhibitor kazal type 7 (SPINK7) is sufficient to unleash uncontrolled proteolytic activity and proinflammatory responses in esophageal epithelial cells.[80] The serine protease kallikrein-5 (KLK5), a direct target of SPINK7, can proteolytically degrade DSG1, causing epithelial barrier dysfunction.[79] Similarly, increased expression of the intracellular protease calpain-14 (CAPN14) by IL-13 in EoE results in impaired barrier function and loss of DSG1.[82]

The impaired esophageal barrier seen in EoE patients allows molecules of up to 40 kDa, which is of similar size as common food allergens, to pass through the epithelium.[83] Indeed, food antigens are present in the esophageal epithelium of EoE patients,[84,85] which may enhance allergic sensitization and type 2 inflammation, forming a pathogenic cycle to further exacerbate allergic inflammation (Figure 2). Whether the disrupted epithelial integrity is restricted to the esophagus in EoE is not yet clear, as there is contradictory literature demonstrating either unaffected[86,87] or impaired[88] epithelial integrity of the small intestine as well in EoE patients.

1.4.3 Localized type 2 inflammation

Allergen sensitization

Multiple lines of evidence support an allergic etiology for EoE that is induced primarily by food allergens and mediated by type 2 inflammation. This is supported by several studies that showed that EoE patients respond to dietary elimination of food antigens, and relapse when the same food antigens are reintroduced, underscoring the importance of specific antigens.[87,89,90] In addition, elemental and empiric elimination diets are highly effective in both children and adults.[41] The majority of the EoE patients (50-80%) have concurrent atopic disorders, such as food allergy, oral allergy syndrome, atopic dermatitis, asthma, and allergic rhinitis. Notably, the presence of IgE-mediated food allergy, atopic dermatitis, and asthma are associated with a later diagnosis of EoE.[91] In addition to food allergens, aeroallergens have also been implicated to contribute to EoE development.[92,93]

Although there is clear evidence that food (and to a lesser extent aero-) allergens induce inflammation that leads to esophageal eosinophilia and ensuing esophageal symptoms, it is currently not fully understood where and how allergen sensitization occurs in EoE. Hypersensitivity reactions can occur via multiple immune mechanisms including IgE-mediated (immediate type) and/or T cell-mediated (delayed type) mechanisms. EoE is associated with elevated total and allergen-specific IgE levels in serum.[94] However, elimination diets solely based on IgE sensitization to food allergens as determined by skin prick testing or serum allergen-specific IgE measurements could not improve symptoms.[40] In addition, anti-IgE (omalizumab) treatment was not better than placebo in inducing EoE remission.[95,96] Thus, while often associated with IgE sensitization, EoE is not simply an IgE-mediated food allergy. Delayed-type, T cell-mediated reactions are also involved, as evidenced by increased local Th2 cell numbers and type 2 cytokine levels.[97] It is therefore possible that EoE is mediated by both IgE- and non-IgE-mediated immune mechanisms.

Immune cells in EoE

Epithelial cells

Besides their barrier function, esophageal epithelial cells can also induce inflammation. IL-33 and TSLP, both increased in EoE, are released by the epithelium upon response to allergen exposure, damage, or stress, and are therefore also known as epithelial 'alarmins'. IL-33 and TSLP are prominent inducers of type 2 immune responses by activating various immune cells, including infiltrating basophils, Th2 cells, mast cells and group 2 innate lymphoid cells (ILC2s) to produce type 2 cytokines or by promoting the Th2-polarizing capacity of dendritic cells, respectively.[98-101] IL-25 is also a well-known epithelial alarmin, but there is no data on its role in EoE thus far. Furthermore, the key EoE and type 2 cytokine IL-13 can induce eotaxin-3 release by esophageal epithelial cells, which signals eosinophil trafficking to the esophagus.[102] A recent single-cell RNA sequencing study[103] speculated that esophageal epithelial cells maintain epithelial inflammatory memory similar to skin[104] and nasal[105] epithelial cells and, in this way, contribute to disease relapses by enhancing sensitivity to subsequent stressors. Interestingly, esophageal epithelial cells are also capable of functioning as nonprofessional antigen-presenting cells (APCs).[106]

Eosinophils

Although eosinophils are not pathognomonic of EoE, they are its most easily recognizable pathologic feature.[107] Mature eosinophils contain granules that are primarily composed of highly charged basic proteins including eosinophil major basic proteins, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase, each with their own cytotoxic and proinflammatory effects, such as increasing smooth muscle activity or triggering mast cell and basophil degranulation.[108,109] In EoE, there is evidence of eosinophil activation and release of granule components such as major basic protein.[110] Activated eosinophils are also capable of generating a large number of proinflammatory cytokines, including IL-1, IL-3, IL-4, IL-5, IL-13, GM-CSF, and TGF-β1, suggesting that eosinophils have the potential to sustain or enhance multiple aspects of the immune response and tissue repair process.[108] In addition, eosinophils can initiate antigen-specific responses by acting as nonprofessional APCs, as eosinophils express relevant costimulatory molecules (CD40, CD28, CD86, B7), can be induced to express major histocompatibility complex class II molecules, and produce cytokines capable of inducing T cell proliferation and maturation.[111,112] The most studied chemotactic factor for eosinophils in EoE is eotaxin-3 (encoded by *CCL26*), which is mainly produced by esophageal epithelial cells upon IL-13 stimulation.[113] The *CCL26* gene is the most upregulated gene in the esophagus of EoE patients and correlates with disease activity.[102]

Mast cells

Mast cells are tissue-resident granulocytes that can be found near sites of environmental interactions (e.g. mucosa), muscles, blood vessels, and nerves.[114] In EoE, mast cells are increased in density and activation in the esophageal epithelium.[115-117] Multiple mast cell marker genes are enriched in the EoE transcriptome, including tryptase (*TPSAB1*), carboxypeptidase A3 (*CPA3*) and histidine decarboxylase (*HDC*).[118] Cross-linking of membrane-bound IgE by specific antigens is the classic form of mast cell activation, and IgE-bearing mast cell levels are elevated in biopsies from EoE patients.[117] However, therapeutic targeting of IgE with omalizumab was

largely unsuccessful in EoE.[95,96] Other mast cell stimuli include cytokines, pathogen-associated molecular patterns, complement, neuropeptides, physical/mechanical stress, and temperature or pH changes.[119] Upon activation, mast cells can rapidly release preformed histamine and proteases and newly synthesized lipid mediators through a process called degranulation in addition to the slower release of newly synthesized cytokines and chemokines. Although mast cells undergo substantial degranulation in the esophageal epithelium of EoE patients,[115] it is currently unknown which stimuli are responsible. In EoE, mast cells have been suggested to contribute to fibrosis, smooth muscle contraction, and nerve signaling.[120,121] A recent transcriptomics study[116] assessed esophageal mast cells at the single-cell level, and identified multiple types of mast cells – resident, transient, and persistent – as well as IL-13-expressing mast cells in EoE. In addition, the study reported mast cell expansion by local proliferation. Notably, the persistent mast cell type was still present in patients with inactive disease, and its transcriptome was enriched for genes associated with activation processes and immune effector processes, suggesting that these mast cells are prepared to be activated despite inactive disease.[116]

Basophils

Basophils are the least abundant granulocyte population in the blood and share functional and morphological similarities with tissue-resident mast cells, such as the expression of FcεRI on the cell membrane.[122] Basophils can be activated by an array of signals, including those mediated by proteases, antibodies, cytokines, and antigens.[123] Although basophils are known to be important in allergic inflammation, their role in EoE remains poorly defined. Siracusa *et al*.[124] demonstrated that TSLP can promote basophil hematopoiesis and surface expression of the IL-33R. These IL-33R-expressing basophils are increased in the esophagus from patients with EoE, where they may encounter IL-33, triggering the production of multiple proinflammatory cytokines and chemokines.[124] Shortly after, Noti *et al*.[125] showed that EoE-like disease in mice can develop in a TSLP- and basophil-dependent, but IgE-independent manner. Another study demonstrated that epicutaneous sensitization to egg protein followed by repeated intranasal administration induced EoE-like inflammation in mice, which was critically mediated by the IL-33–IL-33R–basophil axis.[126]

Dendritic cells

Dendritic cells are professional APCs with an important role in sensitization, but there is a paucity of data on dendritic cell function in EoE. The Langerhans cell, a type of dendritic cell found in squamous epithelia, particularly the skin, seems to be the primary professional APC in the esophagus.[127-130] Esophageal dendritic cells express the high affinity IgE receptor FcεRI in both healthy controls and EoE patients.[131] In EoE, IgE bound to FcεRI on the dendritic cell may facilitate antigen uptake by increasing the efficiency of antigen uptake and presentation by a factor of 100-1000,[132,133] enhancing the development of allergen-specific T cells.[133] Local antigen presentation in EoE likely depends not only on Langerhans cells,[134] as it may also occur via nonprofessional APCs such as epithelial cells and eosinophils.[106,111,112]

Group 2 innate lymphoid cells

ILC2s are a relatively newly discovered immune cell type, and therefore less studied to date, that are not antigen specific. ILC2s are elevated in esophageal biopsies from patients with active EoE

compared with patients with inactive EoE, PPI-responsive esophageal eosinophilia, and control subjects, and strongly correlated with esophageal eosinophil numbers.[135] ILC2s contribute to allergic inflammation by the rapid and robust production of type 2 cytokines, including IL-4, IL-5, IL-9, and IL-13 in response to epithelial cytokines IL-33 and TSLP or mast cell-derived leukotriene D4,[136-140] which may represent an antigen-independent mechanism for the propagation of inflammation in EoE.[135]

T cells

Studies using murine models of EoE lacking various components of the adaptive immune system have demonstrated a critical role for T cells in EoE.[141,142] Similar to other allergic diseases, tissue inflammation in EoE patients is characterized by a type 2 inflammatory response. A recent transcriptome study[97] that analyzed tissue-residing $CD3^+$ T cells on the single cell level demonstrated that two T cell populations, so-called T7 and T8 clusters, were increased in patients with EoE compared with controls. These populations represented regulatory T cell (Treg)-like cells and Th2 effector cells, respectively. Type 2 cytokine production was largely confined to this Th2 cell population, with robust IL-13 expression and IL-4 and IL-5 to a lesser extent. In addition, despite the enrichment of the Treg-like cell population in EoE, they were ineffective in suppressing the adaptive immune response.[97] Recently, esophageal T cells expressing the pathogenic cytokine TNFSF14/LIGHT were found to induce a proinflammatory phenotype in fibroblasts in EoE.[143]

Furthermore, invariant natural killer T (iNKT) cells may have a pathogenic role in EoE.[144-147] iNKT cells are a T cell subset that respond to lipid and glycolipid antigens presented by the MHC class I-like protein CD1d, and are another source of type 2 cytokines upon activation.[148,149] Peripheral blood iNKT cells from children with EoE have been shown to be activated by milk sphingolipids.[145] In addition, iNKT cell-deficient mice are protected from allergen-induced EoE.[147]

B cells, IgE, and IgG4

A process central to many allergic disorders is the Th2 cell-mediated class switching of B cells to IgE. As noted above, IgE can bind the FcεRI on mast cells and basophils to induce degranulation upon cross-linking by antigen. Although IgE sensitization is common in EoE, not all patients show high total or specific IgE levels in serum.[94] However, there is evidence of local rather than systemic IgE involvement in EoE, as B cells in esophageal tissue are increased in number, undergo IgE class switching, and produce IgE regardless of the atopic status of the patient.[150] A role for local IgE has been demonstrated in the colon of patients with inflammatory bowel syndrome and nasal mucosa of patients with seasonal idiopathic rhinitis.[151,152] Though local B cells appear to be generating IgE in EoE, murine models of EoE do not require B cells or IgE to induce esophageal eosinophilia,[125,142] suggesting that IgE may not be involved in the initiation of EoE. In addition, anti-IgE biologicals lack efficacy in clinical trials.[95,96] Thus, the role of IgE in EoE remains unclear.

Besides IgE, tissue IgG4 levels are elevated in adults and children with EoE patients compared with healthy controls, and correlate with disease activity.[96,153] Similarly, tissue food allergen-specific IgG4 is increased in adults with EoE, and – in contrast with plasma allergen-specific IgG4 levels

– decrease when food triggers are eliminated from the diet,[154] suggesting that local IgG4 could play a role in EoE pathogenesis. Recently, a novel IgG4-expressing tissue-infiltrating B cell subset was identified in EoE that promotes angiogenesis and associates with tissue remodeling.[155] These observations challenge the dogma that IgG4 is an anti-inflammatory immunoglobulin isotype as it cannot fix complement, binds weakly to IgG receptors, and has reduced ability to cross-link receptors and form immune complexes due to Fab-arm exchange.[156,157] Furthermore, IgG4 competes with IgE for allergen binding and therefore may also function as a blocking antibody. High IgG4 levels are generated in response to high dose allergen exposure, either naturally (e.g. in beekeepers and cat owners) or following allergen-specific immunotherapy, and are associated with allergen tolerance.[158,159] Notably, oral allergen-specific immunotherapy, in which the goal is a tolerance-inducing IgG4 immune response, induces EoE in 2.7% of the patients undergoing this therapy.[160] It is currently unknown whether IgG4 has a pathogenic role in EoE or is just a result of the ongoing immune response due to chronic allergen exposure.

Cytokines in EoE

Well-known cytokines in EoE

Studies examining the molecular and cellular underpinning of EoE have demonstrated the involvement of proinflammatory epithelium-derived cytokines (e.g. IL-33 and TSLP), chemokines related to eosinophilia (e.g. eotaxin-3, encoded by *CCL26*), and type 2 cytokines (e.g. IL-4, IL-5 and IL-13). Transcriptomic analysis of EoE biopsies revealed that epithelium-derived *CCL26* is the most dysregulated gene with a 279-fold increase in EoE patients compared with controls, and strongly correlated with disease severity.[102] The type 2 cytokine IL-4 mediates B cell class switching to IgE and Th2 cell differentiation.[161] TSLP-elicited basophils, Th2 cells, and iNKT cells are important sources of IL-4 in EoE. Furthermore, IL-5 plays an important role in EoE and mainly affects eosinophils. IL-5 is produced by eosinophils, mast cells, and Th2 cells. It regulates eosinophil expansion, survival, and migration to the esophagus, and primes eosinophils to respond to specific activating signals.[141] Similarly, IL-13 is highly expressed in the esophagus of EoE patients and seems to be central to EoE. Interestingly, IL-13-overexpressing mice develop an EoE-like inflammatory esophageal response.[162] The importance of IL-13 in EoE is further substantiated by the ability of IL-13 to directly induce a large number of EoE-associated genes, including *CCL26*, in esophageal epithelial cells,[113] and damage the esophageal epithelial barrier via a CAPN14-dependent mechanism involving downregulation of DSG1.[77,78,82] Th2 cells, mast cells, eosinophils and potentially ILC2s are important sources of IL-13 in EoE. TGF-β plays a critical role in in EoE-related esophageal remodeling, and will be further discussed later in this chapter.[120,163]

Less-known cytokines in EoE

In addition to the well-known type 2 cytokines (IL-4, IL-5, and IL-13), epithelial alarmins (IL-33 and TSLP) and pro-fibrotic cytokine TGF-β, recent studies have investigated other cytokines that are highly produced in the esophagus of EoE patients. IL-9, most likely produced by ILC2s and mast cells in EoE, is a type 2 cytokine that promotes mast cell expansion and function.[164] In addition, IL-9 was shown to directly disrupt the function of the esophageal epithelial barrier.[165]

While type 2 inflammation represents an important subset of the immune pathways activated within the esophageal mucosa, there is also a role for non-type 2 inflammatory mediators in EoE pathophysiology. First, IL-15 has the ability to stimulate the proliferation and differentiation of activated T cells in an antigen-independent manner.[166,167] IL-15 mRNA levels correlate with esophageal eosinophil count in EoE.[168] Furthermore, IL-15 can amplify type 2 immune responses in EoE by priming $CD4^+$ T cells to produce type 2 cytokines, by promoting the production of eotaxin-3 by esophageal cells, and by activating iNKT cells.[168,169] Second, IL-18, a member of the IL-1 cytokine family, and its receptor IL-18Rα are increased in the blood and esophagus of EoE patients, respectively. In EoE, IL-18 may play in important role by activating iNKT cells to produce type 2 cytokines, including IL-5 and IL-13, without T cell receptor engagement.[170] IL-18 overexpression promotes esophageal eosinophilia and mast cell inflammation in a mice, potentially via an iNKT-mediated pathway.[171] Third, there is a conserved IFN gene expression signature in esophageal biopsies from children and adults with EoE, but a causal link between IFN and pathophysiologic features in EoE remain to be studied.[172] Fourth, TNF-α may be involved in esophageal remodeling and angiogenesis.[173,174] Finally, TNFSF14/LIGHT, a TNF superfamily member, was attributed a role in EoE pathophysiology,[143,175,176] as its overexpression induced a pro-inflammatory phenotype in fibroblasts in EoE,[143] while its deficiency protected mice from developing EoE-like inflammation.[175]

1.4.4 Esophageal remodeling

Uncontrolled and persistent esophageal inflammation almost uniformly progresses to a fibrostenotic disease, resulting in stricture formation, esophageal stiffness, increased smooth muscle mass with smooth muscle dysfunction, and ultimately symptoms of esophageal dysfunction including dysphagia and food impactions.[177,178] Esophageal remodeling occurs in the epithelial and subepithelial layers, and includes basal zone hyperplasia, epithelial-to-mesenchymal transition, fibrosis, angiogenesis, and esophageal smooth muscle hypertrophy/hyperplasia.[179] Signs of fibrosis in esophageal biopsies are found in up to ~90% of children and adults with EoE.[180,181] Nonetheless, strictures are not commonly seen in children, likely due to shorter untreated disease duration.[15] The likelihood of fibrostenotic disease increases with age, and, therefore, it is hypothesized that EoE progresses from an inflammatory to a fibrostenotic disease.[177]

On a molecular level, eosinophil- and mast cell-derived TGF-β1 may be a central regulator of EoE tissue remodeling and esophageal dysmotility,[120,163] and is increased in both pediatric and adult EoE.[163,182] TGF-β1 is known to directly regulate profibrotic processes, as it induces fibroblast activation and epithelial-mesenchymal transition in EoE, thereby promoting the production and deposition of extracellular matrix proteins (e.g. periostin and collagen) in subepithelial layers.[120,183-186] Periostin may in turn induce eotaxin-3-mediated eosinophil recruitment and adhesion to the esophagus, along with tissue remodeling.[187] In addition, TGF-β1 is involved in acute esophageal smooth muscle contraction associated with immediate symptoms of dysphagia.[120]

The fibrostenotic phenotype associates with a distinct EoE endotype that is enriched for downregulated epithelial genes, particularly *ACPP*, *CTNNAL1*, *CITED2*, *FLG*, *EML1*, *MT1M*,

GRPEL2, *PNLIPPR3*, and, *TSPAN12*.[188] Recently, *TSPAN12*, a tetraspanin protein that regulates cell development, activation, growth and motility, was identified as the most dysregulated gene in fibrostenotic EoE regardless of age group or gender.[189] Patients with fibrostenotic EoE express decreased levels of endothelial *TSPAN12*, which is negatively regulated by IL-13 but not TGF-β1. Loss of endothelial *TSPAN12* may contribute to tissue remodeling in EoE by promoting endothelial dysfunction and endothelial cell-fibroblast crosstalk.[189]

1.5 AIM AND OUTLINE OF THIS THESIS

Major advances in the understanding of EoE disease course and pathophysiology have been made over the past 25-30 years. Nonetheless, many unmet needs associated with the prevention, phenotyping, diagnosis, and management of the disease remain. It is evident that there is a demand to better understand the (food-induced) local immune responses that lead to eosinophilic inflammation of the esophagus to enable future therapies to be more effective. Therefore, this thesis aims to investigate these local immune mechanisms that underly EoE. Furthermore, due to the need for novel (dietary) treatment protocols, we studied the potential of local esophageal food challenge to identify causative foods and make a personalized diet possible, and assessed the therapeutic potential of short-chain fatty acids on the esophageal epithelial barrier.

The interest in dietary therapies for EoE has recently emerged as a result of the limitations associated with other therapies, and its effectiveness in achieving and maintaining clinical remission while avoiding the need for drugs. **Chapter 2** describes the effects of the most abundantly produced short-chain fatty acids – acetate, propionate and butyrate – on the esophageal epithelial barrier. In this chapter, we used an *in vitro* air-liquid interface culture of differentiated human esophageal epithelial cells to study whether short-chain fatty acids could restore barrier function after IL-13-induced impairment, including the mechanisms involved.

EoE is mainly driven by food allergens. However, conventional allergy tests using skin and serum are poorly predictive of the foods that cause esophageal symptoms in EoE patients, likely because the allergic inflammation is restricted to the esophagus. In **Chapter 3**, we describe three local food challenge methods using esophageal tissue that may be used to identify causative foods and guide elimination diets, and to study the local food-induced immune response in EoE.

To date, several studies have provided insight into transcriptional changes associated with active EoE, but little emphasis has been placed on characterizing genes that mediate the acute esophageal response triggered by food. **Chapter 4** describes the molecular processes associated with acute mucosal responses to food injections. We performed bulk RNA-sequencing on esophageal biopsies collected before and 20 minutes after local esophageal food injections to characterize changes in the esophageal transcriptome that occur during an acute esophageal response to food.

Accumulation of mast cells in the esophageal epithelium is an important feature of EoE. In the final experimental chapter of this thesis, **Chapter 5**, we established an *in vitro* coculture system of primary human mast cells and differentiated esophageal epithelial cells cultured at the air-liquid interface to study the effect of mast cells and their products on the function of the esophageal epithelial barrier.

The findings described in this thesis are discussed in **Chapter 6**, and future directions are presented.

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CHAPTER 2

Butyrate and propionate restore interleukin 13-compromised esophageal epithelial barrier function

ABSTRACT

BACKGROUND: Eosinophilic esophagitis (EoE) is a food allergen driven disease that is accompanied by interleukin (IL) 13 overexpression and esophageal barrier dysfunction allowing transepithelial food allergen permeation. Nutraceuticals, such as short-chain fatty acids (SCFAs) that restore barrier function and increase immune fitness may be a promising tool in the management of EoE.

OBJECTIVE: To investigate the effects of the SCFAs acetate, propionate, and butyrate on an IL-13-compromised human esophageal epithelial barrier, including the mechanisms involved.

METHODS: An air-liquid interface culture model of differentiated human EPC2-hTERT (EPC2) was used to study whether SCFAs could restore barrier function after IL-13-induced impairment. Esophageal epithelial barrier function was monitored by transepithelial electrical resistance (TEER) and FITC-dextran paracellular flux, and was further examined by qPCR and immunohistochemical analysis. G protein-coupled receptor (GPR) GPR41, GPR43, GPR109a, or histone deacetylase (HDAC) (ant)agonists were used to assess mechanisms of action of SCFAs.

RESULTS: IL-13 stimulation decreased TEER and increased FITC flux, which was counteracted by butyrate and propionate, but not acetate treatment. Barrier proteins FLG and DSG1 mRNA expression was upregulated following butyrate and propionate treatment, whereas expression of eosinophil chemoattractant CCL26 and protease CAPN14 was downregulated. Similarly, butyrate and propionate restored FLG and DSG1 protein expression. Similar effects were observed with an HDAC antagonist but not with GPR agonists.

CONCLUSION: Nutraceuticals butyrate and propionate restore the barrier function of esophageal epithelial cells after an inflammatory insult and may be of therapeutic benefit in the management of EoE.

KEYWORDS: barrier function; dietary intervention; eosinophilic esophagitis; interleukin 13; short-chain fatty acids.

2.1 INTRODUCTION

The epithelial barrier of the esophagus forms the first line of chemical, physical and immunologic defenses, and provides a protective wall against environmental factors including microbes and food allergens.[1] In eosinophilic esophagitis (EoE), a chronic food allergen-mediated disease of the esophagus, the esophageal barrier is frequently disrupted, leading to exposure to food allergens in the esophageal mucosa and the subsequent induction of a local type 2 immune response.[2,3] Current treatment options for EoE consist of topical steroids and dietary restrictions,[4,5] but are sometimes unpopular with patients. Thus, there is a demand for novel treatment protocols that restore esophageal barrier function and mitigate esophageal inflammation to re-establish esophageal immune fitness.

Recent studies have demonstrated a link between the type 2 cytokine interleukin (IL) 13 in esophageal epithelial proliferation and esophageal barrier dysfunction.[3,6,7] In fact, esophageal epithelial cells express each subunit of the IL-13 receptor including IL-4Rα, IL-13Rα1 and IL-13Rα2.[8] Transcriptomics studies have shown that IL-13 is overexpressed during active EoE, but its major cellular source or sources remain to be elucidated.[8] Subsequently, IL-13 disrupts the esophageal barrier, mediated in part by the loss of the epithelial barrier proteins desmoglein-1 (DSG1) and filaggrin (FLG).[3,9] In addition, IL-13 induces marked overexpression of eosinophil chemoattractant chemokine (C-C motif) ligand 26 (CCL26, encoding eotaxin-3) and protease calpain-14 (CAPN14).[10,11] Notably, the EoE transcriptome can be partially reproduced in IL-13-treated immortalized esophageal epithelial cells cultured under air-liquid interface (ALI) conditions, indicating that IL-13-induced gene expression in esophageal epithelial cells may make an important contribution to the EoE pathogenesis.[6]

Short-chain fatty acids (SCFAs) -in particular acetate, propionate and butyrate- are produced by bacterial fermentation of dietary fiber in the gut, where they serve as an energy source for colonocytes, maintain intestinal homeostasis, and promote gut barrier function.[12-14] SCFAs are agonists of G protein-coupled receptor (GPR) 41, GPR43, and GPR109a, inducing anti-inflammatory pathways upon binding.[15-18] In addition, butyrate and propionate influence the activity of histone deacetylase (HDAC), a class of histone modification enzymes that regulates gene transcription and has the potential to influence biological processes.[19-22] Although mainly produced in the gut, SCFAs have also been shown to have immunomodulatory effects in other barrier organs such as the lungs and skin.[23-29]

In this study, we use a model that resembles differentiated (i.e. stratified squamous) human esophageal epithelium to investigate the potential barrier-restorative effects of the SCFAs acetate, propionate, and butyrate on an IL-13-compromised barrier. In addition, we aimed to determine the underlying mechanisms of the observed functional effects.

2.2 MATERIALS AND METHODS

2.2.1 EPC2-hTERTt culture

The immortalized human esophageal epithelial cell line EPC2-hTERT (EPC2) was a kind gift from Dr. Anil Rustgi (University of Pennsylvania, Philadelphia, PA, USA).[30-32] EPC2 were cultured in low-calcium (0.09 mM) keratinocyte serum-free medium (KSFM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with bovine pituitary extract (50 µg/ml), epidermal growth factor (1 ng/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml).

2.2.2 Air-liquid interface (ALI) culture system and SCFA treatment

The 3D ALI culture protocol was adapted from Kc *et al*.[6] A schematic representation of the experimental timeline is shown in Figure 1A. Briefly, EPC2 were grown to confluence on semi-permeable membranes (0.4 µm; Corning Incorporated, Corning, NY, USA) in low-calcium KSFM for three days. Initial differentiation of confluent monolayers was induced by switching to high-calcium (1.8 mM) KSFM from culture day 3 to 7. Terminal epithelial differentiation and stratification were induced by removing the media from the apical chamber and exposing the cells to the ALI from culture day 7 to 14. Cells were exposed to IL-13 (100 ng/ml; Prospec, Rehovot, Israel) in the basolateral chamber at the start of ALI culture.

Sodium acetate was purchased from BDH Laboratory Supplies (Poole, England, cat. no. 102364Q). Sodium propionate and sodium butyrate were purchased from Sigma-Aldrich (Saint-Louis, MO, USA, cat. no. P1880 (propionate) and 303410 (butyrate)). All SCFAs were used in preliminary work in a range of concentrations from 5 to 20 mM (acetate and propionate) and 2 to 10 mM (butyrate) (Figure S1). In final experiments, acetate (10 mM), propionate (10 mM) or butyrate (5 mM) was added to the basolateral chamber of IL-13-stimulated EPC2 ALI cultures from day 10 to 14. EPC2 ALI cultures were also treated with SCFAs in the absence of IL-13 (Figure S2). Media plus IL-13 and SCFAs were refreshed every other day. ALI cultures were then collected for total RNA isolation and immunohistochemistry.

2.2.3 Assessment of mechanisms of action of SCFAs

The following (ant)agonists were used to investigate the involvement of GPR41, GPR43, GPR109a and HDAC in the barrier-restorative effects of SCFAs: GPR41 agonist AR420626 (1 µM), GPR43 agonist 4-CMTB (10 µM), GPR109A agonist niacin (10 mM) and HDAC antagonist Trichostatin A (TSA, 2 µM). All (ant)agonists were dissolved in DMSO or 1M NaOH according to the manufacturer's instructions and were purchased from Sigma-Aldrich (cat. no. SML1339 (AR420626), SML0302 (4-CMTB), N4126 (niacin) and T8552 (TSA)). All (ant)agonists were used in preliminary ALI experiments in a range of concentrations from 1 to 100 µM (AR420626), 0.1 to 50 µM (4-CMTB), 1 to 20 mM (niacin) and 0.1 to 10 µM (TSA) (Figure S4, S5). In final experiments, (ant)agonists were added to the basolateral chamber of the IL-13-stimulated EPC2 ALI cultures from day 10 to 14. Media plus IL-13 and (ant)agonist were refreshed every other day. ALI cultures were then collected for total RNA isolation and immunohistochemistry.

2.2.4 Transepithelial electrical resistance (TEER), paracellular flux assays and LDH toxicity test

TEER was measured during ALI culture using a Millicell ERS-2 Voltohmmeter (Merck Millipore, Burlington, MA, USA). High-calcium KSFM was added to the apical chamber 1 h prior to TEER measurement. Paracellular flux assays were performed 1 h after TEER measurement on day 14. 4-kDa fluorescein isothicyanate (FITC)-dextran (0.1 mg/µl; Sigma-Aldrich) was added to the apical chamber, and fluorescein levels in the basolateral chamber were detected after 15, 30, 60, 90, 120 and 180 min using a Glomax Discover Microplate Reader (Promega, Madison, WI, USA) at Ex/Em = 492/518. Cytotoxicity was measured in 50 µL supernatant collected at day 14 using the Cytotoxicity Detection Kit (LDH) (Roche, Basel, Switzerland) (Figure S7) per manufacturer's instructions.

2.2.5 Quantitative real-time PCR

Total RNA was treated with DNase I (Qiagen, Hilden, Germany) and isolated from EPC2 ALI cultures using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was generated from 500 ng RNA using the iScript™ cDNA synthesis kit (BioRad, Hercules, CA, USA). qPCR was performed using SYBR Green (BioRad). All primers used for amplification were purchased from BioRad (Unique Assay ID qHsaCID0017001 (CAPN14), qHsaCED0041923 (CCL26), qHsaCED0044569 (DSG1) and qHsaCED0036604 (FLG)). Results were normalized to ribosomal protein S13 (RPS13; Unique Assay ID qHsaCID0038672) expression for each sample. mRNA expression levels were calculated using the following formula: fold change = $2^{-\Delta\Delta Ct}$ and were normalized to the untreated control.

2.2.6 Histology and immunofluorescence

Formalin-fixed paraffin-embedded EPC2 ALI cultures were cut into 5 µm sections and deparaffinized using xylene followed by graded ethanol washes. For histology, sections were stained in hematoxylin, rinsed in tap water and then stained in eosin, followed by dehydration in graded ethanol washes and xylene before mounting with Pertex (Histolab, Askim, Sweden) and xylene (1:1). For immunofluorescence, heat-induced antigen retrieval in sodium citrate buffer (10 mM citric acid, pH 6.0) was used on deparaffinized sections and endogenous peroxidase activity was quenched using 3% H_2O_2 in methanol. After rinsing in 0.2% Tween in PBS, sections were blocked in 3% BSA in PBS containing 5% normal goat serum (Dako, Jena, Germany) for 90 min, followed by overnight incubation at 4 °C with rabbit anti-DSG1 (1 µg/ml; Abcam, Cambridge, UK, cat. no. ab209490) or rabbit anti-FLG (1 µg/ml; Abcam, cat. no. ab234406). Sections were rinsed and incubated for 1 h with goat anti-rabbit AF594 (10 µg/ml; Invitrogen, Carlsbad, CA, USA, cat. no. A11072). Sections were mounted with ProLong™ Gold antifade reagent with DAPI (Invitrogen) for nuclei staining. Immunofluorescent images were acquired using the Keyence Fluorescence Microscope BZ-9000, and immunofluorescence intensity was quantified using ImageJ software.

2.2.7 Nuclear extract preparation and HDAC activity

EPC2 were grown in 12 wells culture plates (Costar) in low-calcium KSFM until confluent, followed by stimulation with acetate (10 mM), propionate (10 mM), butyrate (5 mM) or TSA (2 µM) in high-calcium KSFM. Cytoplasmic and nuclear extracts were isolated 48 h after stimulation. Briefly, EPC2 were trypsinized, collected by centrifugation (1000 rpm, 4 min, 4 °C) and washed twice in ice-chilled PBS. EPC2 were resuspended in 100 µL ice-chilled Buffer 1 (Table S1) and incubated on a rotator for 10 min at 4 °C. After vortexing, lysates were centrifuged (12,000 rpm; 1 min; 4 °C) and the cytoplasmic protein fractions were collected and stored at -80 °C. Nuclear pellets were washed twice with ice-chilled PBS, disrupted with 40 µL Buffer 2 (Table S1), and incubated on ice for 30 min with regular vortexing followed by sonication for 3 x 10 seconds. The suspension was centrifuged(12,000 rpm; 15 min; 4 °C) and the nuclear fractions were collected and stored at -80 °C. Total protein content was quantified using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). HDAC activity was measured in 2 ng nuclear extract using the colorimetric Epigenase HDAC Activity/Inhibition Direct Assay Kit (EpiGentek, Farmingdale, NY, USA) according to the manufacturer's instructions.

2.3 RESULTS

2.3.1 SCFAs butyrate and propionate restored esophageal barrier resistance and permeability in IL-13-stimulated EPC2 ALI cultures

We used an ALI culture model that resembles human differentiated esophageal epithelium to investigate the potential barrier-restorative effects of SCFAs as depicted schematically in Figure 1A. Following seven days of differentiation at the ALI, EPC2 formed a stratified squamous epithelial layer indicating the development of differentiated esophageal epithelium (Figure 1B). Prolonged IL-13 exposure resulted in marked morphological changes including decreased epithelial differentiation and expansion of the epithelial layer (Figure 1B). Furthermore, IL-13 induced a significant decrease in TEER from day 10 and onwards (Figure 1C), and a significant increase in FITC-dextran paracellular flux (FITC flux) on day 14 (Figure 1D). Together, these results indicate that IL-13 induces barrier dysfunction in EPC2 ALI cultures as reported previously.[3,6]

To study the barrier-restorative effects of SCFAs on IL-13-stimulated EPC2 ALI cultures, acetate, propionate or butyrate were added to the basolateral chamber from day 10 to 14. At day 14, IL-13 stimulation showed a 2.2-fold decrease in TEER compared to untreated cultures. Propionate and butyrate counteracted the effect of IL-13 on TEER as shown by a 2.7-fold increase in propionate-treated ALI cultures and 3.8-fold increase in butyrate-treated cultures compared to IL-13-stimulated EPC2 ALI cultures (Figure 1E). FITC flux assays confirm these findings. IL-13-stimulated EPC2 ALI cultures had a significantly increased FITC flux at day 14, which was counteracted by propionate and butyrate treatment (Figure 1F). In addition, SCFAs -in particular butyrate- restored IL-13-induced barrier dysfunction measured by TEER and FITC flux in a culture model of apical SCFA treatment, supporting our data on basolateral SCFA treatment (Figure S3). Together, these data show that butyrate and propionate, but not acetate, restore esophageal barrier resistance and permeability after IL-13-induced impairment.

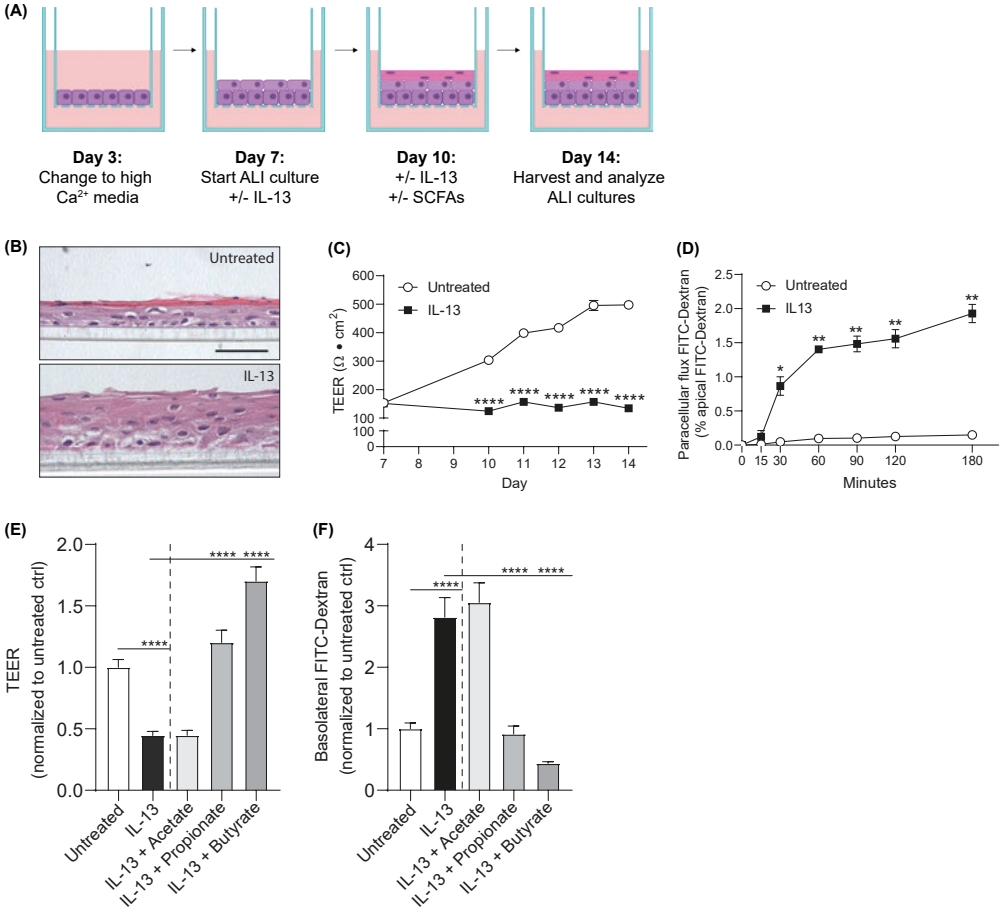

Figure 1. Butyrate and propionate restore IL-13-induced barrier dysfunction in EPC2 ALI cultures. A, Schematic diagram of the ALI culture model. Culture day 1 to 7 allows initial differentiation, and culture day 7 to 14 (ALI) induces terminal differentiation and stratification of the EPC2. EPC2 are stimulated with IL-13 (100 ng/ml) from day 7 to 14. EPC2 are treated with SCFAs acetate (10 mM), propionate (10 mM), or butyrate (5 mM) from day 10 to 14. **B**, Hematoxylin and eosin staining of EPC2 differentiated at the ALI in the absence (untreated) or presence of IL-13 (100 ng/ml). Scale bar = 50 µm. **C**, TEER development of EPC2 in the absence (untreated) or presence of IL-13 (100 ng/ml) during differentiation under ALI conditions. **D**, Kinetic FITC flux analysis of EPC2 differentiated at the ALI in the absence (untreated) or presence of IL-13 (100 ng/ml). **E** and **F**, Day 14 TEER (**E**) and FITC flux (180 min) (**F**) of IL-13-stimulated EPC2 ALI cultures treated with acetate (10 mM), propionate (10 mM) or butyrate (5 mM). Images and data in panels B-D are representative of twelve independent experiments (n = 6 wells/group). Data in panels E and F are representative of 2-8 independent experiments (n = 4 wells/group). Data are presented as mean + SEM. Asterisks represent statistical significance: *p < 0.05, ** p < 0.01, ****p < 0.0001, by one-way ANOVA followed by Dunnett's post hoc test.

2.3.2 Butyrate and propionate restored mRNA expression of key EoE genes

qPCR analysis was used to assess whether SCFAs changed mRNA expression of proinflammatory factor CCL26, protease CAPN14, and barrier proteins DSG1 and FLG. IL-13 treatment significantly increased CCL26 and CAPN14 mRNA expression, and significantly decreased DSG1 and FLG mRNA expression by day 14. This was counteracted by propionate and butyrate as they decreased the expression of CCL26 and CAPN14, while increasing the expression of FLG and DSG1 compared to IL-13-stimulated EPC2 ALI cultures (Figure 2A, B). These results correspond with the observed improved barrier function after butyrate and propionate treatment and further indicate that treatment with these SCFA have an anti-inflammatory action.

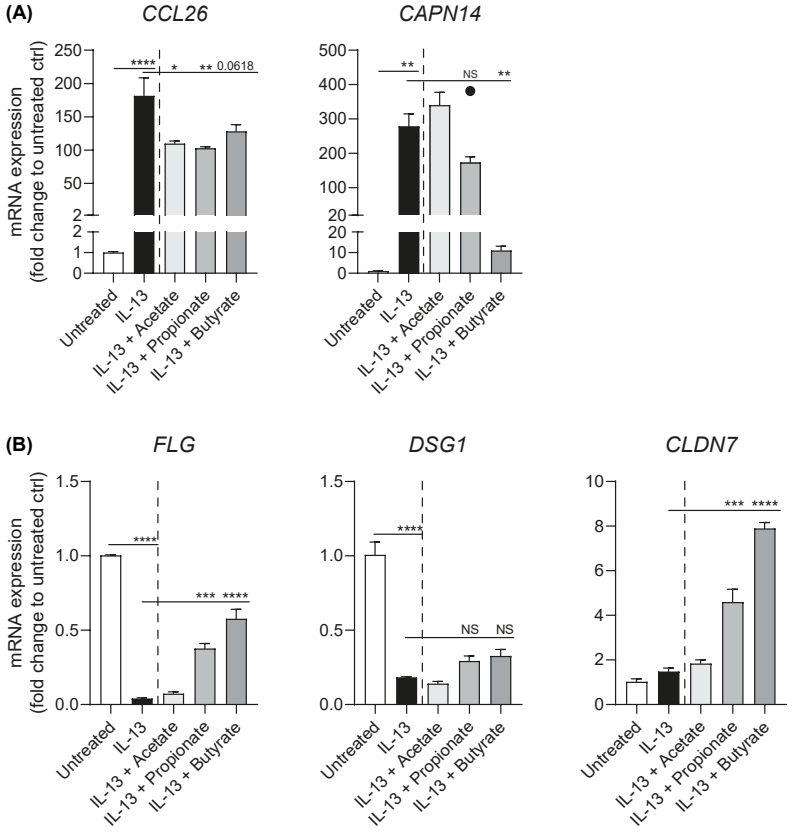

Figure 2. Butyrate and propionate restore mRNA expression of EPC2 ALI cultures. A, mRNA expression of proinflammatory factor CCL26 and protease CAPN14 in IL-13-stimulated EPC2 ALI cultures treated with acetate (10 mM), propionate (10 mM), or butyrate (5 mM). **B**, mRNA expression of esophageal barrier proteins FLG and DSG1 in IL-13-stimulated EPC2 ALI cultures treated with acetate (10 mM), propionate (10 mM), or butyrate (5 mM). Data are representative of 2-6 independent experiments (n = 3 wells/group). Outlier is shown as a separate data point but is included in the statistical analysis. Data are presented as mean + SEM. Asterisks represent statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, by one-way ANOVA followed by Dunnett's post hoc test. NS, not significant.

2.3.3 Butyrate and propionate restored DSG1 and FLG protein expression

To test the effects of SCFA treatment on esophageal barrier protein expression, we examined day 14 DSG1 and FLG expression by immunofluorescent staining. DSG1 and FLG expression was decreased in IL-13-stimulated EPC2 ALI cultures compared to untreated ALI cultures. Consistent with the mRNA expression data, butyrate, and to a lesser extent propionate, restored the expression of DSG1 and FLG in IL-13-stimulated EPC2 ALI cultures (Figure 3A). Quantification of fluorescence intensity confirms the upregulation of DSG1 and FLG in IL-13-stimulated EPC2 ALI cultures after butyrate and propionate treatment (Figure 3B, C).

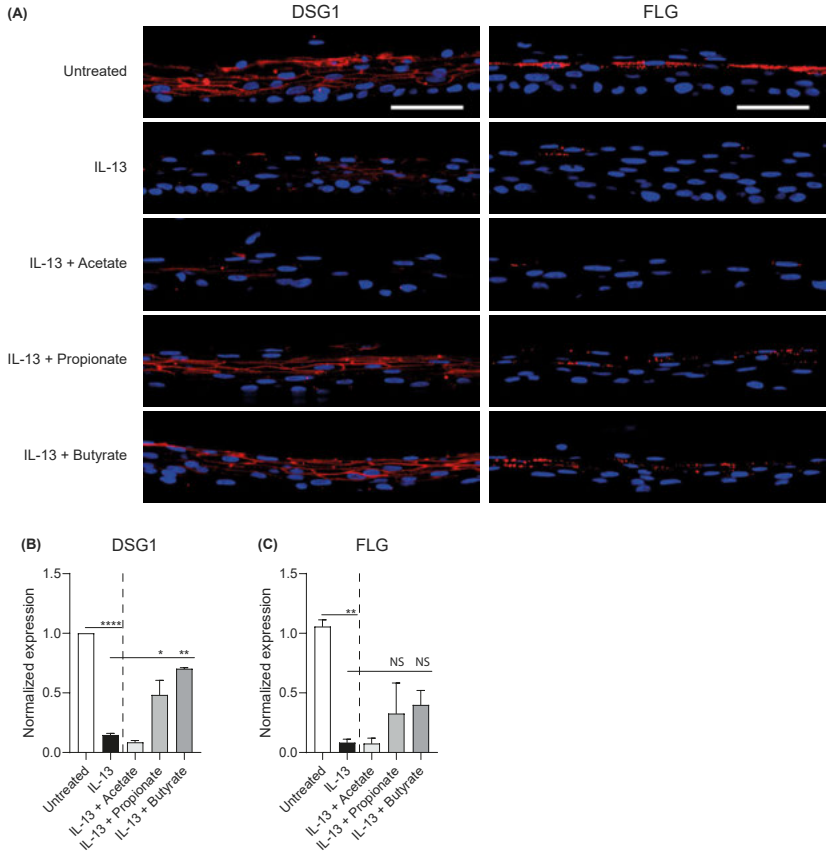

Figure 3. Butyrate and propionate upregulate DSG1 and FLG protein expression in ALI cultures of EPC2 treated with IL-13. A, Immunofluorescent staining for barrier proteins DSG1 (left) and FLG (right) in red with a blue DAPI nuclear counterstain in IL-13-stimulated EPC2 ALI cultures treated with acetate (10 mM), propionate (10 mM), or butyrate (5 mM). Scale bar =50 µm. **B** and **C**, Quantification of DSG1 (**B**) and FLG (**C**) expression in IL-13-stimulated EPC2 ALI cultures treated with SCFA. Images in panel A are representative of three independent experiments performed in duplicate or triplicate and are taken at 40x magnification. Data in panel B and C are pooled from three independent experiments performed in duplicate or triplicate and are presented as mean + SEM. Asterisks represent statistical significance: *$p < 0.05$, **$p < 0.01$; ****$p < 0.0001$, by one-way ANOVA followed by Dunnett's post hoc test. NS, not significant.

2.3.4 The barrier-restorative effects of butyrate and propionate are independent of the free fatty acid receptors GPR41, GPR43, and GPR109a

Next, we investigated whether the barrier-restorative effects of butyrate and propionate depend on signaling through the free fatty acid receptors GPR41, GPR43, and GPR109a. All three GPRs were found expressed in EPC2 on mRNA and protein level (data not shown). Direct stimulation of GPRs with specific agonists did not affect TEER (Figure 4A) nor FITC flux (Figure 4B). In line with these observations, expression of genes associated with EoE and altered by IL-13 stimulation of EPC2 grown under ALI conditions was unaffected by GPR stimulation (Figure 4C).

To confirm the ability of these agonist to stimulate GPRs and reduce inflammation, human umbilical vein endothelial cells (HUVECs) were stimulated with lipopolysaccharide (LPS) following treatment with GPR agonists. Stimulation of GPRs with specific agonists decreased LPS-induced IL-6 and IL-8 release in a concentration-dependent manner (Figure S6), confirming the ability of the GPR agonists used in this study to stimulate GPR signaling. Together, these data indicate that the barrier-restorative effects of butyrate and propionate measured by TEER, FITC flux, and mRNA and protein expression are most likely not mediated via stimulation of GPR41, GPR43 or GPR109a in EPC2.

2.3.5 HDACs may be involved in the barrier-restorative effects of butyrate and propionate

It has been demonstrated that SCFAs are also effective inhibitors of HDAC activity.[19,21,22] Since the effects of butyrate and propionate are independent of GPR signaling, we investigated if the barrier-restorative effects of SCFAs may be related to inhibition of HDAC. To study the functional effects of HDAC inhibition, TSA, a potent and specific inhibitor of HDAC activity was added to EPC2 ALI cultures. Despite the minimal effect on TEER (Figure 5A), TSA significantly decreased FITC flux in IL-13-stimulated EPC2 ALI cultures, although with a smaller impact than butyrate (Figure 5B). *CAPN14* and *FLG* mRNA expression were not affected by TSA (Figure 5C). Furthermore, butyrate and propionate treatment led to attenuated HDAC activity in EPC2 (Figure 6). These data suggest that inhibition of HDAC activity can partly mimic the restorative effects on epithelial barrier function as observed by butyrate and propionate.

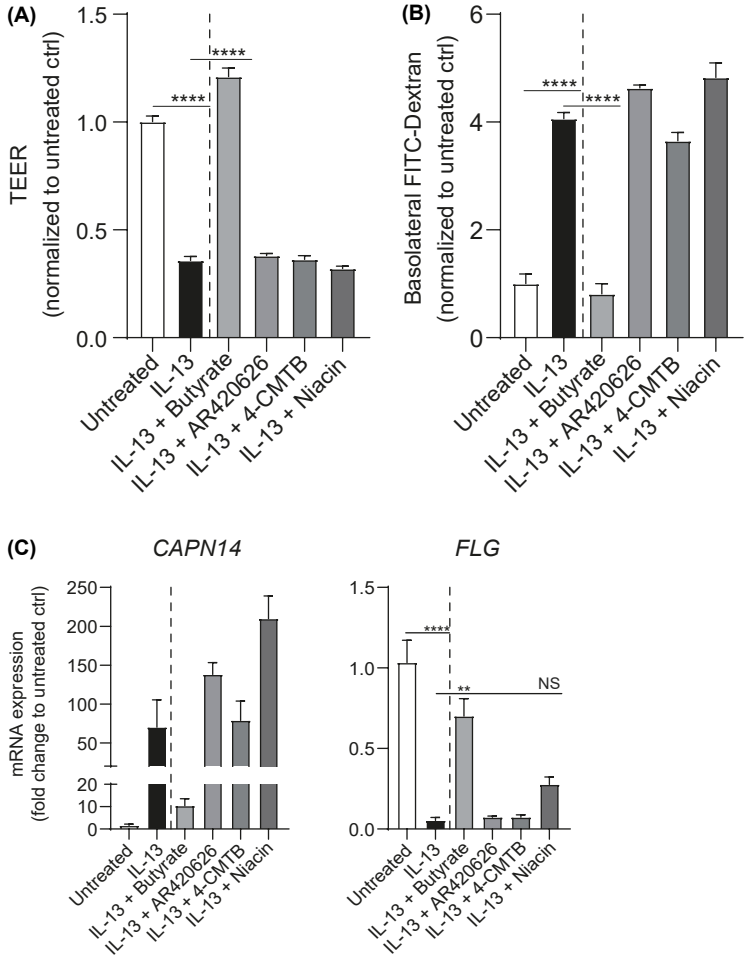

Figure 4. The effects of SCFAs are independent of GPR41, GPR43, and GPR109a stimulation. A and **B**, day 14 TEER (**A**) and FITC flux (180 min) (**B**) of IL-13-stimulated EPC2 ALI cultures treated with GPR agonists AR420626 (1 μM, GPR41), 4-CMTB (10 μM, GPR43), or niacin (10 mM, GPR109a). **C**, CAPN14 and FLG mRNA expression in IL-13-stimulated EPC2 ALI cultures treated with GPR agonists. Data are representative of two independent experiments (n = 3 wells/group) and are presented as mean + SEM. Asterisks represent statistical significance: $^{**}p < 0.01$; $^{****}p < 0.0001$, by one-way ANOVA followed by Dunnett's post hoc test. NS, not significant.

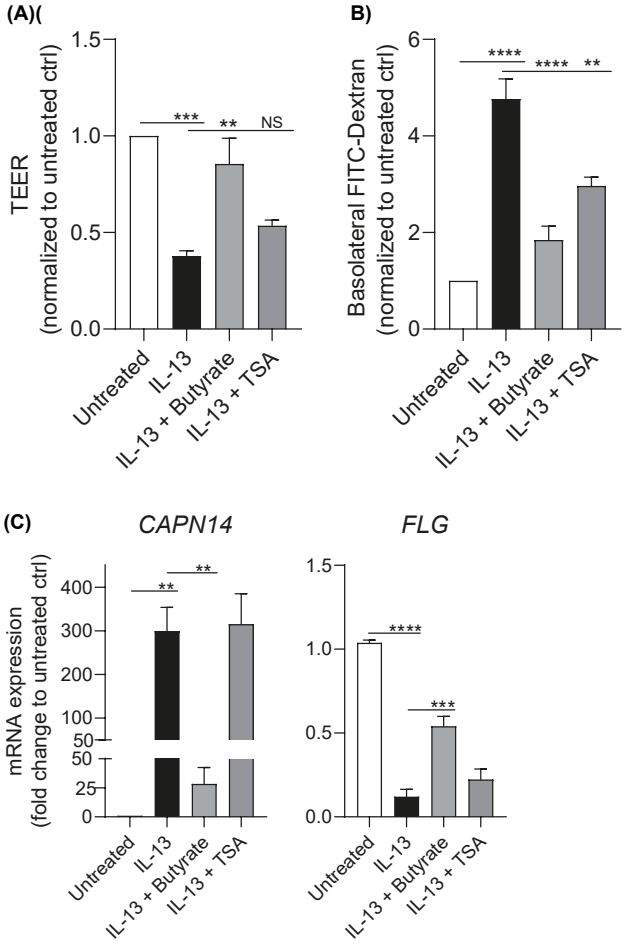

Figure 5. The HDAC inhibitor TSA partially mimics effects in EPC2 ALI cultures. A and **B**, day 14 TEER (**A**) and FITC flux (180 min) (**B**) of IL-13-stimulated EPC2 ALI cultures treated with TSA (2 μM). **C**, CAPN14 and FLG mRNA expression in IL-13-stimulated EPC2 ALI cultures treated with TSA (2 μM). Data are pooled from four independent experiments performed in duplicate, triplicate, or quadruplicate and are presented as mean + SEM. Asterisks represent statistical significance: $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA followed by Dunnett's post hoc test. NS, not significant.

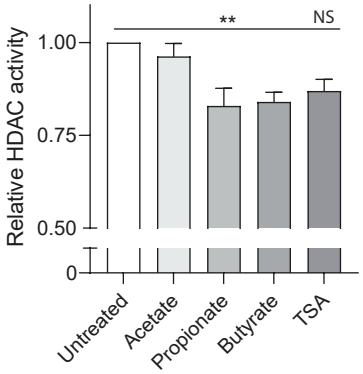

Figure 6. Butyrate and propionate decrease HDAC activity in EPC2. HDAC activity was measured in 2 ng nuclear proteins after treating confluent EPC2 for 48 h with acetate (10 mM), propionate (10 mM), butyrate (5 mM), or TSA (2 μM) and was normalized to the untreated control. Data are pooled from three independent experiments performed in triplicate, and are presented as mean + SEM. Asterisks represent statistical significance: *$p < 0.05$, by one-way ANOVA followed by Dunnett's post hoc test.

2.4 DISCUSSION

The data presented in this study demonstrate that the SCFAs butyrate and propionate, but not acetate, restore esophageal epithelial barrier function after IL-13-induced impairment using an ALI culture model resembling differentiated human esophageal epithelium. First, we demonstrate that butyrate and propionate restore epithelial barrier resistance and permeability, as assessed by TEER and FITC flux. Second, we show that butyrate and propionate restore mRNA expression of genes associated with inflammation in EoE, such as *CCL26*, and barrier function, such as *CAPN14*, *DSG1* and *FLG*. Third, we show that butyrate and propionate increase DSG1 and FLG protein expression. Fourth, our studies suggest that the barrier-restorative effects of butyrate and propionate are independent of GPR signaling, but may -in part- be dependent on inhibition of nuclear HDAC activity.

Although acetate is the most abundant SCFA in the gut and periphery, butyrate is the most potent immunomodulatory SCFA.[33] Indeed, we observed that butyrate has the highest potency to enhance esophageal barrier function after IL-13-induced impairment. Also propionate, but not acetate, significantly augmented barrier function, although with a lower activity than butyrate. Our data add to the growing body of literature linking SCFAs to immunomodulation and epithelial barrier function. Nonetheless, Wen *et al.*[34] have reported a potential proinflammatory effect of SCFAs in Th2 cell-associated responses, indicating that immunomodulatory effects of SCFAs are cell type-dependent. Our findings are consistent with effects of SCFAs on cytokine-compromised monolayers of Caco-2 and T84 human colorectal carcinoma cells and 16HBE human bronchial

epithelial cells, where butyrate enhanced barrier function and tight junction protein expression at millimolar level.[35-38] We used relatively high SCFA concentrations compared to these studies, which could be attributed to characteristics of the stratified esophageal epithelial layer that may contribute to SCFA sensitivity.

Here, we focused on the response of CAPN14 protease and esophageal barrier proteins DSG1 and FLG expression to SCFA treatment because of their suggested role in esophageal barrier function.[3,9,11,39] Expression of the epithelium-derived proinflammatory factor CCL26 was studied because of its strong correlation with disease severity.[10] The increase in TEER and decrease in FITC flux induced by butyrate and propionate was associated with a decrease in mRNA expression of *CCL26* and *CAPN14*, and an increase in mRNA and protein expression of DSG1 and FLG. CAPN14 activity is specific for esophageal tissue and its overexpression results in loss of epithelial barrier function.[11,40-42] Furthermore, previous studies have shown that DSG1 and FLG are downregulated in inflamed esophageal mucosa of EoE patients,[3,6,9] but are restored after successful therapeutical treatment and are associated with improved mucosal integrity.[43,44] Whereas DSG1 is specifically linked to EoE pathology,[3] IL-13-mediated downregulation of FLG has also been described in atopic dermatitis.[45,46] The role of other epithelial barrier proteins including claudins, occludin, involucrin, E-cadherin and keratins in maintaining esophageal epithelial integrity is less evident.[6,47] Interestingly, rather than changes in tight junction proteins, DSG1 and FLG dysregulation contributes to esophageal barrier dysfunction.[44] Current findings indicate that SCFAs can restore dysregulated expression of DSG1 and FLG leading to restoration of esophageal barrier function. In addition to IL-13, transforming growth factor (TGF) β1 and IL-9 have also been found to diminish esophageal barrier function of esophageal epithelial cells grown under ALI conditions.[39,48] Further studies characterizing the effects of SCFAs on TGF-β1 and IL-9-induced barrier dysfunction will clarify the full impact of SCFA treatment on the compromised esophageal barrier.

We considered signaling via free fatty acid receptors GPR41, GPR43 and GPR109a as a potential mechanism for the barrier-restorative effects of butyrate and propionate. AR420626, 4-CMTB and niacin, agonists for GPR41, GPR43 and GPR109a, were used to investigate if activation of these receptors could mimic the effects of SCFAs on EPC2. GPR agonists did not increase epithelial integrity as measured by TEER and FITC flux in IL-13-stimulated EPC2 ALI cultures contrasting the effects of butyrate and propionate. Also, *CAPN14* and *FLG* mRNA expression was unaffected by GPR agonists, indicating that the barrier-restorative effects of SCFAs are independent of GPR stimulation. Furthermore, as a positive control for GPR stimulation, we studied the effect of GPR agonists on LPS-induced IL-6 and IL-8 production by HUVECs, since it has been shown that this is partially mediated via GPRs.[21] We observed a dose-dependent decrease in LPS-induced IL-6 and IL-8 production, indicating that the lack of a response in EPC2 ALI cultures is not caused by biologically inactive GPR agonists but by the inability of these GPR agonists to induce SCFA-like effects. Our findings are in line with other studies demonstrating that SCFAs can exert their effects independent of free fatty acid receptors GPR41, GPR43 and GPR109a.[49-51]

Alternatively, SCFAs can directly act as nuclear HDAC inhibitors.[19,21,22] Indeed, both butyrate and propionate attenuated HDAC activity in EPC2. To further investigate if HDAC inhibition could

potentially contribute to the barrier-restorative effects of butyrate and propionate the pan-HDAC inhibitor TSA was used.[21,49,50] TSA is structurally unrelated to butyrate and propionate but is 1000 times more potent in inhibiting HDAC than these SCFAs.[52] HDAC inhibition results in histone hyperacetylation, leading to changes in chromatin structure that facilitate access for transcription factors to the promotor region of certain genes which then induces gene transcription. However, despite the overall correlation between histone acetylation and transcriptional activity, active gene transcription rather relates to the transcriptional competence of the gene than the high levels of histone acetylation.[53-55] This could explain why the effects of TSA on barrier function in EPC2 ALI cultures measured by TEER and FITC flux were modest compared to those of butyrate and propionate. Thus, the ability of these SCFAs to directly inhibit HDAC activity may only be in part involved in their barrier-restorative effects.

Nevertheless, our studies have some limitations. Exposure to air in the ALI culture is essential for terminal epithelial differentiation and stratification. EPC2 ALI cultures were therefore treated with SCFAs in the basolateral compartment, but similar high concentrations of SCFAs may be difficult to reach systemically.[56] However, our data on apical SCFA treatment suggest that SCFA exposure from the apical side of the epithelium also supports the restoration of the esophageal epithelial barrier. Interestingly, it has been shown that increased dietary fiber intake influences the esophageal microbiome, which might lead to increased local SCFA concentrations in the esophagus.[57] Furthermore, we used the immortalized human esophageal epithelial cell line EPC2-hTERT to study the effects of SCFA treatment on an IL-13-compromised barrier. It may support our study to confirm our findings in primary human esophageal epithelial cells derived from EoE patients despite the marked transcriptional and morphological overlap between IL-13-stimulated EPC2 ALI cultures and inflamed esophageal tissue.[3,6]

The esophageal epithelial barrier during active EoE is impaired and selectively permeable to food allergens that can remain in the esophageal epithelium for up to 4 days.[58] The presence and subsequent recognition of food allergens in the esophageal mucosa generates a local type 2 immune response,[59-61] forming a pathogenic cycle to further exacerbate allergic inflammation. Butyrate and propionate may break this cycle by restoring barrier function and thus preventing the penetration of food allergens into the esophageal mucosa and subsequent inflammation.

The interest in dietary therapies for EoE has recently emerged as a result of the limitations associated with other therapies, and its effectiveness in achieving and maintaining clinical remission while avoiding the need for drugs.[62] A recent meta-analysis has shown that empiric elimination diets have moderate response rates (71%), but require a large number of endoscopies, whereas the efficacy of skin allergy testing-directed food elimination is questionable (45%).[63] Interestingly, complete dietary allergen avoidance using an elemental diet is highly effective in both children and adults (90.8%),[63] and restores esophageal mucosal integrity.[44,64] It would be interesting to investigate whether a dietary intervention with SCFA formulations could restore esophageal immune fitness and improve symptoms.

In conclusion, our findings demonstrate that butyrate and propionate restore esophageal barrier function after IL-13-induced impairment, and that this is at least in part mediated by their ability to directly inhibit HDAC activity. Deeper knowledge of the mechanisms underlying the beneficial effects of butyrate and propionate could lead to novel approaches to restore esophageal barrier function. Our data highlight a potential role for butyrate and propionate in the management of EoE.

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SUPPLEMENTARY METHODS

Apical SCFA treatment of EPC2 cultures

EPC2 were cultured on semi-permeable membranes as described previously in the Material and Methods section. Following three days of differentiation under ALI conditions in the absence or presence of IL-13 (100 ng/ml), apical media was reintroduced from culture day 10 to 14 containing acetate (10 mM), propionate (10 mM) or butyrate (5 mM). Media plus IL-13 and SCFAs were refreshed every other day. TEER was measured from culture day 7 to 14 and FITC flux experiments were performed on culture day 14. Results are shown in Figure S3.

Bioactivity tests GPR agonists in human umbilical vein endothelial cells (HUVECs)

HUVECs were cultured and stimulated with SCFAs and LPS as previously described.[21] Briefly, 5×10^3 HUVECs were seeded in a 96-well plate in EGM-2 supplemented with 2% FCS and VEGF (Lonza, Basel, Switzerland). Confluent HUVECs were treated for 24 h with acetate (10 mM), butyrate (0.1 mM), propionate (0.3 mM), AR420626 (0.1-50 µM), 4-CMTB (0.1-50 µM) or niacin (0.1-20 mM), followed by LPS stimulation (1 µg/ml) for 24 h. Supernatant was then collected to measure IL-6 and IL-8 release by ELISA (Invitrogen) according to the manufacturer's instructions. Results are shown in Figure S6.

SUPPLEMENTARY FIGURES

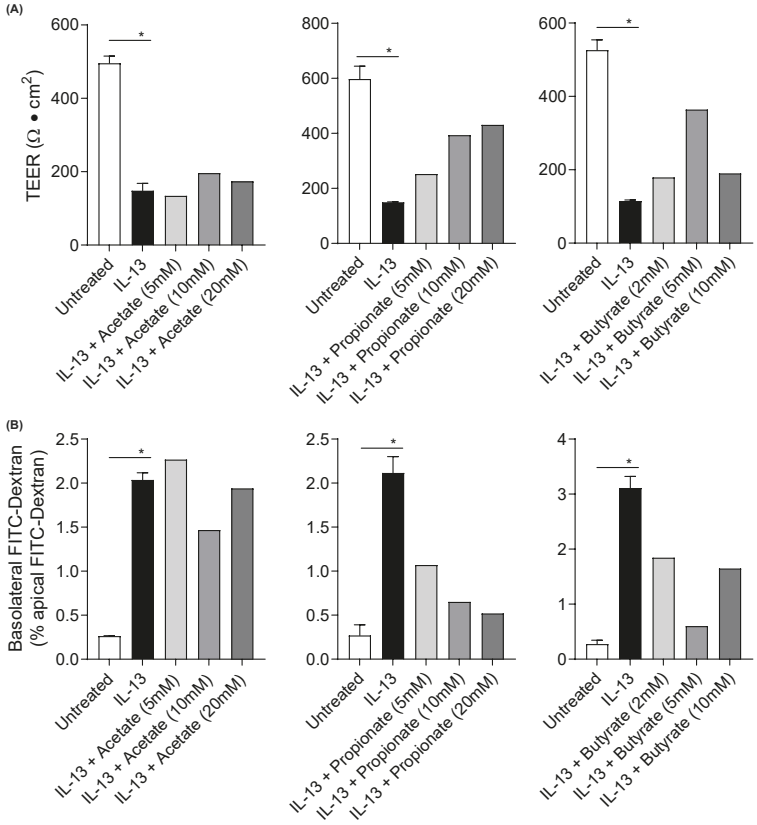

Figure S1. Effects of graded SCFA concentrations on barrier function of IL-13-stimulated EPC2 ALI cultures. Day 14 TEER (**A**) and FITC flux (180 min) (**B**) of IL-13-stimulated EPC2 ALI cultures treated with acetate (5-20 mM), propionate (5-20 mM) or butyrate (2-10 mM). Data are from one experiment performed in single or duplicate. Asterisks represent statistical significance: *$p < 0.05$, by one-way ANOVA followed by Dunnett's post hoc test

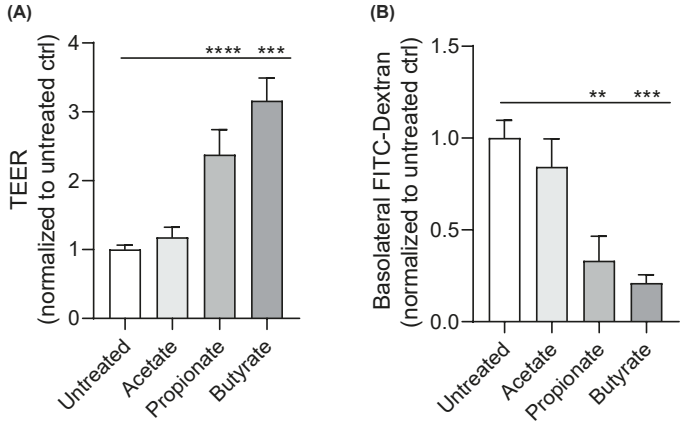

Figure S2. Butyrate and propionate treatment augment barrier function of EPC2 ALI cultures. Day 14 TEER (**A**) and FITC flux (180 min) (**B**) of EPC2 ALI cultures treated with acetate (10 mM), propionate (10 mM) or butyrate (5 mM) alone. Data are representative of two to eight independent experiments (n = 4 wells/group) and are presented as mean + SEM. Asterisks represent statistical significance: **p < 0.01, ***p < 0.001, ****p < 0.0001, by one-way ANOVA followed by Dunnett's post hoc test.

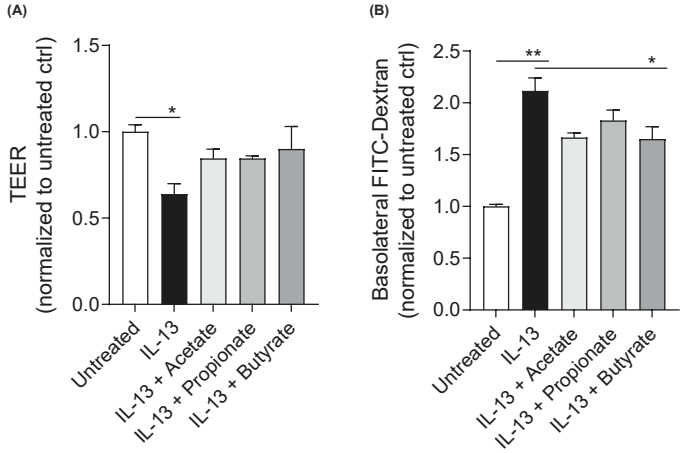

Figure S3. Apical SCFA treatment restores barrier function of IL-13-stimulated EPC2 ALI cultures. Day 14 TEER (**A**) and FITC flux (180 min) (**B**) of EPC2 cultures treated apically with acetate (10 mM), propionate (10 mM) or butyrate (5 mM) following three days of differentiation under ALI conditions in the absence or presence of IL-13. Data are from one experiment performed in duplicate and are presented as mean + SEM. Asterisks represent statistical significance: *p < 0.05, **p < 0.01, by one-way ANOVA followed by Dunnett's post hoc test.

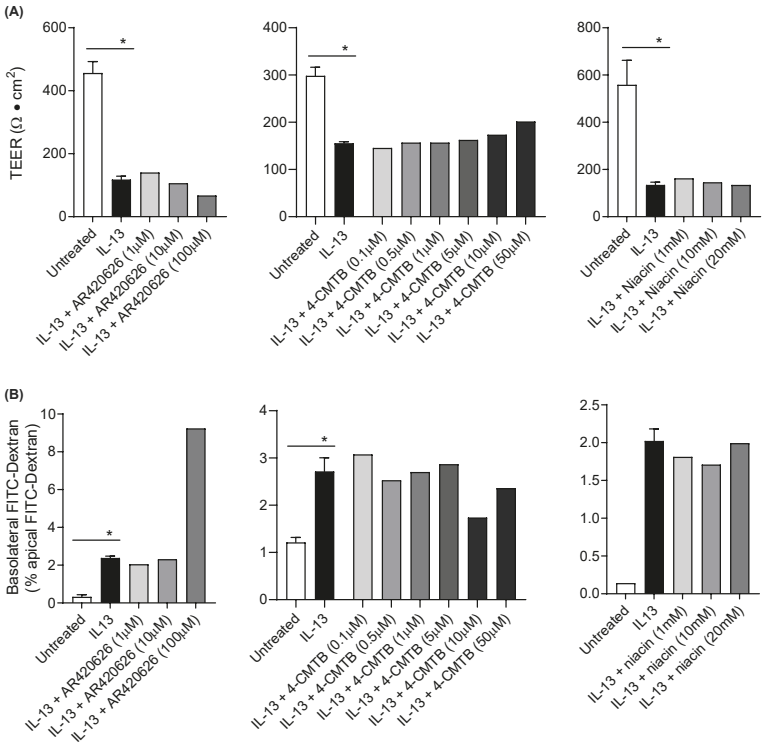

Figure S4. Effects of graded GPR agonist concentrations on barrier function of IL-13-stimulated EPC2 ALI cultures. Day 14 TEER (**A**) and FITC flux (180 min) (**B**) of IL-13-stimulated EPC2 ALI cultures treated with AR420626 (1-100 μM), 4-CMTB (0.1-50 μM) or niacin (1-20 mM). Data are from one experiment performed in single or duplicate. Asterisks represent statistical significance: *p < 0.05, by one-way ANOVA followed by Dunnett's post hoc test.

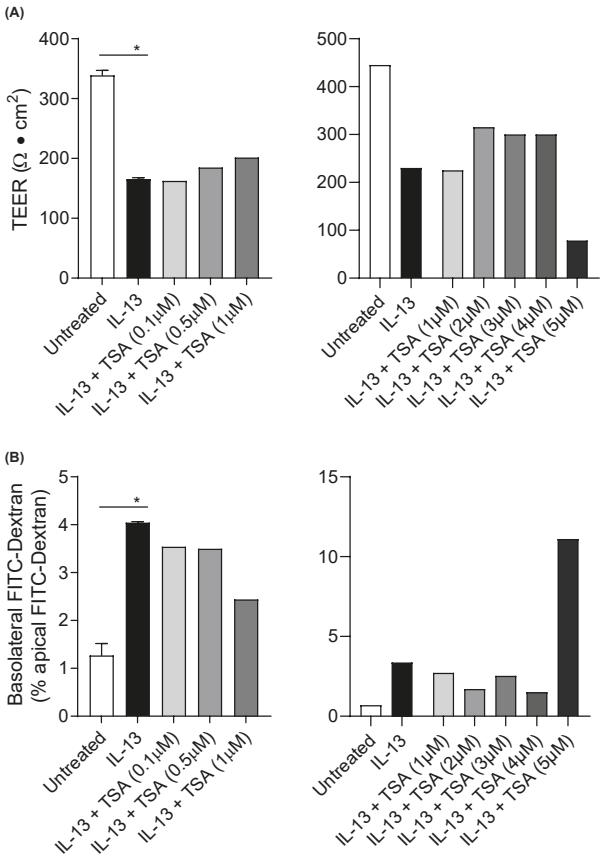

Figure S5. Effects of graded TSA concentrations on barrier function of IL-13-stimulated EPC2 ALI cultures. Day 14 TEER (**A**) and FITC flux (180 min) (**B**) of IL-13-stimulated EPC2 ALI cultures treated with TSA (0.1-5 μM). Data are from one experiment performed in single or duplicate. Asterisks represent statistical significance: *$p < 0.05$, by one-way ANOVA followed by Dunnett's multiple comparisons test.

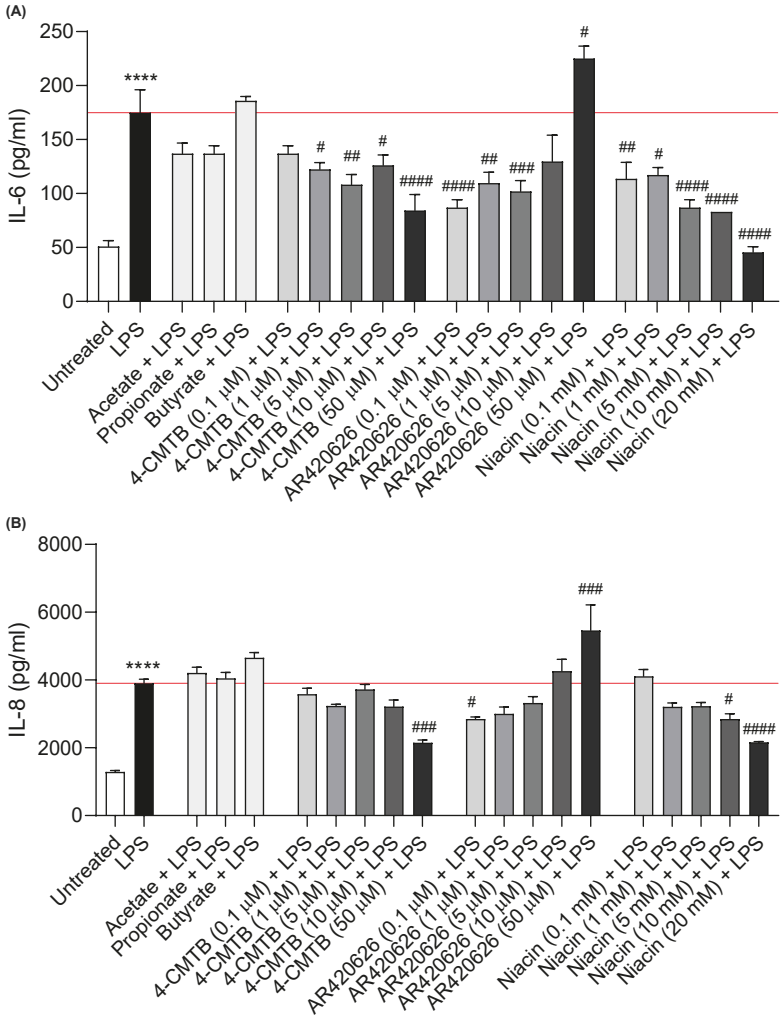

Figure S6. GPR agonists reduce LPS-induced IL-6 and IL-8 production in HUVECs in a dose-dependent manner. IL-6 (**A**) and IL-8 (**B**) release by HUVECs treated with acetate (10 mM), propionate (0.3 mM), butyrate (0.1 mM) or graded concentrations of AR420626 (0.1-50 μM), 4-CMTB (0.1-50 μM) or niacin (0.1-20 mM) before LPS stimulation (1 μg/ml). Data are from one experiment performed in triplicate and are presented as mean + SEM. Asterisks and hashtags represent statistical significance: $^{****}p < 0.0001$ compared to untreated, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ compared to LPS, by one-way ANOVA followed by Dunnett's post hoc test.

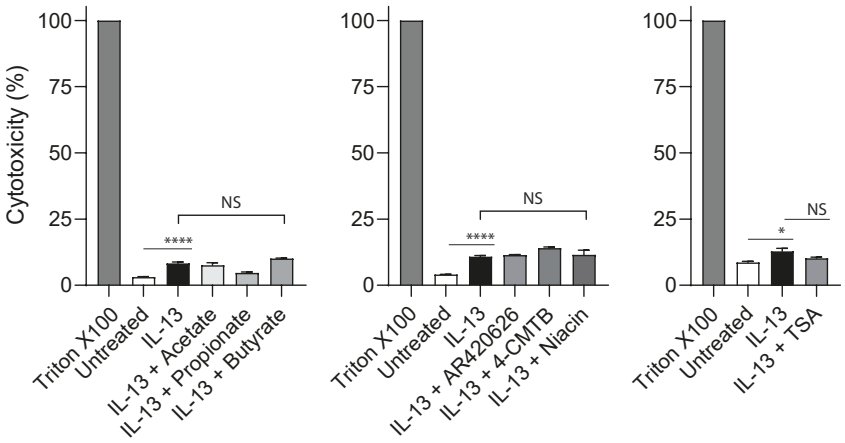

Figure S7. IL-13, SCFA, GPR agonists and TSA treatments were non-toxic. Day 14 LDH release by EPC2 ALI cultures after IL-13 (100 ng/ml), SCFA (10 mM acetate, 10 mM propionate, 5 mM butyrate), GPR agonist (1 μM AR420626, 10 μM 4-CMTB, 10 mM niacin) or TSA (2 μM) treatment. Asterisks represent statistical significance: *$p < 0.05$, ****$p < 0.0001$, by one-way ANOVA followed by Dunnett's post hoc test. NS, not significant.

SUPPLEMENTARY TABLES

Table S1. Buffers used for cytoplasmic and nuclear extract preparation.

Buffer 1	**For 10 mL**
MilliQ	6.5 mL
100 mM HEPES, pH 7.5	1 mL HEPES, pH 7.5 (= 10 mM)
30 mM $MgCL_2$	0.5 mL $MgCL_2$ (= 1.5 mM)
100 mM KCl	1 mL KCl (= 10 mM)
5% NP40	1 mL NP40 (= 0.5%)
Protease inhibitor cocktail tablets	1 tablet
Buffer 2	**For 5 mL**
RIPA Lysis and Extraction Buffer	5 mL
200 mM $MgCL_2$	25 µL $MgCl_2$ (= 1 mM)
10 KU Benzonase Nuclease	10 µL Benzonase Nuclease (= 0.25 KU)
Protease inhibitor cocktail tablets	1 tablet

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CHAPTER 3

In vivo and ex vivo inflammatory responses of the esophageal mucosa to food challenge in adults with eosinophilic esophagitis

ABSTRACT

BACKGROUND: Skin and serum IgE tests do not reliably identify foods that should be eliminated from the diets of patients with eosinophilic esophagitis (EoE).

OBJECTIVE: To study whether *in vivo* and *ex vivo* challenge of the esophageal mucosa with whole food extracts could yield clinically and immunologically relevant information about esophageal responses to specific foods.

METHODS: The esophageal mucosa of adult EoE patients (n = 12) was challenged *in vivo* by food injections and flush. Esophageal biopsies from EoE patients and controls (n = 6) were cultured *ex vivo* with or without food extracts to analyze inflammatory proteins in 24-h culture supernatants. Skin prick tests (SPT) and serum IgE tests were also conducted.

RESULTS: Acute esophageal responses (edema, erythema or smooth muscle contraction) were observed in 8/11 patients to mucosal injections, and in 4/11 patients to flush. Of the positive injections, 53% corresponded with patient's history, 35% with SPT and 35% with serum IgE results. Increased IL-5, IL-6, IL-8, IL-13, MCP-1 and total IgE levels in non-challenged biopsy culture supernatant distinguished EoE from controls. Several foods -particularly apple- induced IL-9 production in 7/10 EoE patients following *ex vivo* food challenge. A panel comprising of *ex vivo* food-induced IL-5, IL-8, MCP-1 and TNF outperformed the conventional SPT and serum IgE in distinguishing suspected from non-suspected foods as determined by machine learning.

CONCLUSION: Challenge of esophageal tissue better reflects clinical response to foods than SPT and serum IgE. Esophageal biopsy tissue culture is a functional model of EoE and could potentially be used as an *ex vivo* model for esophageal food challenge to identify causative foods and study the food-induced immune response.

KEY WORDS: Food challenge test, eosinophilic esophagitis, food allergens, intervention study

Elimination diets without the causative foods induce histological and clinical remission in patients with eosinophilic esophagitis (EoE), an allergen-driven type 2 inflammatory disease of the esophagus.[1] However, current tests using skin or serum are poorly predictive of the causative foods,[2] likely because the allergic inflammation may be restricted to the esophagus. We aimed to determine whether in vivo and ex vivo challenge of the esophageal mucosa with whole food extracts could yield clinically and immunologically relevant information about esophageal responses to specific foods.

During endoscopy, the esophageal mucosa of 12 EoE patients was challenged by local injection of three common food triggers (cow's milk, wheat, and apple) and three foods based on patient's clinical history, and by local flush (i.e., spray) of a mixture of the six foods. Acute local responses were monitored for 20 min. Skin prick tests (SPT) and serum IgE measurements were also performed. Esophageal biopsies were exposed to foods in culture to analyze inflammatory mediator production, which was compared with six non-EoE controls. Methods are fully described in the Supplementary Methods. Patient characteristics are provided in Table 1.

All patients were previously diagnosed with EoE and presented with the typical symptoms and endoscopic signs of EoE at time of endoscopy. Of the 11 patients that underwent injections, acute responses characterized by edema, erythema, or smooth muscle contraction (determined by the formation of a muscular ring) at the injection site were observed in eight patients after injection with apple (n = 4), peanut (n = 4), wheat (n = 3), milk (n = 2), tomato (n = 1), egg (n = 1), and mango (n = 1) (Table 1; Figure 1A). In addition, after the end of the endoscopy, four patients experienced dysphagia, cramping retrosternal pain or burning sensation that was similar to pain occurring after ingesting those foods. Of the in total 17 foods that induced acute responses following local injections, 9 foods (53%) corresponded with patient's clinical history, 6 (35%) with SPT results and 6 (35%) with serum IgE results. The local flush with a mixture of foods also induced acute responses but, unlike the injections, these responses were barely notable and were observed in only four patients (Table S1). Our results confirm the observations of our previous study that esophageal food challenge can trigger local responses in adult EoE patients.[3] However, there was no clear relation between foods that induced a response by mucosal injection, and SPT or serum IgE. The fact that the foods that induce mucosal responses do not necessarily show positive SPT and/or serum IgE results, and the fact that SPT and serum IgE are poorly predictive of the causative foods,[2] indicate that local esophageal challenge may indeed be needed for a better prediction of the causative foods. Nonetheless, given the moderate responsiveness to challenge by flush, the clinical challenges associated with injections, and the invasiveness for patients as endoscopic challenge can induce short lasting but severe symptoms, both challenge tests will not likely become a useful test in clinical practice.

In contrast, a less invasive biopsy-based *ex vivo* food challenge test may be considered a promising tool for the identification of causative foods in EoE patients. Non-challenged EoE esophageal biopsies maintained in culture for 24 h showed increased production of total IgE (13.7 vs. 0.1 ng/mg, $p = 0.0002$), IL-5 (12.5 vs. 1.1 pg/mg, $p = 0.0288$), IL-6 (29.8 vs. 1.5 ng/mg, $p = 0.0047$), IL-8 (86.6 vs. 23.2 ng/mg, $p = 0.0069$), IL-13 (28.6 vs. 0.0 pg/mg, $p = 0.0080$), and MCP-1 (659 vs. 112 pg/

mg, $p = 0.0320$) compared with non-challenged biopsies from controls (Figure 1B). Eotaxin, IL-9, and IFN-γ were below the detection limit. Analysis of protein levels based on peak eosinophil count did not provide additional insights (data not shown). Furthermore, when exposing biopsies to food in culture, an immunological response is triggered that may reflect the inflammatory cascade seen in EoE. Interestingly, IL-5 levels were increased after *ex vivo* exposure to milk (89.8 vs. 12.5 pg/mg, $p = 0.0195$), and IL-9 was increased after exposure to apple (132.3 vs. 0.0 pg/mg, $p = 0.0039$; Figure 1C). To our knowledge, we are the first to report food-specific induction of IL-5, an important factor in eosinophil trafficking,[4] and IL-9, a promotor of mast cell expansion and function,[5] in the inflamed esophagus of EoE patients, highlighting a potential role for both cytokines in the allergen-specific immune response in EoE.

Lastly, we used a machine learning approach[6] to study whether the *ex vivo* challenge test can better discriminate clinically suspected (as provided in Table 1) from non-suspected foods than the conventional SPT and serum IgE. Indeed, the *ex vivo* challenge test outperformed SPT/serum IgE with an area under the curve (AUC) of 0.64 vs. 0.5 (Figure S1), evidencing sufficient discriminative scores.[7] Performing food re-challenges based on the *ex vivo* results was beyond the scope of this study.

This study has limitations. Our study was conducted in a small cohort, and the tested foods were not proven by elimination diets. Extending the current study in a larger cohort of EoE patients in which causative and safe foods have been identified is needed to shed more light on the usefulness of the *ex vivo* test to identify causative foods and guide elimination diets. Furthermore, EoE is patchy in biopsies. Normalization of cytokine levels for epithelial/immune cell composition of the biopsies is therefore needed for standardization of the *ex vivo* test.

In conclusion, we demonstrated that results of food challenge using esophageal tissue provide distinct results from tests using skin and serum and may better reflect clinical response to food exposure. Esophageal biopsy tissue culture is a functional model of EoE and could potentially be used as an ex vivo model for esophageal food challenge to a) study the food-induced immune response and b) identify causative foods to guide elimination diets, and therefore warrants further validation and development.

Table 1. EoE patient characteristics and sensitization patterns.

Patient ID	Sex	Age *(y)*	Atopic comorbidity	PEC	Patient's history *Clinically suspected foods*	Selected foods *In addition to milk, wheat and apple*	Skin prick test *Positive responses*	Serum sIgE test *Positive responses (kU/L)*	Esophageal mucosal injections *Positive responses*
1	M	26	cat*, dog*, OAS *, RhC*	45	wheat, milk, apple, peanut, soy, tomato,	soy, peanut, tomato	wheat, soy, peanut, apple	wheat (0.44), apple (6.54), soy (0.66), peanut (1.16)	moderate narrowing/edema **tomato**/**peanut**
2	F	21	-	24	milk, apple, hazelnut, cashew, galia melon	cashew, hazelnut, galia melon	-	-	-
3	M	48	-	5	wheat, apple, chicken	soy, peanut, chicken	wheat	milk (1.48), wheat (0.49)	moderate edema/rings **apple**/ peanut, questionable milk
4	M	52	-	100	wheat, apple, milk, orange, beer	soy, orange, beer	milk, beer	milk (2.93)	moderate edema **wheat**, questionable **apple**
5	M	44	RhC*	50	-	soy, peanut, tomato**	-	-	-
6	M	48	OAS*, RhC*	52	wheat, apple, beer	soy, peanut, beer	peanut, soy, wheat, milk	peanut (0.72), malt (0.42)	strong response **peanut**/**beer,** moderate **wheat**
7	M	37	hives*, OAS*, RhC*	14	-	soy, peanut, egg	inconclusive due to hives	wheat (0.55), apple (0.66), peanut (1.11)	-
8	M	26	OAS*, RhC*	30	wheat, apple, grape, tomato, mango	mango, grape, tomato	grape, mango, wheat, apple, tomato	-	moderate edema **mango**, questionable edema milk
9	F	28	OAS*, RhC*	NA	wheat, milk, apple, banana	soy, peanut, banana	wheat, banana	-	not performed***
10	F	22	RhC*	50	wheat, milk, egg	soy, peanut, egg	milk, egg	milk (2.85), wheat (0.40), egg (0.66)	moderate edema apple
11	M	41	asthma, OAS*, RhC*	20	milk	soy, peanut, egg	apple, soy, milk, wheat	milk (3.08), apple (3.11)	contractile, muscular ring: apple, wheat and egg
12	M	30	asthma, OAS*, RhC*	12	milk, peanut, wheat	soy, peanut egg	milk, soy, wheat	milk (2.38), wheat (1.27), apple (0.59), soy (0.84), peanut (0.85), egg (0.64)	questionable **peanut**

*SPT and/or serum sIgE proven; ** Tomato instead of egg was used because egg extract was not available; *** Not performed because the patient withdrew consent for the endoscopy. Foods presented in bold correspond with patient's history. Abbreviations: -, none; F, female; M, male; NA, not available; OAS, oral allergy syndrome; PEC, peak eosinophil count; RhC, rhinoconjunctivitis; sIgE, allergen-specific IgE.

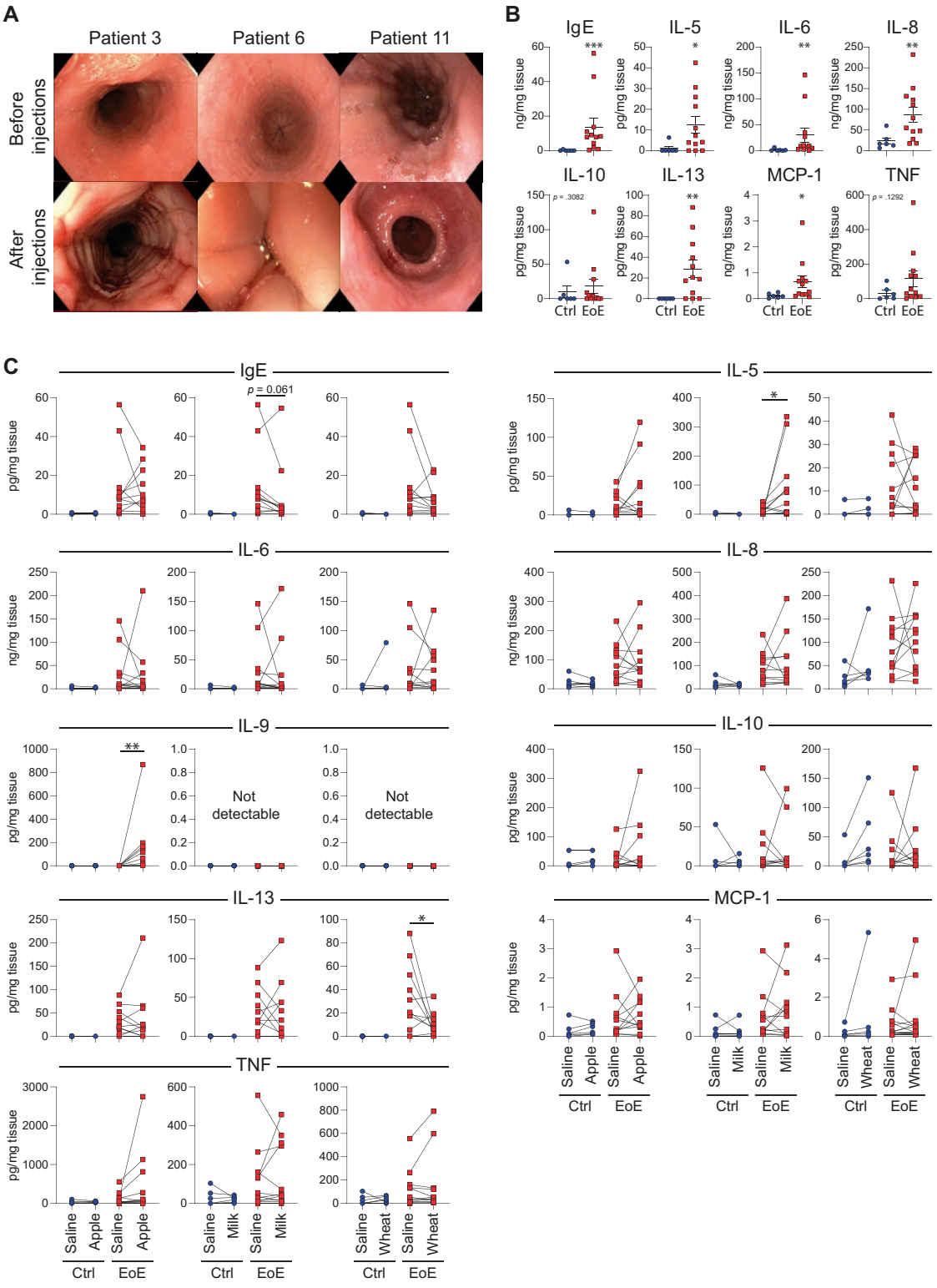
A
Patient 3
Patient 6
Patient 11
Before injections
After injections
B
IgE
IL-5
IL-6
IL-8
IL-10
IL-13
MCP-1
TNF
ng/mg tissue
pg/mg tissue
p = .3082
p = .1292
Ctrl
EoE
C
IgE
p = 0.061
IL-5
IL-6
IL-8
IL-9
IL-10
IL-13
MCP-1
TNF
pg/mg tissue
ng/mg tissue
Not detectable
Not detectable
Saline
Apple
Milk
Wheat
Ctrl
EoE

Figure 1. In vivo and ex vivo responses to food challenge. **A**, Acute responses to mucosal food injections. Patient 3 showed increased edema and more visible rings and furrows after injection of apple and peanut. Patient 6 showed increased edema and erythema after injection of wheat. Patient 11 showed a contractile muscular ring after injection of apple, wheat and egg. **B,** Inflammatory protein levels in culture supernatant of non-challenged esophageal biopsies from EoE patients (EoE, n = 12) and controls (Ctrl, n = 6) cultured for 24 h. Asterisks represent statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, by Mann–Whitney test. **C,** Inflammatory protein levels in culture supernatant of esophageal biopsies from EoE patients (EoE, n = 12) and controls (Ctrl, n = 6) exposed to saline (negative control) or the common EoE triggers apple, cow's milk or wheat extract for 24 h. Asterisks represent statistical significance: *p < 0.05, **p < 0.01, by Wilcoxon matched-pairs signed rank test.

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SUPPLEMENTARY METHODS

Study subjects

In this prospective intervention study, adult EoE patients (aged 18-75 years) were included from the outpatient clinic of the Amsterdam University Medical Center (UMC), location AMC, between August 2019 and 2021. Adults were eligible for enrollment if EoE was previously diagnosed according to current guidelines, defined as the presence of >15 eosinophils per high-power field and typical symptoms of EoE (e.g. dysphagia and food impaction). Exclusion criteria were: (i) inability to stop topical corticosteroids, beta-blockers or ACE inhibitors, (ii) use of oral or systemic antihistamines, oral cromoglicates, systemic corticosteroids, leukotriene inhibitors or monoclonal antibodies in the month preceding the study, (iii) proven gastroesophageal reflux disease or other cause for esophageal eosinophilia, (iv) history of peptic ulcer disease, Barrett's esophagus or gastrointestinal cancer, (v) severe comorbidity scored as ASA class III, VI or V, and (vi) history of anaphylaxis or a severe systemic reaction to previous allergy tests (grade 3 or 4). For the *ex vivo* challenge test, adult subjects who underwent endoscopy for other reasons than esophageal complaints were included as a control. These controls were approached through their treating physician and did not suffer from any known atopic comorbidity. Signed informed consent to participate in the study was obtained from patients and controls. The study protocol was approved by the Medical Ethics Committee of the Amsterdam UMC and registered in the International Clinical Trial Registry Platform (trialsearch.who.int, ID: NL7781).

Study design

Consented patients were not allowed to use any immunosuppressive drugs during the trial. Before the initial endoscopy, serum was collected for allergen-specific IgE measurements (cut-off: > 0.35 kU/L; ImmunoCAP, ThermoFisher Scientific), and SPTs (cut-off: wheal diameter ≥ 3 mm) were performed for the same foods that were tested by the esophageal challenge tests.

During endoscopy, the esophageal mucosa of EoE patients was challenged with foods in two different ways: *in vivo* mucosal injections or *in vivo* mucosal flush. Patients underwent these endoscopies 6 weeks apart in randomized order. Endoscopies were performed under mild or deep sedation, and heart rates, blood pressure and oxygen saturations were monitored during the entire procedure. Before the start of each in vivo challenge test, baseline biopsies were taken to evaluate histologic disease activity (3 biopsies) or for *ex vivo* food challenge. These areas were not exposed to foods during subsequent in vivo challenge tests. A maximum of six foods could be tested in each patient due to the limited available esophageal area. Three foods were selected based on the most prevalent sensitizations in EoE: cow's milk, wheat, and apple.[1] In addition, all patients reported clinically suspected foods of which the three most suspected were included in this study. These suspected foods were identified by the patients as triggers for their typical esophageal symptoms after consumption. If the patient's history did not reveal any, then soy, peanut and egg, which are other common causative foods,[2,3] were selected.

In vivo mucosal challenge by injections

Food extracts were prepared from fresh foods as previously described,[4] because the sensitivity of allergy tests decrease significantly when commercial food extracts are used. During endoscopy, 0.2 ml of six food extracts (1 mg/ml) and a negative control (0.9% NaCl, saline) were injected into the esophageal mucosa using a sclerotherapy needle through the endoscope. Compared to our previous study,[4] we used more concentrated solutions (1 mg/ml instead of 0.3 mg/ml) since no systemic reactions occurred previously, and we wanted to increase the sensitivity to reduce potential false-negative results. The injections were done in a pre-specified order at the 3 and 9 o'clock and 6 and 12 o'clock positions at 3 cm distance in axial length. Acute local responses were monitored for up to 20 min and were captured on still images and video. Endoscopic signs were scored blindly.

In vivo mucosal challenge by flush

Because mucosal injections are rather invasive, we also studied the potential of the less invasive mucosal flush to trigger acute responses, in which a food mixture was sprayed on the esophageal mucosa during upper endoscopy. The selection of foods for the mucosal flush was the same as used for mucosal injections. However, instead of individual food extracts, a mixture of homogenized fresh foods was used to increase allergenicity. Fresh foods were homogenized (for solids), mixed, and diluted with saline until the consistency of the mixture was suitable to be flushed through the working channel of the endoscope. Between 50-100 ml of this mixture was sprayed on the esophageal mucosa until the entire mucosa of the most distal 15 cm of the esophagus had been flushed. Acute local responses were monitored for up to 20 min and were captured on still images and video. Endoscopic signs were scored blindly.

Ex vivo challenge of esophageal biopsies

Biopsies were directly collected into ice-chilled Gibco™ Roswell Park Memorial Institute (Thermo Fisher Scientific, Waltham, MA, USA) 1640 medium without phenol red supplemented with 1% fetal calve serum, penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml) (culture medium), and were transported on ice to the laboratory of Utrecht University within 80 min for *ex vivo* experiments. Briefly, biopsies were cut into two equal parts, placed in 100 µl culture medium in a 96 wells cell culture plate (Greiner bio-one, Kremsmünster, Austria) and incubated for 30 min in a humidified CO_2 incubator at 37 °C on a rocker (30 rpm). Next, supernatants were discarded and 100 µl fresh culture medium was added. Biopsies were cultured with 50 µL food extract (1 mg/ml) or 0.9% NaCl (saline; B. Braun, Melsungen, Germany). The selection and preparation of food extracts was the same as used for mucosal allergen injections. For non-EoE controls, the following food extracts were used: apple, cow's milk, wheat, soy, peanut and egg. Supernatants were collected 24 h after challenge and stored at -80 °C until further analysis. Biopsies were then transferred to homogenization tubes (VWR, Radnor, PA, USA) and incubated in 200 µl ice-chilled lysis buffer (RIPA lysis and extraction buffer supplemented with 1 mM $MgCl_2$, 0.25 KU Benzonase Nuclease and protease inhibitors) for 30 min on ice with regular vortexing. Biopsies were homogenized using Precellys® homogenisator (VWR) and lysates were collected by centrifugation (10 min; 14,000 rpm; 4 °C) and stored at -80 °C. Total protein content of the lysates was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). An

inflammatory protein panel consisting of eotaxin (detecting eotaxins 1-3), interferon (IFN) γ, total IgE, interleukin (IL) 5, IL-6, IL-8, IL-9, IL-10, IL-13, monocyte chemoattractant protein (MCP) 1 and tumor necrosis factor (TNF) was measured in 24-h biopsy culture supernatants using Cytometric Bead Array (CBA; BD Biosciences, Franklin Lakes, NJ, USA) on BD FACSCanto™ II (BD Biosciences) per manufacturer's instructions. Protein levels were quantified using Flow Cytometric Analysis Program Array™ software (BD Biosciences) and corrected for total protein content of the lysates.

Statistical analysis

Statistical analyses on clinical data were performed using IBM SPSS Statistics version 26. Descriptive statistics were used to assess clinical characteristics. Normally distributed variables were described as means and standard deviation. Non-normally distributed variables were described as medians and interquartile range (IQR). Categorical variables were expressed as absolute (n) and relative (%) frequencies. Statistical analysis of *ex vivo* data were performed using GraphPad Prism v 9.4.1 (GraphPad Software Inc., La Jolla, CA, USA). Mann-Whitney test or Wilcoxon matched-pairs signed rank test was used, as appropriate. Results were considered statistically significant if $p < 0.05$.

Machine learning-based recursive ensemble feature selection

A previously established machine learning-based recursive ensemble feature selection (REFS)[5,6] was used to assess the potential of the ex vivo challenge test to better predict suspected and non-suspected foods than the conventional SPT and serum IgE. This ensemble overcomes the bias of using a single machine learning algorithm, thus allowing for a robust selection of features for classification. REFS was used on two datasets: (1) SPT + serum IgE results, and (2) ex vivo food-induced inflammatory protein levels. In each dataset, REFS determined the most important feature(s) to achieve robust food classification. Patients were excluded from the analysis if there was no non-suspected food tested (n = 1) or if suspected foods were unknown (n = 2), yielding n = 9 EoE patients for further analysis.

Lopez-Rincon *et al.*[6] described the ensemble ranking process in detail. Briefly, REFS uses 8 classification algorithms: Stochastic Gradient Descent, Support Vector Machine Classifier, Gradient Boosting, Random Forest, Logistic Regression, Passive Aggressive Classifier, Ridge Classifier and Bagging. Each algorithm was run in 10-fold using nested cross-validation to score features on their importance for classifying a food as suspected or non-suspected. The ranking of each feature was based on how often it appeared within the top classifying features. Having determined the panel of features that allows for most robust classification in each of 2 datasets, five other classification algorithms (Ada Boost Classifier, Extra Trees Classifier, K-Nearest Neighbors Classifier, Lasso CV, Multi-Layer Perception Classifier) were run to validate the (panel of) feature(s) – to avoid overfitting – and generate receiver operating characteristic (ROC) curves.

SUPPLEMENTARY TABLE

Table S1. Acute responses to esophageal mucosal flush.

Patient ID	PEC	Esophageal mucosal flush
1	0	no response
2	39	no response
3	43	no response
4	100	no response
5	55	no response
6	4	increased edema flushed area, furrows more visible
7	5	no response
8	65	no response
9	90	possible increased edema flushed area
10	48	possible increased edema flushed area
11	NA	not performed*
12	52	possible increased edema flushed area

*Not performed because the patient withdrew consent for the endoscopy.

Abbreviations: NA, not available; PEC, peak eosinophil count

SUPPLEMENTARY FIGURE

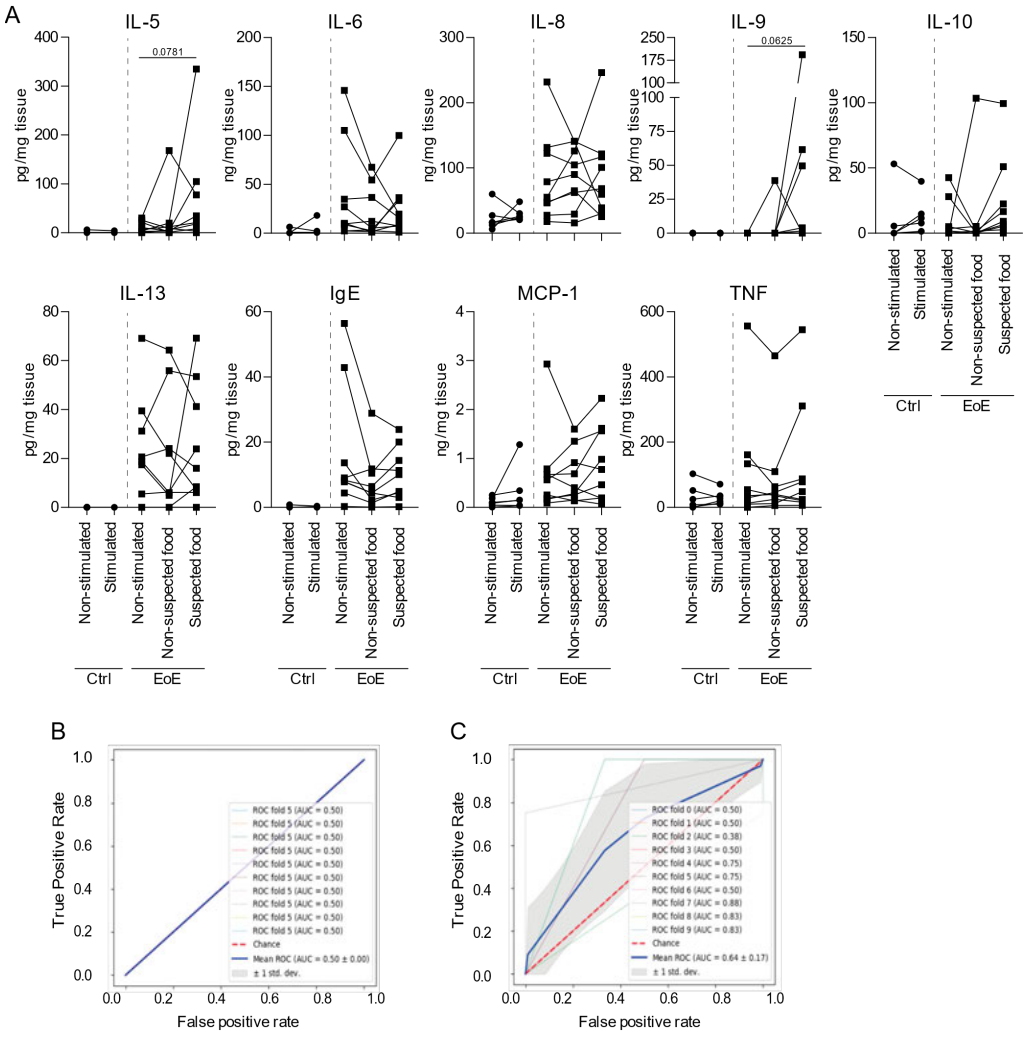

Figure S1. Univariate and multivariate analysis of the cytokine response to ex vivo food exposure. A, Inflammatory protein levels in culture supernatant of esophageal biopsies from EoE patients (EoE, n=9) and controls (Ctrl, n=6) at baseline (non-stimulated) or stimulated with food extracts. For the EoE patients, the 6 tested foods were classified as non-suspected food or suspected food based on the patient's clinical history of foods that trigger their typical esophageal symptoms after consumption. Averages of stimulated (for Ctrl), clinically non-suspected, and suspected foods (for EoE) are plotted, resulting in one data point per condition per control/patient. Wilcoxon matched-pairs signed rank test was used. **B-C,** Machine learning-based recursive ensemble feature selection[5,6] was used on datasets from the ex vivo challenge test and SPT/serum IgE results to assess cytokines or tests to predict whether a food is suspected or non-suspected. Receiver operating characteristic curves from multi-layer perception classifier to separate suspected from non-suspected foods based on the SPT/serum IgE (feature identified by REFS: SPT) (**B**) or ex vivo challenge test (features identified by REFS: IL-5, IL-8, MCP-1, TNF) (**C**) are shown.

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CHAPTER 4

Transcriptomic profiling of the acute mucosal response to local food injections in adults with eosinophilic esophagitis

ABSTRACT

BACKGROUND: Exposure of the esophageal mucosa to food allergens can cause acute mucosal responses in patients with eosinophilic esophagitis (EoE), but the underlying local immune mechanisms driving these acute responses are not well understood.

OBJECTIVE: To gain insight into the early transcriptomic changes that occur during an acute mucosal response to food allergens in EoE.

METHODS: Bulk RNA-sequencing was performed on esophageal biopsies from adult EoE patients (n = 5) collected before and 20 minutes after local challenge by esophageal injection of various food extracts. Baseline biopsies from non-EoE controls (n = 5) were also included.

RESULTS: At baseline, the transcriptome of the EoE patients showed increased expression of genes related to an EoE signature. After local food injection, we identified 40 genes with a potential role in the early immune response to food allergens (most notably *CEBPB*, *IL1B*, *TNFSF18*, *PHLDA2*, and *SLC15A3*). These 40 genes were enriched in processes related to immune activation, such as the acute phase response, cellular responses to external stimuli, and cell population proliferation. *TNFSF18* (also called GITRL), a member of the TNF superfamily that is best studied for its co-stimulatory effect on T cells, was the most dysregulated early EoE gene showing a 12-fold increase compared with baseline and an 18-fold increase compared with a negative visual response. Further experiments showed that the esophageal epithelium may be an important source of TNFSF18 in EoE, which was rapidly induced by stimulating differentiated esophageal epithelial cells with the key EoE cytokine interleukin-13.

CONCLUSION: Our data provide unprecedented insight into the transcriptomic changes that mediate the acute mucosal immune response to food allergens in EoE, and implicate TNFSF18 as an important effector molecule in this response. As such, the TNFSF18 pathway may become a new therapeutic target for EoE.

KEYWORDS: acute response, eosinophilic esophagitis, esophagus, food challenge, GITRL, RNA-sequencing, TNFSF18.

4.1 INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic, allergen-driven disorder of the esophagus characterized by the infiltration of eosinophils in the esophageal mucosa and symptoms related to esophageal dysfunction.[1] The prevalence of EoE is approximately 1 in 3000, with a male-to-female ratio of 3:1.[2] Food allergens play an important role in the pathogenesis of EoE, as demonstrated by endoscopic and clinical resolution of EoE once the causative food is removed from the diet, and exacerbation when the same food is reintroduced.[3] Similarly, amino acid-based elemental diets are effective in both adults and children with EoE.[4-8] Type 2 inflammation represents an important subset of the relevant immune pathways activated during EoE. This is supported by studies that show local expression of cytokines such as interleukin (IL)-4, IL-5 and IL-13,[9] increased numbers of esophageal T helper 2 cells, mast cells, eosinophils, basophils, B cells and group 2 innate lymphoid cells (ILC2s),[10-14] and an association of EoE with other atopic disorders.[15,16] However, the exact mechanism by which food allergens can initiate inflammation in EoE is still unknown, as there is limited data available on the early local esophageal immune response after challenge with a specific food trigger.

Previous studies have provided insight into transcriptional changes associated with active EoE.[17-19] The EoE transcriptome is enriched in genes functionally involved in eosinophilia, immunity and atopy.[19] The IL-13-induced gene chemokine (C-C motif) ligand 26 (CCL26, encodes eotaxin-3) is the most upregulated gene in EoE patients compared with controls (279-fold) and strongly correlates with disease severity.[17] Other highly induced genes include the extracellular matrix protein periostin (POSTN), protease calpain-14 (CAPN14), leucine-rich repeat-containing protein 31 (LRRC31) and the calcium-activated chloride channel anoctamin-1 (ANO1).[17,20-22] Downregulated genes in EoE are related to epithelial homeostasis,[17] such as the desmosome desmoglein-1 (DSG1).[23] Furthermore, long non-coding RNAs, a type of RNA that are not translated into protein, have been shown to play a role in EoE pathophysiology and may help in diagnosis and monitoring disease activity.[19]

However, little emphasis has been placed on characterizing genes that mediate the acute esophageal immune response to food allergens. Recently, we challenged the esophageal mucosa of adult EoE patients by local food injections during upper endoscopy, and monitored acute mucosal responses for 20 min.[24] Local challenge of the esophageal mucosa induced acute responses, such as edema, erythema and smooth muscle contraction, in various degrees of severity in EoE patients. The fact that these food-induced acute esophageal responses could be responsible for painful esophageal symptoms and potentially exacerbate esophageal inflammation, stresses the need for better understanding the cellular and molecular processes mediating such reactions. This was also stressed in a recent paper describing these symptoms as food-induced immediate response of the esophagus (FIRE).[25] Therefore, in this study, we aimed to gain insight into the early transcriptomic changes that occur during an acute mucosal response to food allergens in EoE. For this purpose, we performed bulk RNA-sequencing (RNA-seq) on esophageal biopsies collected before and 20 min after local challenge by esophageal food injections in adult EoE patients.

4.2 METHODS

4.2.1 Study design and sample collection

Detailed methods on study subjects and design were previously described.[24] Briefly, adult patients (18-75 y) with previously diagnosed EoE (i.e., ≥ 15 eos/hpf and clinical signs of esophageal dysfunction) were included from the outpatient clinic of the Amsterdam UMC between August 2019 and 2021. In addition, adult subjects who underwent endoscopy for other reasons than esophageal complaints were included as controls. This study was approved by the Medical Ethics Committee of the Amsterdam UMC, and all subjects provided written informed consent (trialsearch.who.int, ID: NL7781).

During endoscopy, the esophageal mucosa of EoE patients was locally challenged by mucosal injections with six different foods and a negative control (0.9% NaCl). Three foods were selected based on the most prevalent sensitizations in EoE (cow's milk, wheat and apple),[26] and another three foods were included based on patient's history of clinically suspected foods. The injections were done in a pre-specified order at 3 cm intervals in axial length, alternating at the 3 and 9 o'clock and 6 and 12 o'clock positions. Acute local visual responses were monitored by endoscopy for up to 20 min.[24] Baseline biopsies were collected prior to the injections and 20 min after the injections, biopsies were collected from each of the seven injection sites. These biopsies and baseline biopsies from five non-EoE controls were collected in RNAlater (Qiagen, Hilden, Germany) and stored at -80 °C until further use.

Biopsies from five EoE patients were used for RNA-seq. From each of the five patients, we included one baseline biopsy, one biopsy from a positive visual response to food injection, and one biopsy from a negative visual response to food injection, totaling 15 samples (3 biopsies × 5 patients). In this way, each patient served as its own control. If a patient had positive visual responses to multiple injections with different food extracts, the most severe response was used for the analysis. Biopsies from a negative visual response to injections were obtained at most distant to sites with a positive visual response to prevent possible interference.

4.2.2 Sample library preparation, RNA sequencing, and data analysis

Esophageal biopsies stored in RNAlater at -80 °C were homogenized in 600 µl RLT buffer (Qiagen) + 1% β-mercaptoethanol using the Precellys homogenisator (VWR). Homogenates were centrifuged (2 min; 14,000 rpm; RT) and DNA, RNA and protein was extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) per manufacturer's instructions. DNA, RNA and protein were stored at -80 °C until further use.

Sample quality control measures were provided by Novogene (Novogene, Beijing, China), and libraries were constructed from samples of acceptable quality using the Novogene NGS RNA Library Prep Set (PT042). Library quantification was performed using Qubit and real-time PCR, and size distribution selection was performed using the Bioanalyzer system. Quantified libraries were sequenced on Illumina NovaSeq6000 (sequencing strategy PE150) at the Novogene sequencing lab in Cambridge, UK, and paired-end reads were generated. For quality control of the raw data,

raw reads of FASTQ format were first processed through in-house Perl scripts to obtain clean reads. Reads containing adapter, reads containing ploy-N and low-quality reads were removed from the raw data. In addition, Q20, Q30 and GC content of the clean data were calculated. All downstream analyses were based on the high-quality clean data. Paired-end clean reads were aligned against the GRCh38 human reference genome using Hisat2 v 2.0.5. FeatureCounts v1.5.0-p3 was used to generate read counts mapped to each gene. Read counts data were analyzed using iDEP v 0.96 (available at http://ge-lab.org/idep/).[27] The expression threshold for downstream analysis was set at a minimum of 1 count per million (CPM) in at least two samples to remove low abundance genes, and counts data were transformed using EdgeR:[28] log2(CPM + c), where constant "c" = 4. Differentially expressed genes (DEG) were identified by DESeq2.[29] Fold changes (FC) were assessed, and *p* values were corrected for multiple testing using false discovery rate (FDR), generating adjusted *p* values. Genes were considered differentially expressed if FC > 1.5 and FDR < 0.05. For the analysis of food injections, DESeq2 ran paired tests by using the following statistical model: *Gene expression ~ Response + Patient ID*, where "Response" (baseline, negative, positive) is the factor variable, and "Patient ID" is the fixed factor to pair samples.

Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of specific gene clusters were performed using ShinyGO v 0.76.1 (available at http://ge-lab.org/go/) with the full set of expressed genes as background.[30] Enriched GO terms or KEGG pathways were considered significant if FDR < 0.05.

4.2.3 Cell culture

The immortalized human esophageal epithelial cell line EPC2-hTERT[31-33] (EPC2; provided by Dr. Anil Rustgi, University of Pennsylvania, PA, USA) was cultured in low-calcium ([Ca^{2+}] = 0.09 mM) keratinocyte serum-free media (KSFM; Gibco, Waltham, MA, USA) supplemented with bovine pituitary extract (50 µg/ml), epidermal growth factor (1 ng/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cultures were tested every month for mycoplasma contamination.

For the air-liquid interface (ALI) culture system, EPC2 were grown to confluence in low-calcium KSFM on permeable inserts with 0.4 µm pores (Corning Incorporated, Corning, NY, USA). Confluent monolayers were switched to high-calcium ([Ca^{2+}] = 1.8 mM) KSFM for an additional five days. Epithelial differentiation and stratification were induced by removing media from the apical compartment and exposing the EPC2 to air. Five days after the start of ALI culture, differentiated EPC2 were exposed to IL-13 (100 ng/ml) in the basolateral compartment. At 0, 1, 6 and 24 h after IL-13 stimulation, EPC2 ALI cultures were lysed in 350 µL RLT buffer (Qiagen) + 1% β-mercaptoethanol for RNA isolation using RNeasy Mini Kit (Qiagen) per manufacturer's instructions.

4.2.4 RT-qPCR

Total RNA was subjected to reverse transcription using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RT-qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). PrimePCR SYBR Green assays *CEBPB* (Unique assay ID: qHsaCED0019041), *IL1B* (qHsaCID0022272), *PHLDA2* (qHsaCED0047473), *SLC15A3*

(qHsaCED0001796) and *TNFSF18* (qHsaCED0043856) were purchased from BioRad. Results were normalized to ribosomal protein S13 (*RPS13*; qHsaCID0038672). mRNA expression levels were calculated by subtracting *RPS13* cycle threshold (Ct) from the gene of interest Ct to obtain ΔCt. For gene expression analysis in biopsies, the relative mRNA expression was calculated using the following formula: mRNA expression = 100,000 × ($2^{-\Delta Ct}$). For gene expression analysis in EPC2 *in vitro* experiments, the control ΔCt (T = 0 h) was subtracted from the treatment condition ΔCt (T = 1, 6, 24 h) to obtain ΔΔCt. mRNA expression was calculated using the following formula: Fold change = $2^{-\Delta\Delta Ct}$.

4.2.5 Statistical analysis

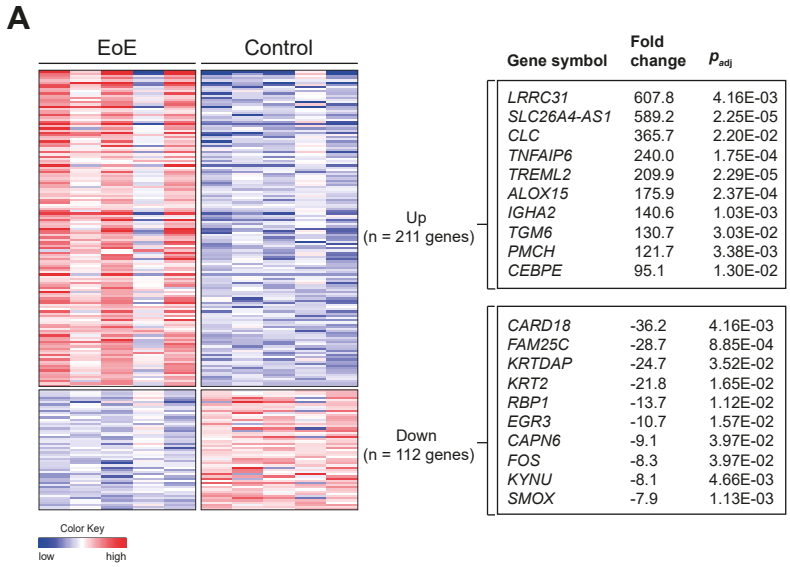
RNA-seq data were analyzed using iDEP v 0.96 as described above. Further statistical analyses were performed using GraphPad Prism v 9.4.1 (GraphPad Software, San Diego, CA, USA). Statistical significance was determined by unpaired t test (normal distribution, equal variance, 2 groups), Welch's t test (normal distribution, unequal variance, 2 groups), or (repeated measures) one-way ANOVA followed by Bonferroni's multiple comparisons test (normal distribution, equal variance, ≥ 3 groups). P values were considered significant if $p < 0.05$.

4.3 RESULTS

4.3.1 Transcriptomic characteristics of the EoE patient cohort

First, we aimed to determine the baseline inflammatory status of the esophagus of the five EoE patients included in this study and how it compares to previous reports. Patient characteristics are provided in Table S1. We subjected biopsies collected prior to the food injections from each of the five patients and baseline biopsies from five non-EoE controls to bulk RNA-seq. A total of 15,203 genes passed the expression threshold of at least 1 CPM in two samples. Among these expressed genes, 323 genes (2.12%) were dysregulated (FC > 1.5, FDR < 0.05) and showed high similarity in transcript expression patterns among EoE patients. Of the 323 dysregulated genes, 211 genes (65.3%) were upregulated and 112 genes (34.7%) were downregulated in EoE patients compared with non-EoE controls (Figure 1A). Similar to previous EoE transcriptome studies,[17,19,20,22] we found robust upregulation of *LRRC31* (608-fold, $p = 0.004$), *POSTN* (43-fold, $p = 0.014$), *CCL26* (41-fold, $p = 0.002$), *ANO1* (8.5-fold, $p = 0.020$) and *CAPN14* (3.4-fold, $p = 0.006$), and downregulation of *DSG1* (5-fold, $p = 0.024$) in EoE patients compared with controls (Figure 1C). All 323 DEGs along with their FC and adjusted *p* value are provided in Table S2.

Consistent with the distinct transcriptional signatures, GO enrichment analysis showed that the overexpressed genes were primarily involved in immune cell activation and (regulation of) the immune response. The downregulated genes related to a variety of functional/homeostatic processes (Figure 1B). Altogether, the transcriptome of our EoE patient cohort shows inter-individual similarities and compares with previously published EoE transcriptome studies, setting a solid basis for further analyses.



Gene symbol	Fold change	p_{adj}
LRRC31	607.8	4.16E-03
SLC26A4-AS1	589.2	2.25E-05
CLC	365.7	2.20E-02
TNFAIP6	240.0	1.75E-04
TREML2	209.9	2.29E-05
ALOX15	175.9	2.37E-04
IGHA2	140.6	1.03E-03
TGM6	130.7	3.03E-02
PMCH	121.7	3.38E-03
CEBPE	95.1	1.30E-02
CARD18	-36.2	4.16E-03
FAM25C	-28.7	8.85E-04
KRTDAP	-24.7	3.52E-02
KRT2	-21.8	1.65E-02
RBP1	-13.7	1.12E-02
EGR3	-10.7	1.57E-02
CAPN6	-9.1	3.97E-02
FOS	-8.3	3.97E-02
KYNU	-8.1	4.66E-03
SMOX	-7.9	1.13E-03

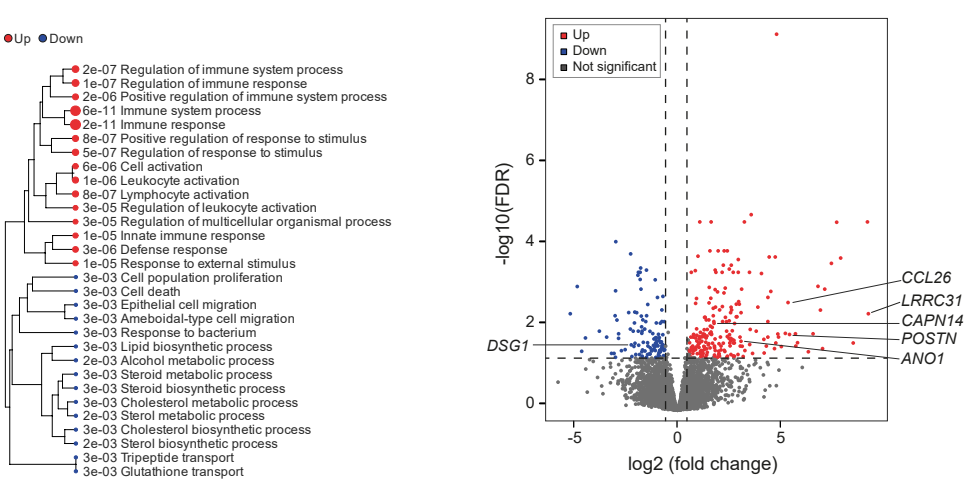


Figure 1. Gene expression analysis by RNA-seq in esophageal biopsies from patients with active EoE and non-EoE controls. A, Heatmap of the 323 genes that were identified as dysregulated (FC > 1.5, FDR < 0.05) in EoE patients (n = 5) compared with controls (n = 5). The 10 most dysregulated genes in each cluster along with their fold change (EoE vs. Ctrl) and adjusted p value are indicated on the right. Each column represents an individual patient or control, and each row represents a gene. **B**, Hierarchical clustering tree of enriched biological processes that are up- (red) or downregulated (blue) in EoE patients vs. non-EoE controls, with dot size inversely corresponding to the adjusted p value. **C**, Volcano plot showing log2 fold change values by -log10 FDR values for all 15,203 expressed genes. Significantly upregulated genes (n = 211 genes) are red, significantly downregulated genes (n = 112 genes) are blue, and non-significant genes are grey. Dashed lines represent the thresholds used for FDR (< 0.05) and fold change (> 1.5). Genes that were previously identified as part of the EoE transcriptome are indicated.

4.3.2 Identifying genes associated with acute responses of the esophageal mucosa to food injections

The primary aim of this study was to characterize gene expression signatures and functional processes of the acute mucosal response to food injections. To do this, we subjected biopsies collected before (baseline), 20 min after a negative and after a positive visual response to local esophageal challenge by food injections to bulk RNA-seq. The positive visual responses included in the analysis were not induced by the same food extracts in each patient (Table S1). We used 15 biopsy samples for gene expression analysis (3 biopsies × 5 patients). A total of 15,417 genes passed the expression filter of CPM ≥ 1 in at least two samples. Samples were paired by patient ID, and we used an FC > 1.5 and an FDR < 0.05 to define DEGs. When comparing negative visual responses to baseline, 11 genes were differentially expressed (10 up, 1 down) (Figure 2A). For positive visual response versus baseline comparisons, 124 DEGs (76 up, 48 down) were identified. Out of these 124 DEGs, 11 genes overlapped with the negative visual response vs. baseline comparison (Figure 2B). These changes may be the effect induced by the injection itself. Following removal of these 11 genes, 113 genes (66 up, 47 down) were found unique to a positive visual response to food injection (Figure 2B; Table S3). Upregulated genes were related to the cellular response to epidermal growth factor (*ERRFI1*, *SOX9*, *ID1*, *MYC*, *ZFP36L2*), ERK1 and ERK2 cascade (*ERRFI1*, *SOX9*, *IL1B*, *BMP2*, *MYC*, *DUSP6*, *CCN1*, *ZFP36L2*, *ATF3*), and cellular response to external stimulus (*PTGS2*, *HSPA8*, *SRF*, *CDKN1A*, *SOX9*, *IL1B*, *ATF3*, *NUAK2*, *CEBPB*, *FOSL1*) (Table S4). There were no significantly enriched GO terms or KEGG pathways in the downregulated gene cluster. Interestingly, one of 113 genes was also differentially expressed when compared with a negative visual response (Figure 2A, B).

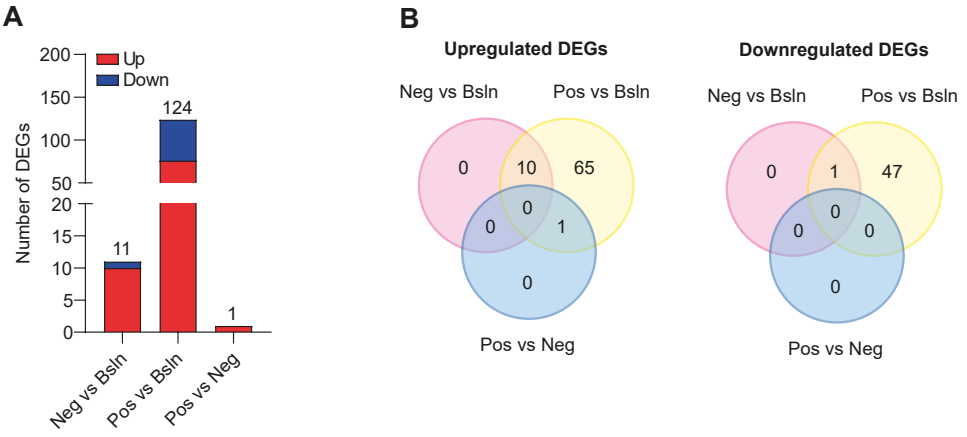
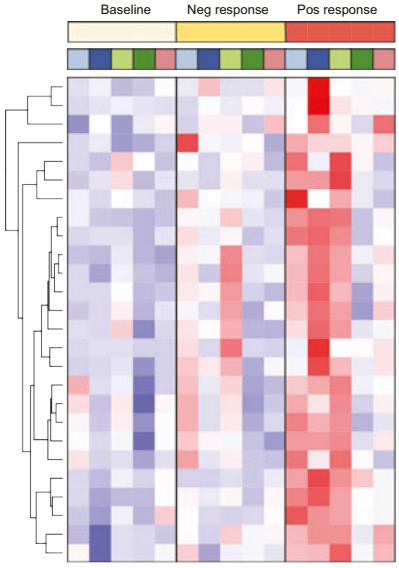


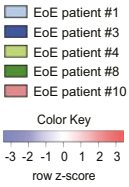
Figure 2. Gene expression analysis by RNA-seq in esophageal biopsies from patients with active EoE 20 min after local challenge by food injections. A, Number of significantly up- and downregulated DEGs (FC > 1.5, FDR < 0.05) in three different comparison groups: negative visual response vs. baseline (Neg vs Bsln), positive visual response vs. baseline (Pos vs Bsln) and positive visual response vs. negative visual response (Pos vs Neg). **B**, Venn diagrams depicting significantly upregulated (left) and downregulated DEGs (right) that are unique to or shared by the different comparison groups.

Of the 113 genes that were unique to a positive visual response to food injection, 40 genes (34.5%) also had an FC > 1.5 in the positive versus negative visual response comparison though did not pass the FDR cutoff of 0.05 (Figure 3; Table S5). Because of the explorative nature of this study and because DEGs are sensitive to arbitrary cutoffs,[34] we continued further downstream analysis with this set of 40 genes. For ease, we refer to this set of 40 genes henceforth as 'early EoE genes'.

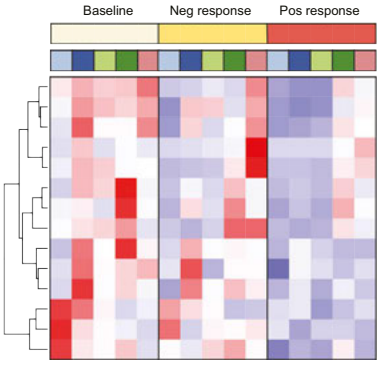
A **Upregulated (n = 26 genes)**



Gene symbol	Function	Fold change Pos vs Bsln	Fold change Pos vs Neg
CAPN6	microtubule-stabilizing	4.45**	1.92
PTGS2	prostaglandin synthesis	2.73*	2.10
SOX9	transcription factor	2.36*	1.56
TBX3	transcription factor	4.03*	1.53
PLA2G4E	phospholipase	3.74*	3.63
IL1B	cytokine	3.26*	2.83
ABCA12	protein transport	3.35*	1.62
LDLR	lipoprotein transporter	2.25**	1.55
ENSG00000275216	lncRNA	6.36***	2.50
HBEGF	growth factor	4.25****	1.78
SLC25A25	protein transport	3.57**	1.57
FOSL1	transcription factor	6.22*	2.28
ERRFI1	reg of cell signaling	2.1*	1.62
RGS1	reg of cell signaling	3.21**	2.05
EGR2	transcription factor	19.16**	2.87
ATF3	transcription factor	5.20***	1.64
RRAD	calmodulin-binding	2.51**	2.13
IFFO2	intermediate filament	2.00*	1.81
KRT16	epithelium related	3.16*	2.45
SLC15A3	protein transport	1.96*	1.63
CDH16	cell adhesion	2.21*	1.66
TNFSF18	cytokine	12.35***	18.27***
GJA3	ion channel	2.99**	1.78
CASC19	lncRNA	3.31*	2.45
PHLDA2	epithelium related	3.36****	1.85
CEBPB	transcription factor	1.75*	1.53



B **Downregulated (n = 14 genes)**



Gene symbol	Function	Fold change Pos vs Bsln	Fold change Pos vs Neg
ENSG00000176593	lncRNA	-2.86*	-1.61
MIR29B2CHG	lncRNA	-2.94*	-2.04
RBM20	reg of mRNA splicing	-2.50*	-1.59
CYP4F35P	NA	-33.33*	-4.35
TNNI2	reg of muscle contraction	-7.69*	-1.61
PRR33	NA	-3.13*	-1.56
STEAP4	metalloreductase	-2.86*	-2.13
SYT8	synaptotagmin	-5.00**	-2.33
HOXB-AS2	lncRNA	-3.70*	-2.70
HOXB3	transcription factor	-2.13*	-1.79
CNTNAP3	nervous system related	-1.92*	-1.79
CEL	esterase enzyme	-2.13*	-1.61
ENSG00000264785	lncRNA	-3.23*	-3.03
NATD1	NA	-1.89*	-1.75

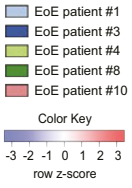


Figure 3. The early EoE genes. A and **B**, Hierarchical clustering heatmap showing z-scores for the 26 upregulated DEGs (**A**) and 14 downregulated DEGs (**B**) for each patient prior to injections (baseline), 20 min after a negative visual response to food injection (Neg response) and 20 min after a positive visual response to food injection (Pos response). Genes are shown on the right along with their function and fold change for the indicated comparison. *$p_{adj} < 0.05$, ** $p_{adj} < 0.01$, *** $p_{adj} < 0.001$, **** $p_{adj} < 0.0001$. Abbreviations: NA, not available; neg, negative response; pos, positive response; reg, regulator.

Six early EoE genes were increased significantly following a positive visual response to food injection when compared to baseline as well as a negative visual response (Figure 4A): *CEBPB* (1.75-fold, $p = 0.021$ for pos vs. baseline; 1.53-fold, $p = 0.035$ for pos vs. neg), *ENSG00000275216* [novel transcript affiliated with lncRNA class] (6.36-fold, $p = 0.015$ for pos vs. baseline; 2.5-fold, $p = 0.049$ for pos vs. neg), *IL1B* (3.26-fold, $p = 0.037$ for pos vs. baseline; 2.83-fold, $p = 0.036$ for pos vs. neg), *PHLDA2* (3.36-fold, $p = 0.0023$ for pos vs. baseline; 1.85-fold, $p = 0.025$ for pos vs. neg), *SLC15A3* (1.96-fold, $p = 0.005$ for pos vs. baseline; 1.63-fold, $p = 0.015$ for pos vs. neg), and most prominently *TNFSF18* (12.4-fold, $p = 0.028$ for pos vs. baseline; 18.3-fold, $p = 0.022$ for pos vs. neg). Furthermore, 3/6 genes (*CEBPB, IL1B* and *TNFSF18)* were confirmed by qPCR (Figure 4B). Plots for the remaining 20 upregulated early EoE genes and 14 downregulated early EoE genes are provided in Figure S1 and S2, respectively.

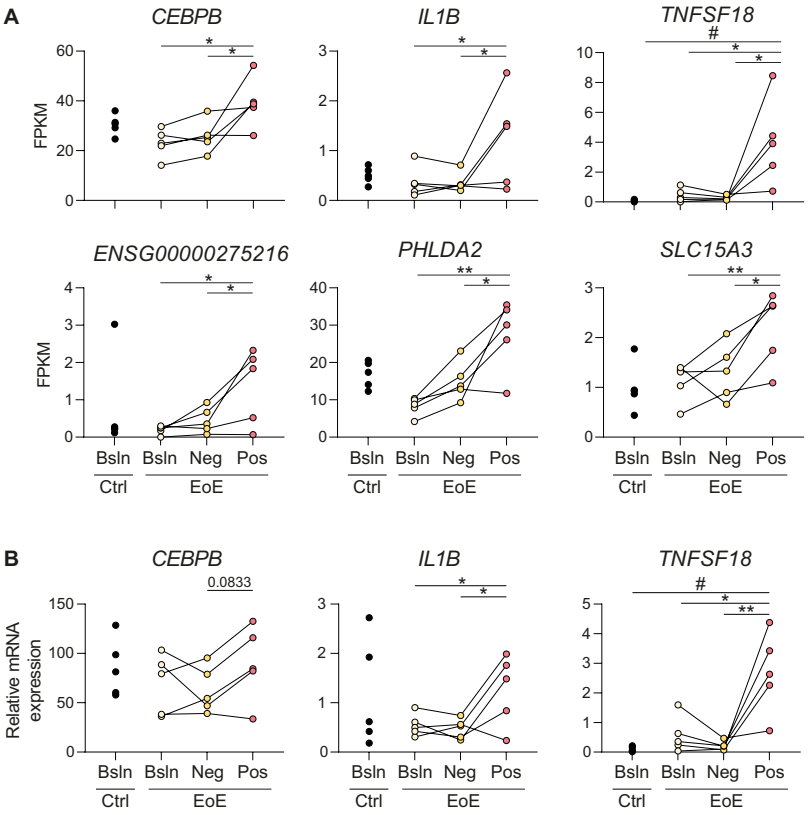


Figure 4. Gene expression pattern of 6 early EoE genes following a visual positive or a negative response to local challenge by food injection. A and **B**, mRNA expression of CEBPB, ENSG00000275216, IL1B, PHLDA2, SLC15A3 and TNFSF18 in esophageal biopsies of non-EoE controls (Ctrl) at baseline (Bsln) and of EoE patients (EoE) at baseline and after a negative (Neg) or positive visual response (Pos) to food injection as measured by RNA sequencing (**A**) and RT-qPCR (**B**). Data are presented as FPKM. Asterisks and hashtags represent statistical significance: *p < 0.05, **p < 0.01, by repeated measures one-way ANOVA and Bonferroni's multiple comparisons test; #p < 0.05, by Welch's t-test.

4.3.3 Early EoE genes are enriched in processes related to immune activation

To gain insight in the collective putative function of the genes that were activated in the early phase of the immune response to food allergens in EoE, we performed GO and KEGG enrichment analysis on the upregulated and downregulated early EoE genes separately. In the upregulated gene cluster (n = 26 genes; Figure 3A), GO analysis demonstrated gene expression related to immune activation, including the neuroinflammatory response (*PTGS2*, *IL1B*, *LDLR*), the acute-phase response (*PTGS2*, *IL1B*, *CEBPB*), positive regulation of the inflammatory response (*PTGS2*, *TNFSF18*, *IL1B*, *LDLR*, *CEBPB*), cellular responses to external stimuli (*PTGS2*, *SOX9*, *IL1B*, *ATF3*, *CEBPB*, *FOSL1*), and cell population proliferation (*PTGS2*, *HBEGF*, *ERRFI1*, *TNFSF18*, *SOX9*, *IL1B*, *TBX3*, *ATF3*, *CEBPB*, *FOSL1*, *PHLDA2*). In addition, KEGG pathway analysis revealed enriched IL-17 signaling (*PTGS2*, *IL1B*, *CEBPB*, *FOSL1*), C-type lectin receptor signaling (*PTGS2*, *EGR2*, *IL1B*), and TNF signaling (*PTGS2*, *IL1B*, *CEBPB*). The GO terms and KEGG pathways associated with the early EoE genes, along with their fold enrichment and adjusted *p* values are shown in Table 1. A complete list of enriched GO terms and KEGG pathways are provided in Table S6. In the downregulated gene cluster (n = 14 genes; Figure 3B) there were no enriched GO terms or KEGG pathways.

Table 1. GO and KEGG profile of the upregulated acute EoE genes (n = 26 genes).

Pathway database	GO term or KEGG pathway	Adjusted *p* value	Fold enrichment	Genes
GO BP	Neuroinflammatory response (GO:0150076)	2.70E-03	66.9	*PTGS2*, ***IL1B***, *LDLR*
GO BP	Acute-phase response (GO:0006953)	2.70E-03	64.3	*PTGS2*, ***IL1B***, ***CEBPB***
GO BP	Positive regulation of inflammatory response (GO:0050729)	4.95E-04	30.6	*PTGS2*, ***TNFSF18***, ***IL1B***, *LDLR*, ***CEBPB***
GO BP	Cellular response to external stimulus (GO:0071496)	2.70E-03	11.2	*PTGS2*, *SOX9*, ***IL1B***, *ATF3*, ***CEBPB***, *FOSL1*
GO BP	Regulation of cell population proliferation (GO:0042127)	2.30E-03	4.9	*PTGS2*, *HBEGF*, *ERRFI1*, ***TNFSF18***, *SOX9*, ***IL1B***, *TBX3*, *ATF3*, ***CEBPB***, *FOSL1*, ***PHLDA2***
KEGG	IL-17 signaling pathway (hsa04913)	5.11E-04	33.8	*PTGS2*, ***IL1B***, ***CEBPB***, *FOSL1*
KEGG	C-type lectin receptor signaling pathway (hsa04625)	1.36E-02	20.2	*PTGS2*, *EGR2*, ***IL1B***
KEGG	TNF signaling pathway (hsa04668)	1.47E-02	17.08	*PTGS2*, ***IL1B***, ***CEBPB***

Fold enrichment is defined as the percentage of genes in the set of interest belonging to a term/pathway, divided by the corresponding percentage of genes in the background set that belong to the same term/pathway. Genes in bold are part of the 6 acute EoE genes shown in Figure 4A. Abbreviations: BP, biological process; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

4.3.4 Esophageal epithelial cells are a potential source of TNFSF18/ GITRL, which is upregulated upon epithelial activation by IL-13

TNFSF18 (TNF superfamily member 18), also known as GITRL (glucocorticoid-induced tumor necrosis factor receptor ligand), was the most prominently upregulated gene in the positive visual response vs. baseline (12.35-fold) as well as the positive visual response vs. negative visual response comparison (18.27-fold) in EoE patients that underwent mucosal food injections (Figure 3A; Figure 4A). To assess the potential cellular source of TNFSF18, we first explored a publicly available single-cell RNA-seq data set of whole EoE esophageal biopsies,[35,36] and found that TNFSF18 was enriched in differentiating epithelial cells compared with other esophageal cells in active EoE (Figure S3). To further narrow down onto the epithelium as a potential source of TNFSF18, we determined whether TNFSF18 expression could be rapidly induced in the esophageal epithelium by an inflammatory trigger. Esophageal epithelial cells grown under ALI conditions are a commonly used *in vitro* model for studying the epithelium of the esophagus because it recapitulates characteristic morphologic features such as the formation of multiple layers of cells.[37,38] We used this model to study the induction of TNFSF18 mRNA in esophageal epithelial cells by IL-13, a key cytokine involved in EoE and an epithelial trigger.[39] A schematic diagram of the experimental time line is provided in Figure 5A. Indeed, differentiated esophageal epithelial cells showed 22-fold ($p < 0.001$) and 31-fold increase ($p < 0.0001$) in TNFSF18 expression at 6 h and 24 h post IL-13 stimulation, respectively, compared with baseline (0 h; Figure 5B).

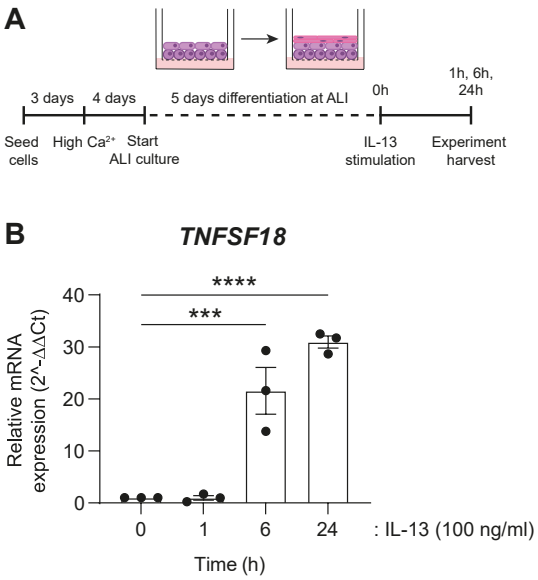


Figure 5. TNFSF18 expression is rapidly induced by IL-13 in differentiated esophageal epithelial cells. A, Schematic overview of the in vitro experimental design. EPC2 differentiated at the air-liquid interface (ALI) were analyzed at baseline (0 h), and 1, 6 and 24h after basolateral IL-13 stimulation (100 ng/ml). **B**, TNFSF18 mRNA expression in EPC2 ALI cultures at various time points post IL-13 stimulation. Data are presented as mean ± SEM from n = 3 independent experiments performed with 2 technical replicates per condition. Asterisks represent statistical significance: ***$p < 0.001$, ****$p < 0.0001$, by one-way ANOVA and Bonferroni's post hoc test.

4.4 DISCUSSION

In this study, we analyzed transcriptomic profiles of esophageal biopsies from adult EoE patients who underwent local food challenge by mucosal injections to characterize gene expression signatures and functional processes associated with the acute esophageal response to a specific food. We identified 113 genes that were unique to a positive visual response to food injections, of which 40 genes were dysregulated by more than 1.5-fold compared with a negative visual response to food injections. These "early EoE genes" were enriched in pro-inflammatory processes, such as the acute-phase response and cellular response to external stimuli. Of the early EoE genes, *TNFSF18* (also called GITRL), a member of the TNF superfamily best studied for co-stimulatory effect on T cells, was most highly induced following a positive visual response to food injection compared with a negative visual response (18-fold) and baseline (12-fold). Interestingly, TNFSF18 appears to play a role in other atopic conditions such as asthma[40] and atopic dermatitis.[41,42] Finally, we show that esophageal epithelial cells may be an early source of TNFSF18. The data presented herein for the first time provide insight into the early transcriptomic changes that are associated with an acute mucosal response to food allergens in EoE.

The increasing knowledge of pathogenic pathways and cytokines in EoE derives mostly from bulk or single-cell RNA-seq studies of esophageal biopsies collected during active and inactive disease.[13,17,19,35,36] However, to date there have been no studies reported that performed RNA-seq on esophageal tissue collected just after food challenge. A major strength of this study is that we could characterize changes in gene expression that occurred during an acute response to food by profiling the transcriptomes of EoE patients before and shortly after local food challenge. Rather than characterizing the active EoE transcriptome, our study design allowed us to leverage data collected at different time points (baseline vs. after injection) and between esophageal responses (negative vs. positive) within the same patient, increasing power by reducing bias due to inter-individual variability. It should be noted that EoE is patchy, resulting in differences in cellular composition of the biopsies from the same patient. By comparing a positive visual response biopsy to both baseline and negative visual response biopsies, we aimed to reduce the effect of patchiness on the precision of the analysis.

The current transcriptome study provides a comprehensive molecular map of immune alterations that occurred in the esophagus of adult patients with EoE following challenge by local food injection. We identified early EoE genes that showed significant changes in expression in the early phase of the immune response to food allergens in EoE. The observed expression signatures were involved in pro-inflammatory processes, such as the acute phase response, cellular response to external stimuli and regulation of cell population proliferation. These functional categories are similar to those identified in a dynamic transcriptome study that characterized changes in peripheral blood samples during an allergic response to peanut.[43]

Six early EoE genes (*ENSG00000275216*, *PHLDA2*, *SLC15A3*, *IL1B*, *CEBPB* and *TNFSF18*) demonstrated increased expression triggered by local food allergen exposure. The role of ENSG00000275216 (novel transcript affiliated with lncRNA class) in immune responses is not established. While IgE

crosslinking on human mast cells is accompanied with increased expression of *PHLDA2* (Pleckstrin Homology Like Domain Family A Member 2),[44] its functional role in allergic inflammation is not clear. Expression of *SLC15A3* (Solute Carrier Family Member 3) has been shown in monocytes where it has a role in driving virus-induced production of type I and III interferons.[45] *IL1B* (interleukin 1 beta), *CEBPB* (CCAAT Enhancer Binding Protein Beta) and *TNFSF18* (TNF Superfamily Member 18) have established and validated roles in allergic inflammation. *IL1B* encodes the pro-inflammatory cytokine IL-1β that is produced by a variety of immune cells, including dendritic cells, macrophages and B cells, as well as non-immune cells such as keratinocytes.[46] In addition, IL-1β has been implicated in the pathogenesis of atopic dermatitis and asthma,[47-49] and drives mast cell hyperactivation in atopic dermatitis-like inflammation in mice.[50] *CEBPB* encodes a transcription factor that regulates genes involved in pro-inflammatory responses,[51] and was found to be upregulated in esophageal eosinophils in IL-13-induced experimental EoE.[52] Interestingly, both *IL1B* and *CEBPB* were increased in peripheral blood leukocytes from subjects admitted to the emergency room with anaphylaxis.[53] *TNFSF18*, which was most prominently upregulated during an acute response to food injection, encodes the TNFSF18/GITRL protein that occurs in transmembrane and soluble forms.[54] While TNFSF18 is expressed on professional antigen-presenting cells (APC), including dendritic cells, macrophages, and B cells, as well as non-professional APCs such as endothelial and epithelial cells, its receptor (TNFRSF18/GITR) is mainly expressed on effector and regulatory T cells, but also ILC2s.[54-56] Ligation of TNFRSF18 by either anti-TNFRSF18 antibodies or its natural ligand TNFSF18 typically results in the activation or enhancement of the immune response,[57] and has been shown to stimulate effector T cells and attenuate regulatory T cell-mediated suppression.[58-62] Co-signaling between TNFRSF18 and IL-33 receptor promotes human ILC2 expansion and expression of type 2 cytokines IL-9, IL-5 and IL-13.[56] Upon transmembrane TNFSF18-TNFRSF18 interaction, TNFSF18 can transduce bidirectional signals, of which most have a pro-inflammatory function.[55,63] So, TNFSF18 does not merely function as a trigger protein for TNFRSF18, but also modulates activity of the cells that express TNFSF18 itself.[64]

TNFSF18-TNFRSF18 interactions hold a critical role in allergic inflammation. Several *in vivo* studies using murine models of allergic asthma have demonstrated a role for TNFSF18 in promoting Th2 cell differentiation and effector functions, and in enhancing lung allergic responses by inducing Th2 cell and ILC2 activity.[40,56,62,65,66] In EoE, TNFSF18 expression is increased in esophageal biopsies and fibroblasts.[67] Recently, another TNF superfamily member, TNFSF14/LIGHT, was attributed a role in EoE pathogenesis,[67-69] as its overexpression induced a pro-inflammatory phenotype in fibroblasts in EoE,[68] while its deficiency protected mice from developing EoE-like inflammation.[69]

Here, we demonstrated that stimulation of differentiated esophageal epithelial cells with the type 2 cytokine IL-13 rapidly induced TNFSF18 expression by these cells, which is in line with previous reports on human keratinocytes and airway epithelial cells.[42,70] In addition, TNFSF18 expression is increased in keratinocytes of acute skin lesions of patients with atopic dermatitis.[42] Of note, human skin and esophageal epithelium share morphologic similarities as both epithelia consist of stratified squamous cells. Ligation of TNFSF18 expressed on human keratinocytes induced an increase in expression of the pro-inflammatory cytokine IL-8 and T cell chemokine

CCL27.[71] It is interesting to speculate that the food-induced increase in TNFSF18 expression in the esophageal epithelium may promote the signaling potential between the epithelium and TNFRSF18-expressing T cells and ILC2s, resulting in the production of a plethora of pro-inflammatory mediators from epithelial cells, proliferation of T cells, and activation of ILC2s. The source of TNFSF18 in EoE still requires further study. While the esophageal epithelium is indeed one of the primary drivers of EoE pathogenesis,[72] and we identified TNFSF18-expressing epithelial cells, we cannot rule out that the increase in TNFSF18 mRNA expression upon food challenge comes from another cellular source, which would require single-cell RNA-seq. If TNFSF18 indeed plays an initial and essential role in the early phase of the food-induced immune response in EoE, blockade of the TNFSF18-TNFRSF18 pathway may provide a new therapeutic target for EoE, as it may become in asthma.[40]

This study has limitations. First, biopsies were collected from the injection sites 20 min after local challenge. While changes in gene expression can be measured within 2 min after stimulation,[73] biopsies taken at a later timepoint would have provided a broader insight into the immune mechanisms underlying a mucosal response to food. However, due to the invasiveness of the procedure and the discomfort that several patients experienced it was ethically not possible to prolong the endoscopy to collect biopsies at a later time point. It would be interesting for future studies to profile the dynamic transcriptome using biopsies collected at multiple later time points following food challenge. Second, this explorative study was conducted in a small cohort and future studies in larger cohorts should be performed to confirm our findings.

In conclusion, we show that esophageal challenge by local food injections in adult EoE patients triggers the expression of genes that are associated with processes related to immune activation. Our study identifies TNFSF18/GITRL as the most upregulated gene during an acute response to food injections. As such, TNFSF18 may mediate interactions between TNFSF18-expressing cells including esophageal epithelial cells, and TNFRSF18/GITR-expressing cells including T cells and ILC2s during an acute mucosal response to food in patients with EoE to promote inflammation. Further in-depth analysis of how TNFSF18 potentiates acute mucosal responses to food and contributes to EoE pathogenesis is needed to determine if the TNFSF18-TNFRSF18 pathway may be a new therapeutic target for EoE.

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SUPPLEMENTARY FIGURES

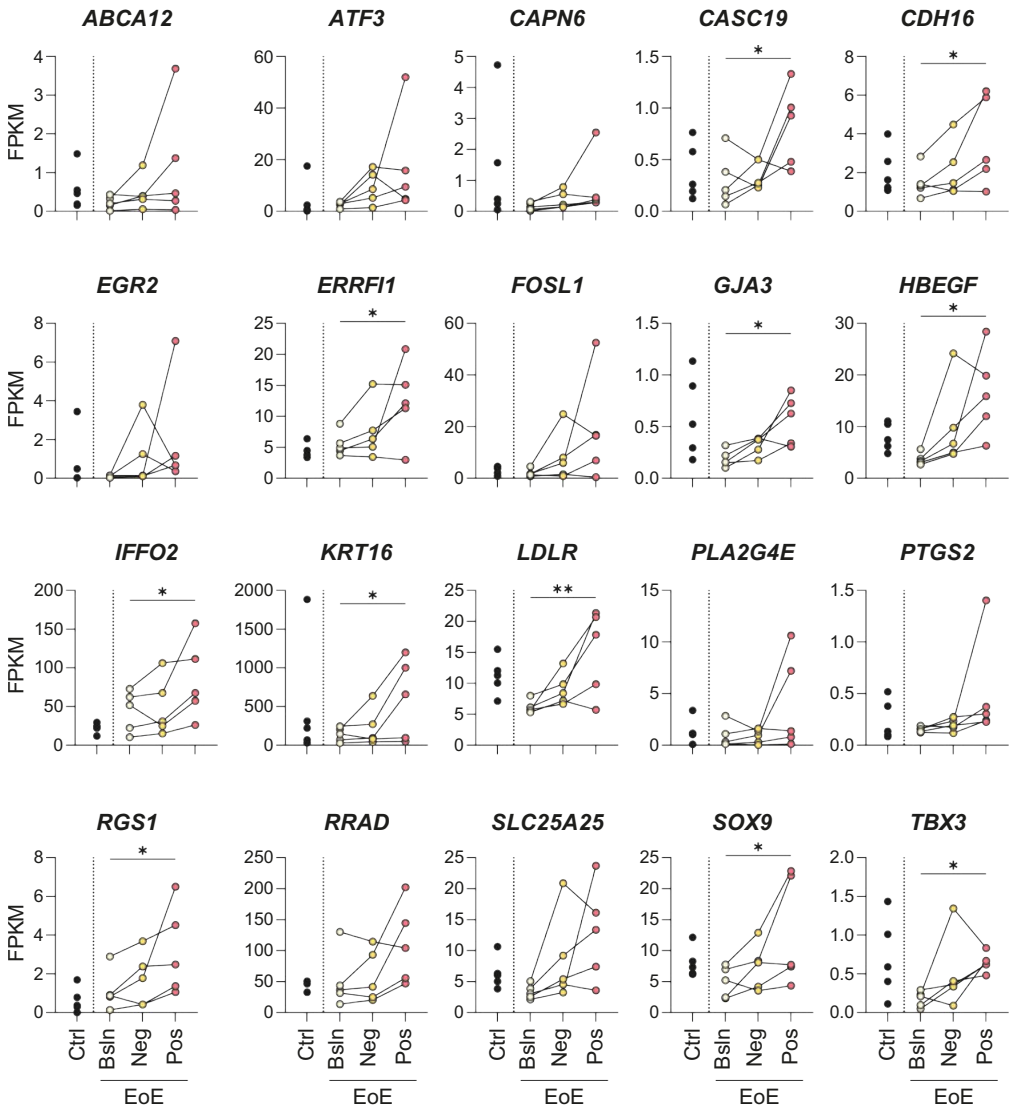

Figure S1. Gene expression pattern of the upregulated early EoE genes. mRNA expression in esophageal biopsies of non-EoE controls (Ctrl) at baseline (n = 5), and of EoE patients (n = 5) at baseline (Bsln) and after a negative (Neg) or positive visual response (Pos) to food injection as measured by RNA-seq. Data are presented in FPKM. Asterisks represent statistical significance: *p < 0.05, **p < 0.01, by repeated measures one-way ANOVA followed by Bonferroni's post hoc test.

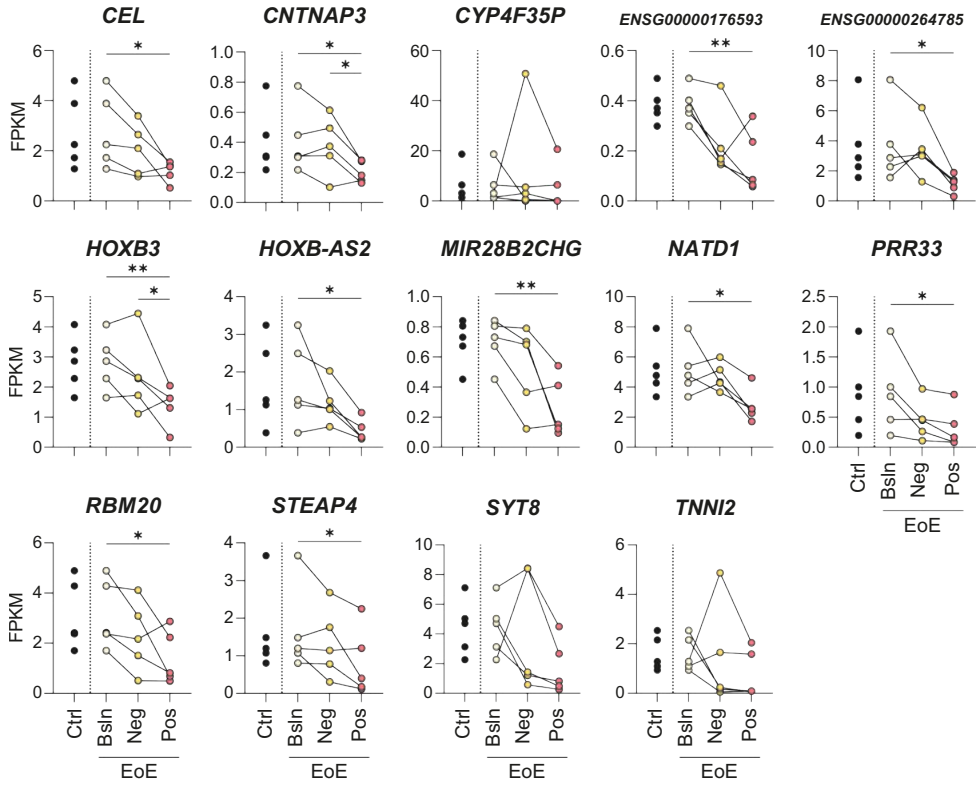

Figure S2. Gene expression pattern of the downregulated early EoE genes. mRNA expression in esophageal biopsies of non-EoE controls (Ctrl) at baseline (n = 5), and of EoE patients (n = 5) at baseline (Bsln) and after a negative (Neg) or positive visual response (Pos) to food injection as measured by RNA-seq. Data are presented in FPKM. Asterisks represent statistical significance: *p < 0.05, **p < 0.01, by repeated measures one-way ANOVA followed by Bonferroni's post hoc test.

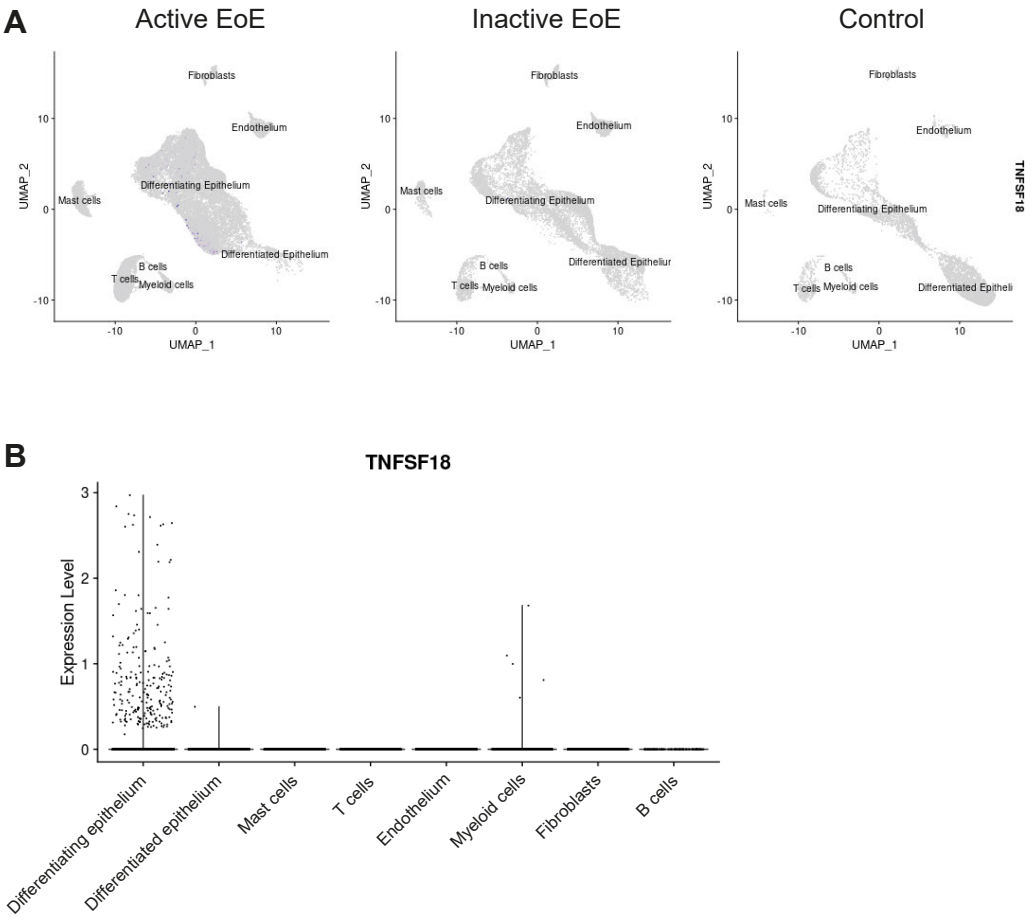

Figure S3. TNFSF18 mRNA expression in esophageal biopsies. A, Feature plot analysis of TNFSF18 of the scRNA-seq of esophageal biopsies from patients with active EoE (n = 5), inactive EoE (n = 3) and non-EoE controls (n = 2). **B**, Scatter plot of TNFSF18 transcript abundance per cell population in esophageal biopsies from active EoE patients. Data are derived from publicly available single-cell RNA-sequencing dataset of esophageal biopsies.[35,36]

SUPPLEMENTARY TABLES

Table S1. Patient characteristics and information on food injections.

Patient ID	Sex (M/F)	Age (y)	PEC	Positive response (cm axial, clock position)	Negative response (cm axial, clock position)
1	M	26	45	Tomato (8, 3)	Milk (11, 12)
3	M	48	5	Peanut (8, 9)	Chicken (14, 3)
4	M	52	100	Wheat (8, 9)	Beer (14, 3)
8	M	26	30	Mango (8, 3)	Grapes (14, 3)
10	F	22	50	Apple (8, 9)	Soy (14, 3)

Abbreviations: F, female; M, male; PEC, peak eosinophil count.

Table S2. Differentially expressed genes in EoE (n = 5) vs. control (n = 5).

Regulation	Ensembl ID	Symbol	Type	EoE vs. Ctrl FC	Adj.Pval
Up	ENSG00000114248	LRRC31	protein_coding	607,81	4,16E-3
Up	ENSG00000233705	SLC26A4-AS1	lncRNA	589,23	2,25E-5
Up	ENSG00000105205	CLC	protein_coding	365,74	2,20E-2
Up	ENSG00000123610	TNFAIP6	protein_coding	240,01	1,75E-4
Up	ENSG00000112195	TREML2	protein_coding	209,94	2,29E-5
Up	ENSG00000161905	ALOX15	protein_coding	175,90	2,37E-4
Up	ENSG00000211890	IGHA2	IG_C_gene	140,64	1,03E-3
Up	ENSG00000166948	TGM6	protein_coding	130,68	3,03E-2
Up	ENSG00000183395	PMCH	protein_coding	121,70	3,38E-3
Up	ENSG00000262539		processed_pseudogene	112,18	8,76E-4
Up	ENSG00000092067	CEBPE	protein_coding	95,07	1,30E-2
Up	ENSG00000198400	NTRK1	protein_coding	81,21	3,62E-2
Up	ENSG00000142224	IL19	protein_coding	56,49	2,16E-2
Up	ENSG00000103355	PRSS33	protein_coding	53,27	2,62E-2
Up	ENSG00000091137	SLC26A4	protein_coding	52,64	1,30E-2
Up	ENSG00000133110	POSTN	protein_coding	43,18	1,35E-2
Up	ENSG00000006606	CCL26	protein_coding	41,07	2,20E-3
Up	ENSG00000181143	MUC16	protein_coding	37,51	1,27E-2
Up	ENSG00000198520	ARMH1	protein_coding	32,80	2,69E-2
Up	ENSG00000226777	FAM30A	lncRNA	29,84	1,56E-2
Up	ENSG00000237560	LINC01497	lncRNA	29,15	1,32E-2
Up	ENSG00000128383	APOBEC3A	protein_coding	27,97	5,20E-10
Up	ENSG00000215853	RPTN	protein_coding	27,24	2,20E-2

Up	ENSG00000102854	MSLN	protein_coding	26,74	1,65E-4
Up	ENSG00000123405	NFE2	protein_coding	26,06	3,02E-2
Up	ENSG00000115602	IL1RL1	protein_coding	23,05	1,17E-3
Up	ENSG00000124215	CDH26	protein_coding	21,83	1,65E-4
Up	ENSG00000184809	B3GALT5-AS1	lncRNA	20,91	1,64E-3
Up	ENSG00000138696	BMPR1B	protein_coding	20,88	6,50E-3
Up	ENSG00000170577	SIX2	protein_coding	20,87	1,57E-2
Up	ENSG00000179915	NRXN1	protein_coding	19,83	2,62E-2
Up	ENSG00000165071	TMEM71	protein_coding	18,43	3,88E-2
Up	ENSG00000236700	LINC01010	lncRNA	18,25	1,79E-2
Up	ENSG00000105675	ATP4A	protein_coding	16,84	4,22E-4
Up	ENSG00000230635	CYP4F60P	unprocessed_pseudogene	14,92	2,86E-3
Up	ENSG00000152463	OLAH	protein_coding	14,12	1,12E-2
Up	ENSG00000140287	HDC	protein_coding	12,49	3,97E-2
Up	ENSG00000166510	CCDC68	protein_coding	11,93	1,49E-5
Up	ENSG00000109684	CLNK	protein_coding	11,54	1,02E-2
Up	ENSG00000105492	SIGLEC6	protein_coding	11,34	2,40E-2
Up	ENSG00000197099		lncRNA	11,26	3,94E-4
Up	ENSG00000157613	CREB3L1	protein_coding	9,66	3,37E-2
Up	ENSG00000090512	FETUB	protein_coding	9,50	2,25E-5
Up	ENSG00000012124	CD22	protein_coding	9,12	4,74E-2
Up	ENSG00000055118	KCNH2	protein_coding	8,83	2,65E-2
Up	ENSG00000237988	OR2I1P	protein_coding	8,66	1,03E-3
Up	ENSG00000134321	RSAD2	protein_coding	8,49	3,92E-3
Up	ENSG00000131620	ANO1	protein_coding	8,48	1,99E-2

Table S2. Continued

Regulation	Ensembl ID	Symbol	Type	EoE vs. Ctrl FC	Adj.Pval
Up	ENSG00000169224	GCSAML	protein_coding	8,27	2,61E-2
Up	ENSG00000149534	MS4A2	protein_coding	8,20	1,57E-2
Up	ENSG00000102243	VGLL1	protein_coding	8,01	2,42E-3
Up	ENSG00000114638	UPK1B	protein_coding	7,83	2,09E-3
Up	ENSG00000173890	GPR160	protein_coding	7,74	1,91E-4
Up	ENSG00000162949	CAPN13	protein_coding	7,73	3,94E-4
Up	ENSG00000263961	RHEX	protein_coding	7,66	4,22E-2
Up	ENSG00000261150	EPPK1	protein_coding	7,40	4,93E-3
Up	ENSG00000148053	NTRK2	protein_coding	7,25	2,42E-3
Up	ENSG00000197083	ZNF300P1	transcribed_unprocessed_pseudogene	7,23	4,96E-3
Up	ENSG00000155307	SAMSN1	protein_coding	6,83	7,23E-3
Up	ENSG00000099954	CECR2	protein_coding	6,78	4,64E-2
Up	ENSG00000171101	SIGLEC17P	transcribed_unprocessed_pseudogene	6,78	2,20E-2
Up	ENSG00000251537		protein_coding	6,69	1,89E-2
Up	ENSG00000271781		lncRNA	6,57	2,20E-2
Up	ENSG00000261821		lncRNA	6,53	3,94E-4
Up	ENSG00000144619	CNTN4	protein_coding	6,52	4,59E-2
Up	ENSG00000255587	RAB44	protein_coding	6,33	1,10E-2
Up	ENSG00000174944	P2RY14	protein_coding	6,32	1,79E-2
Up	ENSG00000140297	GCNT3	protein_coding	6,19	3,35E-3
Up	ENSG00000135426	TESPA1	protein_coding	6,12	2,63E-4
Up	ENSG00000196684	HSH2D	protein_coding	6,07	2,87E-3
Up	ENSG00000108405	P2RX1	protein_coding	6,05	4,22E-2
Up	ENSG00000141574	SECTM1	protein_coding	6,00	1,69E-2

Up	ENSG00000099994	SUSD2	protein_coding	5,99	1,92E-2
Up	ENSG00000128342	LIF	protein_coding	5,98	3,91E-3
Up	ENSG00000124334	IL9R	protein_coding	5,88	2,59E-2
Up	ENSG00000188761	BCL2L15	protein_coding	5,82	2,20E-2
Up	ENSG00000123700	KCNJ2	protein_coding	5,76	1,37E-2
Up	ENSG00000182389	CACNB4	protein_coding	5,72	3,28E-4
Up	ENSG00000100453	GZMB	protein_coding	5,71	6,23E-3
Up	ENSG00000196581	AJAP1	protein_coding	5,67	1,69E-2
Up	ENSG00000140030	GPR65	protein_coding	5,37	4,64E-2
Up	ENSG00000243649	CFB	protein_coding	5,35	1,17E-4
Up	ENSG00000013588	GPRC5A	protein_coding	5,35	1,52E-2
Up	ENSG00000163106	HPGDS	protein_coding	5,28	4,95E-2
Up	ENSG00000163606	CD200R1	protein_coding	5,23	1,12E-2
Up	ENSG00000183778	B3GALT5	protein_coding	5,21	6,53E-3
Up	ENSG00000010671	BTK	protein_coding	5,02	5,11E-3
Up	ENSG00000196639	HRH1	protein_coding	4,99	9,69E-4
Up	ENSG00000124813	RUNX2	protein_coding	4,94	3,55E-2
Up	ENSG00000197635	DPP4	protein_coding	4,83	3,34E-2
Up	ENSG00000157404	KIT	protein_coding	4,82	1,17E-4
Up	ENSG00000068079	IFI35	protein_coding	4,79	1,28E-3
Up	ENSG00000100351	GRAP2	protein_coding	4,68	2,63E-4
Up	ENSG00000165178	NCF1C	unprocessed_pseudogene	4,68	2,22E-2
Up	ENSG00000134326	CMPK2	protein_coding	4,57	6,50E-3
Up	ENSG00000162931	TRIM17	protein_coding	4,54	2,77E-3
Up	ENSG00000143507	DUSP10	protein_coding	4,50	3,94E-4

Table S2. Continued

Regulation	Ensembl ID	Symbol	Type	EoE vs. Ctrl FC	Adj.Pval
Up	ENSG00000257594	GALNT4	protein_coding	4,33	3,25E-2
Up	ENSG00000128268	MGAT3	protein_coding	4,33	2,61E-2
Up	ENSG00000109861	CTSC	protein_coding	4,25	1,56E-2
Up	ENSG00000116741	RGS2	protein_coding	4,14	3,35E-3
Up	ENSG00000159212	CLIC6	protein_coding	4,12	4,64E-2
Up	ENSG00000132205	EMILIN2	protein_coding	4,06	4,03E-2
Up	ENSG00000109819	PPARGC1A	protein_coding	4,05	2,29E-2
Up	ENSG00000187608	ISG15	protein_coding	4,03	4,22E-2
Up	ENSG00000100342	APOL1	protein_coding	4,00	3,49E-2
Up	ENSG00000165949	IFI27	protein_coding	3,94	1,17E-4
Up	ENSG00000262655	SPON1	protein_coding	3,90	4,91E-2
Up	ENSG00000142512	SIGLEC10	protein_coding	3,86	1,20E-3
Up	ENSG00000254535	PABPC4L	protein_coding	3,65	3,94E-4
Up	ENSG00000185736	ADARB2	protein_coding	3,60	2,86E-3
Up	ENSG00000022567	SLC45A4	protein_coding	3,57	3,37E-2
Up	ENSG00000171631	P2RY6	protein_coding	3,56	3,48E-4
Up	ENSG00000173762	CD7	protein_coding	3,56	1,06E-3
Up	ENSG00000028277	POU2F2	protein_coding	3,55	1,75E-2
Up	ENSG00000172578	KLHL6	protein_coding	3,52	4,62E-2
Up	ENSG00000135114	OASL	protein_coding	3,49	2,89E-2
Up	ENSG00000171097	KYAT1	protein_coding	3,48	4,36E-2
Up	ENSG00000213626	LBH	protein_coding	3,48	2,61E-2
Up	ENSG00000153563	CD8A	protein_coding	3,43	7,17E-3
Up	ENSG00000214711	CAPN14	protein_coding	3,41	6,48E-3

Up	ENSG00000118515	SGK1	protein_coding	3,35	4,66E-3
Up	ENSG00000189067	LITAF	protein_coding	3,34	3,05E-3
Up	ENSG00000122862	SRGN	protein_coding	3,26	2,22E-2
Up	ENSG00000271503	CCL5	protein_coding	3,23	9,01E-3
Up	ENSG00000101096	NFATC2	protein_coding	3,15	1,35E-2
Up	ENSG00000009790	TRAF3IP3	protein_coding	3,14	1,30E-2
Up	ENSG00000143851	PTPN7	protein_coding	3,11	2,25E-5
Up	ENSG00000049130	KITLG	protein_coding	3,10	1,18E-2
Up	ENSG00000119917	IFIT3	protein_coding	3,08	1,73E-3
Up	ENSG00000235098	ANKRD65	protein_coding	3,03	8,40E-3
Up	ENSG00000168421	RHOH	protein_coding	3,00	2,91E-2
Up	ENSG00000119922	IFIT2	protein_coding	2,99	1,17E-4
Up	ENSG00000163219	ARHGAP25	protein_coding	2,99	1,28E-2
Up	ENSG00000188641	DPYD	protein_coding	2,92	9,27E-4
Up	ENSG00000125347	IRF1	protein_coding	2,90	2,25E-3
Up	ENSG00000235162	C12orf75	protein_coding	2,88	5,92E-3
Up	ENSG00000129675	ARHGEF6	protein_coding	2,88	2,28E-2
Up	ENSG00000160991	ORAI2	protein_coding	2,83	2,44E-2
Up	ENSG00000145649	GZMA	protein_coding	2,73	3,75E-2
Up	ENSG00000179023	KLHDC7A	protein_coding	2,73	1,02E-2
Up	ENSG00000167851	CD300A	protein_coding	2,70	1,48E-2
Up	ENSG00000161955	TNFSF13	protein_coding	2,68	3,07E-2
Up	ENSG00000107201	DDX58	protein_coding	2,68	4,31E-2
Up	ENSG00000164691	TAGAP	protein_coding	2,64	5,92E-3
Up	ENSG00000111424	VDR	protein_coding	2,63	1,65E-2

Table S2. Continued

Regulation	Ensembl ID	Symbol	Type	EoE vs. Ctrl FC	Adj.Pval
Up	ENSG00000123609	NMI	protein_coding	2,60	2,10E-2
Up	ENSG00000057294	PKP2	protein_coding	2,60	4,95E-2
Up	ENSG00000081320	STK17B	protein_coding	2,55	5,11E-3
Up	ENSG00000108924	HLF	protein_coding	2,53	2,95E-2
Up	ENSG00000169442	CD52	protein_coding	2,50	5,11E-3
Up	ENSG00000172817	CYP7B1	protein_coding	2,49	1,36E-2
Up	ENSG00000147324	MFHAS1	protein_coding	2,47	2,72E-2
Up	ENSG00000162366	PDZK1IP1	protein_coding	2,47	1,52E-2
Up	ENSG00000273033	LINC02035	lncRNA	2,47	1,03E-2
Up	ENSG00000181218	H2AW	protein_coding	2,47	3,21E-2
Up	ENSG00000128284	APOL3	protein_coding	2,44	2,66E-2
Up	ENSG00000116824	CD2	protein_coding	2,44	1,35E-2
Up	ENSG00000105976	MET	protein_coding	2,37	1,32E-2
Up	ENSG00000174808	BTC	protein_coding	2,37	3,97E-2
Up	ENSG00000140391	TSPAN3	protein_coding	2,35	3,99E-2
Up	ENSG00000128335	APOL2	protein_coding	2,35	4,61E-2
Up	ENSG00000197496	SLC2A10	protein_coding	2,30	3,47E-2
Up	ENSG00000176788	BASP1	protein_coding	2,29	3,55E-2
Up	ENSG00000143891	GALM	protein_coding	2,29	4,52E-2
Up	ENSG00000152229	PSTPIP2	protein_coding	2,28	1,55E-2
Up	ENSG00000156463	SH3RF2	protein_coding	2,28	1,99E-2
Up	ENSG00000172785	CBWD1	protein_coding	2,26	3,40E-2
Up	ENSG00000002587	HS3ST1	protein_coding	2,26	4,22E-2
Up	ENSG00000186197	EDARADD	protein_coding	2,24	3,40E-2

Up	ENSG00000162757	C1orf74	protein_coding	2,21	4,79E-2
Up	ENSG00000139324	TMTC3	protein_coding	2,17	1,20E-2
Up	ENSG00000139323	POC1B	protein_coding	2,17	3,57E-2
Up	ENSG00000054219	LY75	protein_coding	2,13	4,03E-2
Up	ENSG00000115267	IFIH1	protein_coding	2,13	2,25E-5
Up	ENSG00000169860	P2RY1	protein_coding	2,11	2,20E-2
Up	ENSG00000122122	SASH3	protein_coding	2,11	1,75E-2
Up	ENSG00000055163	CYFIP2	protein_coding	2,11	1,52E-2
Up	ENSG00000184730	APOBR	protein_coding	2,08	1,71E-2
Up	ENSG00000111144	LTA4H	protein_coding	2,07	2,82E-2
Up	ENSG00000043462	LCP2	protein_coding	2,06	2,20E-2
Up	ENSG00000070061	ELP1	protein_coding	2,01	2,43E-2
Up	ENSG00000221963	APOL6	protein_coding	2,00	1,58E-4
Up	ENSG00000100439	ABHD4	protein_coding	1,95	4,03E-2
Up	ENSG00000138496	PARP9	protein_coding	1,93	1,42E-2
Up	ENSG00000136824	SMC2	protein_coding	1,93	1,96E-2
Up	ENSG00000152484	USP12	protein_coding	1,91	9,83E-3
Up	ENSG00000102805	CLN5	protein_coding	1,91	1,75E-3
Up	ENSG00000112029	FBXO5	protein_coding	1,88	2,77E-2
Up	ENSG00000121858	TNFSF10	protein_coding	1,85	3,16E-2
Up	ENSG00000196154	S100A4	protein_coding	1,85	2,31E-3
Up	ENSG00000008952	SEC62	protein_coding	1,84	1,65E-2
Up	ENSG00000130589	HELZ2	protein_coding	1,83	3,59E-4
Up	ENSG00000188559	RALGAPA2	protein_coding	1,82	2,95E-2
Up	ENSG00000092470	WDR76	protein_coding	1,82	3,03E-2

Table S2. Continued

Regulation	Ensembl ID	Symbol	Type	EoE vs. Ctrl FC	Adj.Pval
Up	ENSG00000185811	IKZF1	protein_coding	1,76	3,34E-2
Up	ENSG00000053254	FOXN3	protein_coding	1,73	1,37E-2
Up	ENSG00000142856	ITGB3BP	protein_coding	1,73	1,35E-2
Up	ENSG00000143119	CD53	protein_coding	1,73	3,11E-2
Up	ENSG00000156802	ATAD2	protein_coding	1,70	1,57E-2
Up	ENSG00000180353	HCLS1	protein_coding	1,69	3,97E-2
Up	ENSG00000080345	RIF1	protein_coding	1,67	2,62E-2
Up	ENSG00000081923	ATP8B1	protein_coding	1,67	3,34E-2
Up	ENSG00000162645	GBP2	protein_coding	1,60	2,86E-2
Up	ENSG00000055332	EIF2AK2	protein_coding	1,60	3,94E-4
Up	ENSG00000106460	TMEM106B	protein_coding	1,58	1,57E-2
Up	ENSG00000143179	UCK2	protein_coding	1,56	4,83E-2
Up	ENSG00000129460	NGDN	protein_coding	1,53	3,49E-2
Up	ENSG00000168961	LGALS9	protein_coding	1,51	2,16E-2
Up	ENSG00000048649	RSF1	protein_coding	1,51	3,34E-2
Up	ENSG00000163840	DTX3L	protein_coding	1,51	4,00E-2
Down	ENSG00000255501	CARD18	protein_coding	-36,23	4,16E-3
Down	ENSG00000276430	FAM25C	protein_coding	-28,67	8,85E-4
Down	ENSG00000188508	KRTDAP	protein_coding	-24,70	3,52E-2
Down	ENSG00000172867	KRT2	protein_coding	-21,78	1,65E-2
Down	ENSG00000114115	RBP1	protein_coding	-13,66	1,12E-2
Down	ENSG00000179388	EGR3	protein_coding	-10,69	1,57E-2
Down	ENSG00000077274	CAPN6	protein_coding	-9,08	3,97E-2
Down	ENSG00000170345	FOS	protein_coding	-8,34	3,97E-2

Down	ENSG00000115919	KYNU	protein_coding	-8,05	4,66E-3
Down	ENSG00000233967		lncRNA	-7,95	4,95E-2
Down	ENSG00000088826	SMOX	protein_coding	-7,93	1,13E-3
Down	ENSG00000283486	FAM95C	protein_coding	-7,87	6,91E-5
Down	ENSG00000076344	RGS11	protein_coding	-7,57	5,96E-3
Down	ENSG00000154917	RAB6B	protein_coding	-7,18	1,69E-2
Down	ENSG00000160221	GATD3A	protein_coding	-7,16	1,30E-2
Down	ENSG00000153404	PLEKHG4B	protein_coding	-6,51	3,40E-2
Down	ENSG00000255346	NOX5	protein_coding	-5,75	3,21E-2
Down	ENSG00000011347	SYT7	protein_coding	-5,10	3,89E-3
Down	ENSG00000176171	BNIP3	protein_coding	-4,83	9,95E-3
Down	ENSG00000136160	EDNRB	protein_coding	-4,78	1,38E-4
Down	ENSG00000134760	DSG1	protein_coding	-4,68	2,44E-2
Down	ENSG00000163221	S100A12	protein_coding	-4,58	4,74E-2
Down	ENSG00000144908	ALDH1L1	protein_coding	-4,39	1,12E-2
Down	ENSG00000172782	FADS6	protein_coding	-4,28	2,67E-2
Down	ENSG00000182379	NXPH4	protein_coding	-4,21	7,03E-3
Down	ENSG00000066248	NGEF	protein_coding	-4,16	3,79E-3
Down	ENSG00000109846	CRYAB	protein_coding	-4,13	2,23E-2
Down	ENSG00000079393	DUSP13	protein_coding	-4,02	4,22E-2
Down	ENSG00000183034	OTOP2	protein_coding	-3,90	3,92E-3
Down	ENSG00000008394	MGST1	protein_coding	-3,75	4,66E-4
Down	ENSG00000168350	DEGS2	protein_coding	-3,70	1,12E-2
Down	ENSG00000182040	USH1G	protein_coding	-3,69	3,94E-4
Down	ENSG00000162040	HS3ST6	protein_coding	-3,65	2,49E-2

Table S2. Continued

Regulation	Ensembl ID	Symbol	Type	EoE vs. Ctrl FC	Adj.Pval
Down	ENSG00000187498	COL4A1	protein_coding	-3,55	4,22E-2
Down	ENSG00000054179	ENTPD2	protein_coding	-3,48	3,94E-4
Down	ENSG00000167315	ACAA2	protein_coding	-3,47	5,93E-4
Down	ENSG00000197859	ADAMTSL2	protein_coding	-3,42	3,09E-4
Down	ENSG00000182809	CRIP2	protein_coding	-3,38	1,03E-3
Down	ENSG00000070087	PFN2	protein_coding	-3,34	4,76E-3
Down	ENSG00000180071	ANKRD18A	protein_coding	-3,31	8,75E-3
Down	ENSG00000196139	AKR1C3	protein_coding	-3,29	1,27E-2
Down	ENSG00000177606	JUN	protein_coding	-3,28	3,61E-2
Down	ENSG00000186442	KRT3	protein_coding	-3,26	1,12E-2
Down	ENSG00000170962	PDGFD	protein_coding	-3,25	4,64E-2
Down	ENSG00000185112	FAM43A	protein_coding	-3,21	3,26E-2
Down	ENSG00000164181	ELOVL7	protein_coding	-3,18	5,90E-3
Down	ENSG00000206072	SERPINB11	protein_coding	-3,13	2,12E-2
Down	ENSG00000182938	OTOP3	protein_coding	-2,98	9,95E-3
Down	ENSG00000107281	NPDC1	protein_coding	-2,96	3,47E-2
Down	ENSG00000073910	FRY	protein_coding	-2,88	6,50E-3
Down	ENSG00000277363	SRCIN1	protein_coding	-2,81	3,48E-4
Down	ENSG00000011677	GABRA3	protein_coding	-2,81	4,95E-2
Down	ENSG00000176903	PNMA1	protein_coding	-2,80	4,66E-3
Down	ENSG00000076864	RAP1GAP	protein_coding	-2,71	4,22E-2
Down	ENSG00000103489	XYLT1	protein_coding	-2,71	2,16E-2
Down	ENSG00000163331	DAPL1	protein_coding	-2,69	2,46E-3
Down	ENSG00000136999	CCN3	protein_coding	-2,68	2,61E-2

Down	ENSG00000152661	GJA1	protein_coding	-2,53	4,04E-2
Down	ENSG00000105520	PLPPR2	protein_coding	-2,50	1,13E-2
Down	ENSG00000171208	NETO2	protein_coding	-2,49	4,13E-2
Down	ENSG00000169169	CPT1C	protein_coding	-2,48	2,89E-2
Down	ENSG00000170899	GSTA4	protein_coding	-2,38	3,40E-2
Down	ENSG00000141655	TNFRSF11A	protein_coding	-2,33	3,40E-2
Down	ENSG00000103222	ABCC1	protein_coding	-2,28	1,29E-2
Down	ENSG00000052802	MSMO1	protein_coding	-2,22	2,52E-2
Down	ENSG00000164403	SHROOM1	protein_coding	-2,19	3,07E-2
Down	ENSG00000113070	HBEGF	protein_coding	-2,19	1,75E-2
Down	ENSG00000065054	SLC9A3R2	protein_coding	-2,10	2,69E-2
Down	ENSG00000151090	THRB	protein_coding	-2,10	6,04E-4
Down	ENSG00000216775		transcribed_unprocessed_pseudogene	-2,09	3,37E-2
Down	ENSG00000134508	CABLES1	protein_coding	-2,07	1,63E-2
Down	ENSG00000181649	PHLDA2	protein_coding	-2,07	2,22E-2
Down	ENSG00000067113	PLPP1	protein_coding	-2,05	4,99E-2
Down	ENSG00000172893	DHCR7	protein_coding	-2,02	7,03E-3
Down	ENSG00000124762	CDKN1A	protein_coding	-2,02	2,22E-2
Down	ENSG00000073060	SCARB1	protein_coding	-2,02	1,63E-2
Down	ENSG00000167508	MVD	protein_coding	-2,01	1,93E-2
Down	ENSG00000125968	ID1	protein_coding	-2,00	2,49E-2
Down	ENSG00000240184	PCDHGC3	protein_coding	-1,94	1,65E-3
Down	ENSG00000169710	FASN	protein_coding	-1,91	2,59E-2
Down	ENSG00000184363	PKP3	protein_coding	-1,88	4,64E-2
Down	ENSG00000079337	RAPGEF3	protein_coding	-1,88	1,34E-2

Table S2. Continued

Regulation	Ensembl ID	Symbol	Type	EoE vs. Ctrl FC	Adj.Pval
Down	ENSG00000116133	DHCR24	protein_coding	-1,88	4,22E-2
Down	ENSG00000053371	AKR7A2	protein_coding	-1,85	3,78E-2
Down	ENSG00000030582	GRN	protein_coding	-1,84	4,74E-2
Down	ENSG00000130164	LDLR	protein_coding	-1,84	1,92E-2
Down	ENSG00000114023	FAM162A	protein_coding	-1,83	1,62E-2
Down	ENSG00000090661	CERS4	protein_coding	-1,80	3,37E-2
Down	ENSG00000109089	CDR2L	protein_coding	-1,79	4,74E-2
Down	ENSG00000134590	RTL8C	protein_coding	-1,79	3,34E-2
Down	ENSG00000130522	JUND	protein_coding	-1,78	3,37E-2
Down	ENSG00000159527	PGLYRP3	protein_coding	-1,77	3,65E-2
Down	ENSG00000109107	ALDOC	protein_coding	-1,77	7,11E-3
Down	ENSG00000136717	BIN1	protein_coding	-1,76	2,15E-2
Down	ENSG00000171056	SOX7	protein_coding	-1,76	3,47E-2
Down	ENSG00000137440	FGFBP1	protein_coding	-1,71	2,06E-2
Down	ENSG00000071242	RPS6KA2	protein_coding	-1,71	2,06E-2
Down	ENSG00000140406	TLNRD1	protein_coding	-1,69	3,03E-2
Down	ENSG00000133935	ERG28	protein_coding	-1,68	3,35E-3
Down	ENSG00000166484	MAPK7	protein_coding	-1,68	6,50E-3
Down	ENSG00000102575	ACP5	protein_coding	-1,68	2,88E-2
Down	ENSG00000184574	LPAR5	protein_coding	-1,67	2,59E-2
Down	ENSG00000183828	NUDT14	protein_coding	-1,62	1,37E-2
Down	ENSG00000091527	CDV3	protein_coding	-1,62	2,42E-2
Down	ENSG00000125266	EFNB2	protein_coding	-1,62	1,55E-3
Down	ENSG00000172375	C2CD2L	protein_coding	-1,61	3,07E-2

Down	ENSG00000107331	ABCA2	protein_coding	-1,58	4,52E-2
Down	ENSG00000103335	PIEZO1	protein_coding	-1,58	4,64E-2
Down	ENSG00000164970	FAM219A	protein_coding	-1,56	3,34E-2
Down	ENSG00000168159	RNF187	protein_coding	-1,55	6,53E-3
Down	ENSG00000139428	MMAB	protein_coding	-1,52	3,22E-2
Down	ENSG00000203950	RTL8A	protein_coding	-1,51	2,60E-2

Abbreviations: Adj. Pval, adjusted P value; ctrl, non-EoE control; down, downregulated; FC, fold change; up, upregulated.

Table S3. Expression patterns of the 113 positive visual response genes

Regulation	ENSEMBL ID	Gene symbol	Neg vs. Bsln FC	Neg vs. Bsln Padj	Pos vs. Bsln FC	Pos vs. Bsln Padj	Pos vs. Neg FC	Pos vs. Neg Padj
Up	ENSG00000124102	PI3	1,82	5,37E-01	2,18	3,03E-02	1,2	8,13E-01
Up	ENSG00000181817	LSM10	1,12	9,60E-01	1,54	1,55E-02	1,38	6,35E-01
Up	ENSG00000120337	TNFSF18	-1,48	9,74E-01	12,35	7,00E-04	18,27	1,00E-04
Up	ENSG00000121743	GJA3	1,68	9,49E-01	2,99	8,70E-03	1,78	6,61E-01
Up	ENSG00000254166	CASC19	1,35	9,61E-01	3,31	2,68E-02	2,45	6,35E-01
Up	ENSG00000188089	PLA2G4E	1,03	9,96E-01	3,74	3,94E-02	3,63	5,23E-01
Up	ENSG00000125538	IL1B	1,16	9,85E-01	3,26	3,94E-02	2,83	6,22E-01
Up	ENSG00000173599	PC	1,16	9,59E-01	1,58	3,03E-02	1,36	6,35E-01
Up	ENSG00000166589	CDH16	1,33	9,59E-01	2,21	2,06E-02	1,66	6,35E-01
Up	ENSG00000144452	ABCA12	2,06	7,40E-01	3,35	1,01E-02	1,62	6,79E-01
Up	ENSG00000166592	RRAD	1,18	9,74E-01	2,51	7,50E-03	2,13	5,23E-01
Up	ENSG00000169991	IFFO2	1,1	9,80E-01	2	1,15E-02	1,81	5,23E-01
Up	ENSG00000186832	KRT16	1,29	9,63E-01	3,16	2,06E-02	2,45	6,35E-01
Up	ENSG00000110446	SLC15A3	1,21	9,59E-01	1,96	1,60E-02	1,63	6,35E-01
Up	ENSG00000203722	RAET1G	1,58	4,31E-01	1,85	1,15E-02	1,17	7,71E-01
Up	ENSG00000139988	RDH12	1,84	4,58E-01	2,07	3,88E-02	1,13	8,87E-01
Up	ENSG00000172893	DHCR7	1,27	9,59E-01	1,71	1,53E-02	1,35	6,61E-01
Up	ENSG00000108106	UBE2S	1,23	8,42E-01	1,53	3,00E-03	1,24	6,61E-01
Up	ENSG00000171223	JUNB	2,5	8,46E-02	2,72	8,70E-03	1,09	9,37E-01
Up	ENSG00000130522	JUND	1,95	2,20E-01	2	4,39E-02	1,03	9,80E-01
Up	ENSG00000255120	OVOL1-AS1	1,96	6,58E-01	2,4	4,58E-02	1,23	8,28E-01
Up	ENSG00000152518	ZFP36L2	1,56	3,98E-01	1,7	3,40E-02	1,09	8,88E-01

Up	ENSG00000182938	OTOP3	1,73	5,20E-01	2,24	7,10E-03	1,3	7,13E-01
Up	ENSG00000140406	TLNRD1	1,37	5,51E-01	1,71	1,50E-03	1,25	6,61E-01
Up	ENSG00000165175	MID1IP1	1,47	1,58E-01	1,62	6,50E-03	1,1	8,23E-01
Up	ENSG00000125398	SOX9	1,51	9,54E-01	2,36	1,56E-02	1,56	6,61E-01
Up	ENSG00000139318	DUSP6	1,32	9,49E-01	1,63	4,58E-02	1,24	6,88E-01
Up	ENSG00000114115	RBP1	3,86	1,20E-01	5,11	5,40E-03	1,32	8,42E-01
Up	ENSG00000077274	CAPN6	2,33	7,33E-01	4,45	4,00E-03	1,92	6,61E-01
Up	ENSG00000073756	PTGS2	1,3	9,63E-01	2,73	4,58E-02	2,1	6,35E-01
Up	ENSG00000010270	STARD3NL	1,43	7,12E-01	1,64	4,28E-02	1,14	7,96E-01
Up	ENSG00000125845	BMP2	2,33	2,49E-01	2,52	4,06E-02	1,08	9,49E-01
Up	ENSG00000135111	TBX3	2,64	5,51E-01	4,03	1,32E-02	1,53	7,34E-01
Up	ENSG00000159840	ZYX	1,27	9,56E-01	1,59	4,06E-02	1,25	6,79E-01
Up	ENSG00000112658	SRF	1,55	7,76E-01	2	2,46E-02	1,3	7,05E-01
Up	ENSG00000136997	MYC	1,79	6,09E-01	2,12	4,39E-02	1,19	8,32E-01
Up	ENSG00000122877	EGR2	6,68	5,51E-01	19,16	6,60E-03	2,87	6,82E-01
Up	ENSG00000125740	FOSB	9,06	1,31E-01	12,34	1,16E-02	1,36	9,09E-01
Up	ENSG00000162772	ATF3	3,16	9,83E-02	5,2	2,00E-04	1,64	6,82E-01
Up	ENSG00000142871	CCN1	3,81	1,74E-01	4,56	1,56E-02	1,19	9,19E-01
Up	ENSG00000130164	LDLR	1,45	8,75E-01	2,25	2,10E-03	1,55	6,35E-01
Up	ENSG00000275216	N/A	2,55	6,09E-01	6,36	1,00E-04	2,5	6,35E-01
Up	ENSG00000113070	HBEGF	2,39	5,11E-02	4,25	0,00E+00	1,78	6,35E-01
Up	ENSG00000148339	SLC25A25	2,27	4,93E-01	3,57	3,70E-03	1,57	6,82E-01
Up	ENSG00000175592	FOSL1	2,73	9,46E-01	6,22	3,88E-02	2,28	6,82E-01
Up	ENSG00000116285	ERRFI1	1,3	9,59E-01	2,1	4,39E-02	1,62	6,61E-01
Up	ENSG00000090104	RGS1	1,57	9,59E-01	3,21	7,00E-03	2,05	6,35E-01

Table S3. Continued

Regulation	ENSEMBL ID	Gene symbol	Neg vs. Bsln FC	Neg vs. Bsln Padj	Pos vs. Bsln FC	Pos vs. Bsln Padj	Pos vs. Neg FC	Pos vs. Neg Padj
Up	ENSG00000163545	NUAK2	1,92	1,94E-01	1,99	3,55E-02	1,04	9,66E-01
Up	ENSG00000189143	CLDN4	1,52	7,00E-01	2,01	6,70E-03	1,32	6,79E-01
Up	ENSG00000141579	ZNF750	1,27	9,59E-01	1,62	4,58E-02	1,28	6,77E-01
Up	ENSG00000067082	KLF6	1,58	9,83E-02	1,7	7,10E-03	1,07	8,97E-01
Up	ENSG00000172818	OVOL1	1,92	4,64E-01	2,8	2,20E-03	1,47	6,77E-01
Up	ENSG00000181649	PHLDA2	1,82	5,51E-01	3,36	0,00E+00	1,85	6,35E-01
Up	ENSG00000125266	EFNB2	1,39	7,33E-01	1,67	1,55E-02	1,21	6,99E-01
Up	ENSG00000172216	CEBPB	1,14	9,61E-01	1,75	2,02E-02	1,53	6,35E-01
Up	ENSG00000185950	IRS2	1,65	5,47E-01	1,87	4,06E-02	1,14	8,55E-01
Up	ENSG00000214318	ATP5MC1P6	3,1	NA	4,54	8,00E-04	1,46	7,14E-01
Up	ENSG00000115963	RND3	2,04	2,25E-01	2,09	4,98E-02	1,02	9,87E-01
Up	ENSG00000087074	PPP1R15A	1,72	2,49E-01	1,85	3,03E-02	1,08	9,21E-01
Up	ENSG00000124762	CDKN1A	1,64	7,92E-01	2,15	3,88E-02	1,31	7,28E-01
Up	ENSG00000125968	ID1	1,99	4,58E-01	2,38	2,39E-02	1,2	8,33E-01
Up	ENSG00000134107	BHLHE40	1,44	7,92E-01	1,79	3,40E-02	1,24	7,23E-01
Up	ENSG00000183010	PYCR1	1,23	9,59E-01	1,58	3,56E-02	1,28	6,61E-01
Up	ENSG00000116649	SRM	1,17	9,59E-01	1,59	1,55E-02	1,35	6,35E-01
Up	ENSG00000109971	HSPA8	1,08	9,74E-01	1,53	2,09E-02	1,42	6,22E-01
Up	ENSG00000130204	TOMM40	1,03	9,88E-01	1,51	4,58E-02	1,47	6,22E-01
Down	ENSG00000229417	NPM1P25	-1,79	2,22E-01	-2,04	1,32E-02	-1,14	8,59E-01
Down	ENSG00000272274	LINC00551	-2,56	6,23E-02	-2,70	8,90E-03	-1,06	9,58E-01
Down	ENSG00000166839	ANKDD1A	-1,27	9,59E-01	-1,82	2,18E-02	-1,45	6,61E-01
Down	ENSG00000274180	NATD1	-1,08	9,85E-01	-1,89	4,39E-02	-1,75	6,35E-01

Down	ENSG00000264785	N/A	-1,06	9,88E-01	-3,23	3,03E-02	-3,03	5,23E-01
Down	ENSG00000125347	IRF1	-1,69	8,08E-01	-2,22	4,58E-02	-1,32	7,50E-01
Down	ENSG00000170835	CEL	-1,32	9,59E-01	-2,13	4,58E-02	-1,61	6,61E-01
Down	ENSG00000265787	CYP4F35P	-9,09	8,89E-01	-33,33	4,58E-02	-4,35	7,14E-01
Down	ENSG00000130598	TNNI2	-5,00	5,51E-01	-7,69	3,98E-02	-1,61	8,33E-01
Down	ENSG00000149043	SYT8	-2,13	9,03E-01	-5,00	7,10E-03	-2,33	6,61E-01
Down	ENSG00000176593	N/A	-1,79	8,88E-01	-2,86	2,66E-02	-1,61	6,82E-01
Down	ENSG00000215012	RTL10	-1,20	9,59E-01	-1,52	3,88E-02	-1,25	6,61E-01
Down	ENSG00000130222	GADD45G	-2,27	5,37E-01	-3,03	1,92E-02	-1,37	7,75E-01
Down	ENSG00000007944	MYLIP	-1,20	9,59E-01	-1,54	3,94E-02	-1,27	6,61E-01
Down	ENSG00000170684	ZNF296	-2,13	6,23E-02	-2,63	7,00E-04	-1,23	7,75E-01
Down	ENSG00000171649	ZIK1	-1,82	4,58E-01	-2,08	4,06E-02	-1,14	8,91E-01
Down	ENSG00000171827	ZNF570	-1,43	9,49E-01	-1,96	3,99E-02	-1,37	6,82E-01
Down	ENSG00000136870	ZNF189	-1,47	3,95E-01	-1,67	1,23E-02	-1,14	7,88E-01
Down	ENSG00000167384	ZNF180	-1,45	7,33E-01	-1,69	4,06E-02	-1,18	7,75E-01
Down	ENSG00000196684	HSH2D	-2,27	1,45E-01	-2,33	3,88E-02	-1,01	9,96E-01
Down	ENSG00000283787	PRR33	-1,96	7,40E-01	-3,13	1,55E-02	-1,56	6,88E-01
Down	ENSG00000127954	STEAP4	-1,35	9,59E-01	-2,86	3,57E-02	-2,13	6,35E-01
Down	ENSG00000197128	ZNF772	-1,16	9,59E-01	-1,54	2,09E-02	-1,33	6,35E-01
Down	ENSG00000239552	HOXB-AS2	-1,33	NA	-3,70	1,15E-02	-2,70	6,22E-01
Down	ENSG00000106714	CNTNAP3	-1,08	9,85E-01	-1,92	4,58E-02	-1,79	6,35E-01
Down	ENSG00000128872	TMOD2	-1,04	9,88E-01	-1,52	4,17E-02	-1,47	6,22E-01
Down	ENSG00000120093	HOXB3	-1,19	9,67E-01	-2,13	4,06E-02	-1,79	6,35E-01
Down	ENSG00000102805	CLN5	-1,49	5,37E-01	-1,89	3,70E-03	-1,28	6,81E-01
Down	ENSG00000198300	PEG3	-10,00	2,22E-01	-14,29	2,00E-02	-1,45	9,00E-01

Table S3. Continued

Regulation	ENSEMBL ID	Gene symbol	Neg vs. Bsln FC	Neg vs. Bsln Padj	Pos vs. Bsln FC	Pos vs. Bsln Padj	Pos vs. Neg FC	Pos vs. Neg Padj
Down	ENSG00000197847	SLC22A20P	-1,69	6,09E-01	-2,08	2,23E-02	-1,25	7,60E-01
Down	ENSG00000089356	FXYD3	-1,85	9,83E-02	-1,96	1,32E-02	-1,05	9,52E-01
Down	ENSG00000198393	ZNF26	-1,39	6,81E-01	-1,67	1,32E-02	-1,20	7,07E-01
Down	ENSG00000198040	ZNF84	-1,35	9,03E-01	-1,92	7,10E-03	-1,41	6,61E-01
Down	ENSG00000186020	ZNF529	-1,18	9,59E-01	-1,69	3,88E-02	-1,45	6,44E-01
Down	ENSG00000104221	BRF2	-1,43	6,71E-01	-1,67	3,81E-02	-1,15	7,93E-01
Down	ENSG00000031003	FAM13B	-1,64	1,24E-01	-1,89	3,40E-03	-1,15	7,96E-01
Down	ENSG00000197937	ZNF347	-1,35	9,59E-01	-1,89	3,60E-02	-1,41	6,61E-01
Down	ENSG00000204519	ZNF551	-1,30	8,42E-01	-1,56	2,68E-02	-1,20	6,87E-01
Down	ENSG00000196214	ZNF766	-1,23	9,56E-01	-1,52	3,33E-02	-1,22	6,77E-01
Down	ENSG00000167380	ZNF226	-1,28	8,89E-01	-1,54	4,41E-02	-1,19	7,12E-01
Down	ENSG00000203867	RBM20	-1,59	9,59E-01	-2,50	3,88E-02	-1,59	6,76E-01
Down	ENSG00000203709	MIR29B2CHG	-1,45	9,59E-01	-2,94	3,88E-02	-2,04	6,61E-01
Down	ENSG00000262966	N/A	-1,47	NA	-3,45	2,39E-02	-2,38	6,35E-01
Down	ENSG00000173875	ZNF791	-1,25	8,88E-01	-1,59	6,50E-03	-1,27	6,61E-01
Down	ENSG00000089335	ZNF302	-1,37	9,56E-01	-1,82	4,39E-02	-1,33	6,82E-01
Down	ENSG00000233030	N/A	-1,41	9,59E-01	-2,08	4,45E-02	-1,47	6,77E-01
Down	ENSG00000177125	ZBTB34	-1,20	9,59E-01	-1,72	3,80E-02	-1,43	6,61E-01

Abbreviations: bsln, baseline; down, downregulated; FC, fold change; neg, negative visual response; Padj, adjusted P value; pos, positive visual response; up, upregulated.

Table S4. Enrichment and pathway analysis of the 113 positive visual response genes.

Database	Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	GO term or KEGG pathway	Genes
GO BP	5,03E-4	5	46	25,69	Cellular response to epidermal growth factor stimulus	ERRFI1 SOX9 ID1 MYC ZFP36L2
GO BP	4,37E-4	7	158	13,14	Reg. of epithelial cell differentiation	ERRFI1 SOX9 IL1B ID1 TBX3 ABCA12 CEBPB
GO BP	4,37E-4	9	350	8,59	ERK1 and ERK2 cascade	ERRFI1 SOX9 IL1B BMP2 MYC DUSP6 CCN1 ZFP36L2 ATF3
GO BP	4,37E-4	10	368	7,22	Cellular response to external stimulus	PTGS2 HSPA8 SRF CDKN1A SOX9 IL1B ATF3 NUAK2 CEBPB FOSL1
GO BP	5,84E-4	14	1042	4,42	Pos. reg. of cell population proliferation	PTGS2 HBEGF CDKN1A EFNB2 SOX9 IL1B BMP2 ID1 TBX3 MYC CCN1 ATF3 FOSL1 IRS2
GO BP	5,84E-4	14	1034	4,27	Response to organic cyclic compound	PTGS2 ERRFI1 CDKN1A IL1B FOSB BMP2 ID1 JUND MYC DUSP6 ZFP36L2 CEBPB FOSL1 CLDN4
GO BP	4,63E-5	22	1817	3,81	Reg. of cell population proliferation	PTGS2 SRF HBEGF ERRFI1 TNFSF18 CDKN1A EFNB2 SOX9 IL1B BMP2 ID1 JUND TBX3 MYC CCN1 ATF3 JUNB CEBPB OVOL1 FOSL1 PHLDA2 IRS2
GO BP	4,02E-4	18	1438	3,78	Reg. of multicellular organismal development	SRF ERRFI1 TNFSF18 EGR2 SOX9 IL1B BMP2 ID1 LDLR BHLHE40 TBX3 MYC DUSP6 CCN1 ABCA12 ZFP36L2 CEBPB PHLDA2
GO BP	7,73E-5	21	1738	3,74	Reg. of cell differentiation	PTGS2 SRF ERRFI1 TNFSF18 EGR2 EFNB2 SOX9 IL1B BMP2 ID1 LDLR JUND BHLHE40 TBX3 MYC ZNF750 CCN1 ABCA12 ZFP36L2 JUNB CEBPB

Table S4. Continued

Database	Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	GO term or KEGG pathway	Genes
GO BP	3,71E-4	20	1769	3,49	Response to endogenous stimulus	PTGS2 SRF ERRFI1 EGR2 CDKN1A SOX9 IL1B FOSB BMP2 ID1 LDLR JUND MYC CCN1 ZFP36L2 ZYX CEBPB FOSL1 IRS2 CLDN4
KEGG	1,27E-2	3	51	22,32	Ovarian steroidogenesis	PTGS2 LDLR PLA2G4E
KEGG	9,05E-5	6	93	19,62	IL-17 signaling pathway	PTGS2 IL1B FOSB JUND CEBPB FOSL1
KEGG	1,64E-2	3	41	19,04	Bladder cancer	HBEGF CDKN1A MYC
KEGG	4,28E-3	5	126	11,24	Osteoclast differentiation	IL1B FOSB JUND JUNB FOSL1
KEGG	2,49E-2	4	112	9,18	TNF signaling pathway	PTGS2 IL1B JUNB CEBPB
KEGG	4,06E-2	4	157	7,44	Hepatitis C	CDKN1A LDLR MYC CLDN4
KEGG	4,28E-3	7	294	6,68	MAPK signaling pathway	HSPA8 SRF IL1B JUND MYC DUSP6 PLA2G4E
KEGG	2,64E-2	5	222	6,31	Human T-cell leukemia virus 1 infection	SRF EGR2 CDKN1A MYC FOSL1

Abbreviations: BP, biological process; FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

Table S5. Expression pattern of the early EoE genes.

Regulation	ENSEMBL ID	Gene symbol	Neg vs. Bsln FC	Neg vs. Bsln Padj	Pos vs. Bsln FC	Pos vs. Bsln Padj	Pos vs. Neg FC	Pos vs. Neg Padj
Up	ENSG00000120337	TNFSF18	-1,48	0,974	12,35	0,001	18,27	0,000
Up	ENSG00000121743	GJA3	1,68	0,949	2,99	0,009	1,78	0,661
Up	ENSG00000254166	CASC19	1,35	0,961	3,31	0,027	2,45	0,635
Up	ENSG00000188089	PLA2G4E	1,03	0,996	3,74	0,039	3,63	0,523
Up	ENSG00000125538	IL1B	1,16	0,985	3,26	0,039	2,83	0,622
Up	ENSG00000166589	CDH16	1,33	0,959	2,21	0,021	1,66	0,635
Up	ENSG00000144452	ABCA12	2,06	0,740	3,35	0,010	1,62	0,679
Up	ENSG00000166592	RRAD	1,18	0,974	2,51	0,008	2,13	0,523
Up	ENSG00000169991	IFFO2	1,1	0,980	2	0,012	1,81	0,523
Up	ENSG00000186832	KRT16	1,29	0,963	3,16	0,021	2,45	0,635
Up	ENSG00000110446	SLC15A3	1,21	0,959	1,96	0,016	1,63	0,635
Up	ENSG00000125398	SOX9	1,51	0,954	2,36	0,016	1,56	0,661
Up	ENSG00000077274	CAPN6	2,33	0,733	4,45	0,004	1,92	0,661
Up	ENSG00000073756	PTGS2	1,3	0,963	2,73	0,046	2,1	0,635
Up	ENSG00000135111	TBX3	2,64	0,551	4,03	0,013	1,53	0,734
Up	ENSG00000122877	EGR2	6,68	0,551	19,16	0,007	2,87	0,682
Up	ENSG00000162772	ATF3	3,16	0,098	5,2	0,000	1,64	0,682
Up	ENSG00000130164	LDLR	1,45	0,875	2,25	0,002	1,55	0,635
Up	ENSG00000275216	N/A	2,55	0,609	6,36	0,000	2,5	0,635
Up	ENSG00000113070	HBEGF	2,39	0,051	4,25	0	1,78	0,635
Up	ENSG00000148339	SLC25A25	2,27	0,493	3,57	0,004	1,57	0,682
Up	ENSG00000175592	FOSL1	2,73	0,946	6,22	0,039	2,28	0,682

Table S5. Continued

Regulation	ENSEMBL ID	Gene symbol	Neg vs. Bsln FC	Neg vs. Bsln Padj	Pos vs. Bsln FC	Pos vs. Bsln Padj	Pos vs. Neg FC	Pos vs. Neg Padj
Up	ENSG00000116285	ERRFI1	1,3	0,959	2,1	0,044	1,62	0,661
Up	ENSG00000090104	RGS1	1,57	0,959	3,21	0,007	2,05	0,635
Up	ENSG00000181649	PHLDA2	1,82	0,551	3,36	0	1,85	0,635
Up	ENSG00000172216	CEBPB	1,14	0,961	1,75	0,020	1,53	0,635
Down	ENSG00000274180	NATD1	-1,08	0,985	-1,89	0,044	-1,75	0,635
Down	ENSG00000264785	N/A	-1,06	0,988	-3,23	0,030	-3,03	0,523
Down	ENSG00000170835	CEL	-1,32	0,959	-2,13	0,046	-1,61	0,661
Down	ENSG00000265787	CYP4F35P	-9,09	0,889	-33,33	0,046	-4,35	0,714
Down	ENSG00000130598	TNNI2	-5,00	0,551	-7,69	0,040	-1,61	0,833
Down	ENSG00000149043	SYT8	-2,13	0,903	-5,00	0,007	-2,33	0,661
Down	ENSG00000176593	N/A	-1,79	0,888	-2,86	0,027	-1,61	0,682
Down	ENSG00000283787	PRR33	-1,96	0,740	-3,13	0,016	-1,56	0,688
Down	ENSG00000127954	STEAP4	-1,35	0,959	-2,86	0,036	-2,13	0,635
Down	ENSG00000239552	HOXB-AS2	-1,33	NA	-3,70	0,012	-2,70	0,622
Down	ENSG00000106714	CNTNAP3	-1,08	0,985	-1,92	0,046	-1,79	0,635
Down	ENSG00000120093	HOXB3	-1,19	0,967	-2,13	0,041	-1,79	0,635
Down	ENSG00000203867	RBM20	-1,59	0,959	-2,50	0,039	-1,59	0,676
Down	ENSG00000203709	MIR29B2CHG	-1,45	0,959	-2,94	0,039	-2,04	0,661

Abbreviations: bsln, baseline; down, downregulated; FC, fold change; neg, negative visual response; Padj, adjusted P value; pos, positive visual response; up, upregulated.

Table S6. Enrichment and pathway analysis of the early EoE genes.

Database	Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	GO term or KEGG pathway	Genes
GO BP	2,70E-3	3	46	66,9	Neuroinflammatory response	PTGS2 IL1B LDLR
GO BP	2,70E-3	3	57	64,3	Acute-phase response	PTGS2 IL1B CEBPB
GO BP	4,95E-4	5	158	30,6	Pos. reg. of inflammatory response	PTGS2 TNFSF18 IL1B LDLR CEBPB
GO BP	8,30E-5	6	158	29,1	Reg. of epithelial cell differentiation	ERRFI1 SOX9 IL1B TBX3 ABCA12 CEBPB
GO BP	2,70E-3	5	273	17	Learning or memory	PTGS2 EGR2 LDLR CEBPB FOSL1
GO BP	2,70E-3	6	368	11,2	Cellular response to external stimulus	PTGS2 SOX9 IL1B ATF3 CEBPB FOSL1
GO BP	2,70E-3	6	437	10,8	Reproductive structure development	PTGS2 SOX9 TBX3 CEBPB FOSL1 PHLDA2
GO BP	2,70E-3	6	440	10,7	Reproductive system development	PTGS2 SOX9 TBX3 CEBPB FOSL1 PHLDA2
GO BP	2,46E-3	10	1438	5,4	Reg. of multicellular organismal development	ERRFI1 TNFSF18 EGR2 SOX9 IL1B LDLR TBX3 ABCA12 CEBPB PHLDA2
GO BP	2,30E-3	11	1817	4,9	Reg. of cell population proliferation	PTGS2 HBEGF ERRFI1 TNFSF18 SOX9 IL1B TBX3 ATF3 CEBPB FOSL1 PHLDA2
KEGG	8,63E-4	3	51	57,7	Ovarian steroidogenesis	PTGS2 LDLR PLA2G4E
KEGG	5,11E-4	4	93	33,8	IL-17 signaling pathway	PTGS2 IL1B CEBPB FOSL1
KEGG	4,21E-2	2	61	28,6	Arachidonic acid metabolism	PTGS2 PLA2G4E
KEGG	1,36E-2	3	104	20,2	C-type lectin receptor signaling pathway	PTGS2 EGR2 IL1B
KEGG	1,47E-2	3	112	17,8	TNF signaling pathway	PTGS2 IL1B CEBPB

Abbreviations: BP, biological process; FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

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CHAPTER 5

Mast cells disrupt the function of the esophageal epithelial barrier

ABSTRACT

BACKGROUND: Mast cells accumulate in the epithelium of patients with eosinophilic esophagitis (EoE), an inflammatory disorder characterized by extensive esophageal eosinophilic infiltration. It has been suggested that epithelial barrier disruption plays an important role in the pathophysiology of EoE.

OBJECTIVE: To investigate the functional characteristics of human esophageal epithelium differentiated under air-liquid interface (ALI) conditions upon coculture with activated primary human mast cells and furthermore identify cytokines that may contribute to the observed effects.

METHODS: Primary human mast cells derived from peripheral blood mononuclear cells were cocultured with esophageal epithelial cells (EPC2) differentiated under ALI conditions. After three days of coculture, mast cell degranulation was induced by crosslinking of membrane-bound immunoglobulin E (IgE). Effects on EPC2 barrier function were assessed by TEER (resistance), FITC-Dextran paracellular flux (permeability) and qPCR analysis of barrier proteins and antiprotease. Crosstalk between mast cells and EPC2 was further examined by targeted proteomic analysis of 45 cytokines and ELISA of mono- and coculture supernatants. Based on proteomics results, oncostatin M (OSM) expression was measured in EoE biopsies, and its effect on EPC2 barrier function was studied.

RESULTS: Coculture of differentiated esophageal epithelial cells with IgE-activated mast cells resulted in a 30% decrease in epithelial resistance ($p = 0.01$) and 22% increase in permeability ($p < 0.0001$) compared with non-activated mast cells. These changes were associated with decreased mRNA expression of barrier proteins filaggrin, desmoglein-1 and involucrin, and antiprotease SPINK7. Using targeted proteomics, we detected various cytokines in coculture supernatants, most notably GM-CSF and oncostatin M (OSM). OSM expression was increased by 12-fold in active EoE ($p < 0.01$) and associated with mast cell marker genes. Furthermore, OSM receptor-expressing esophageal epithelial cells were found in esophageal tissue of EoE patients, suggesting that the epithelial cells may respond to OSM. Stimulation of esophageal epithelial cells with OSM resulted in a dose-dependent decrease in barrier function and expression of filaggrin and desmoglein-1, and an increase in protease calpain-14.

CONCLUSION: Taken together, these data suggest a role for mast cells in decreasing esophageal epithelial barrier function in EoE, which may in part be mediated by the production of OSM.

KEYWORDS: eosinophilic esophagitis, epithelial barrier, IgE, mast cell, oncostatin M

5.1 INTRODUCTION

The epithelium of the esophagus is fundamental to host defense because it protects the deeper mucosal and submucosal layers from infections, environmental toxins, and allergens.[1] The maintenance of the intact esophageal barrier depends on coordinated expression of epithelial differentiation proteins, tight junctions, adherens junctions, and desmosomes.[2] In eosinophilic esophagitis (EoE), a chronic allergen-driven disorder of the esophagus, a defective esophageal barrier is a prominent feature of the underlying pathophysiology.[3] Esophageal barrier dysfunction is mainly driven by the type 2 cytokines interleukin (IL) 4 and IL-13 through effects on epithelial differentiation and causing loss of barrier proteins, such as the desmosome desmoglein-1 (DSG1) and epithelial differentiation proteins filaggrin (FLG) and involucrin (IVL).[4-6] Also, a dysregulated protease/antiprotease response has been demonstrated in the esophageal epithelium in active EoE.[7] In addition to inflammatory mediators, genetic predisposition and environmental factors contribute to the establishment and maintenance of esophageal barrier dysfunction.[3] The loss of barrier function with increased permeability likely enhances the uptake of food antigens with consequent allergic sensitization and a type 2 immune response.[8]

Mast cells are tissue-resident immune effector cells that accumulate in the esophageal epithelium of patients with active EoE but not healthy controls.[9,10] A recent single-cell RNA-sequencing study of esophageal mast cells in active and inactive disease demonstrated that these mast cells exist in subpopulations, proliferate locally, persist during disease remission, and are an important source of IL-13.[11] Interestingly, esophageal mast cells are degranulated in active EoE, and are increased in biopsies with basal zone hyperplasia and dilated intercellular spaces, both characteristics of a defective esophageal barrier.[12,13] Mast cell degranulation is classically induced by cross-linking of membrane-bound immunoglobulin (Ig) E by antigen and results in the release of preformed (e.g. histamine, proteases) and newly synthesized mediators (e.g. lipid mediators, cytokines). While IgE sensitization is common in EoE,[14] the exact mechanism of how food allergens cause allergic inflammation in the esophagus remains not well defined, and both IgE-mediated and non-IgE-mediated mechanisms may be involved in the pathogenesis of the disease.[15]

Herein, we hypothesized that mast cells contribute to esophageal epithelial barrier dysfunction in EoE by the release of mast cell mediators upon degranulation. For this purpose, we investigated the functional characteristics of human esophageal epithelium differentiated under air-liquid interface (ALI) conditions upon coculture with IgE-activated primary human mast cells and furthermore examined the effect of coculture on cytokine production.

5.2 METHODS

5.2.1 Esophageal epithelial cell line and primary human mast cell culture

The immortalized human esophageal epithelial cell line EPC2-hTERT (EPC2) was provided by Dr. Anil Rustgi (University of Pennsylvania, Philadelphia, PA, USA).[16-18] EPC2 were cultured in a humidified incubator at 37 °C with 5% CO_2 in low calcium ([Ca^{2+}] = 0.09 mM) keratinocyte serum-free medium (KSFM; Gibco, Waltham, MA, USA; cat. 10725-018) supplemented with epidermal growth factor (1 ng/ml; Gibco; cat. 10450-013), bovine pituitary extract (50 µg/ml; Gibco; cat. 13028-014), and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco; cat. 15140-122). EPC2 were discarded after 3 months of passages.

Mast cells were generated from human peripheral blood mononuclear cells (PBMCs) as previously described.[19] Briefly, PBMCs were obtained from buffy coats of healthy donors (Dutch Blood Bank, The Netherlands). CD34-enriched precursor cells were isolated using the EasySep Human CD34 Positive Selection Kit II (STEMCELL Technologies, Vancouver, Canada; cat. 17856), and were cultured in a humidified incubator at 37 °C with 5% CO_2 in StemSpan SFEM II medium (STEMCELL Technologies; cat. 09655) supplemented with human recombinant IL-6 (50 ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany; cat. 130-093-934), IL-3 (10 ng/ml; Peprotech, Rocky Hill, CT, USA; cat. 200-03) and stem cell factor (100 ng/ml; Peprotech; cat. 300-07). After four weeks, media was switched to IMDM Glutamax I (Gibco; cat. 31980-030) supplemented with human recombinant IL-6 (50 ng/ml), 3% supernatant of Chinese hamster ovary (CHO) transfectants secreting murine stem cell factor (gift from Dr. P. Dubreuil), 0.5% AlbuMax I (Gibco; cat. 11020-021), β-mercaptoethanol (0.055 mM; Gibco; cat. 21985-023), 1x Insulin-Transferrin-Selenium (Gibco; cat. 41400-45), Ciprofloxacin Hydrochloride (10 µg/ml; Serva, Heidelberg, Germany ; cat. 47977.01) and Amphotericin B (1.25 µg/ml; Gibco; cat. 15290-026). After another eight weeks of culture, mast cell maturity was tested based on the expression of FcεRIa and CD117 (c-KIT) by flow cytometry using BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA), and by degranulation assay (β-hexosaminidase assay) as described previously.[20] Mast cells were then used for experiments.

5.2.2 Human esophageal biopsies and databases

Publicly available bulk RNA-sequencing data set of whole EoE biopsies were obtained from The National Center for Biotechnology Information (data accessible at http://www.ncbi.nlm.nih.gov, GEO accession GSE58640),[21] and a single-cell RNA-sequencing data set of whole EoE biopsies from https://egidexpress.research.cchmc.org/.[11,22] In addition, baseline biopsy specimens from adult EoE patients with clinically and histologically (≥ 15 eos/hpf) active disease (n = 12) and adult non-EoE controls (n = 3) were obtained by endoscopic collection, as previously described.[23] Biopsies were collected in formalin for immunofluorescent staining.

5.2.3 EPC2 and mast cell coculture and barrier assessment

EPC2 were grown to confluence on polyester membrane inserts (0.4 µm pores; Corning Inc., Corning, NY, USA; cat. 3460) while fully submerged in low-calcium KSFM. Confluent monolayers were switched to high-calcium ([Ca^{2+}] =1.8 mM) KSFM for four days to induce initial differentiation.

ALI culture was initiated to induce terminal differentiation and stratification of the EPC2 by removing the media from the apical chamber for six days.

Mast cells were sensitized overnight with human IgE myeloma (1 µg/ml; Sigma-Aldrich; cat. AG30P) one day before the start of ALI culture. Mast cells were washed to remove unbound IgE, and added to the basolateral compartment of the EPC2 ALI cultures at a concentration of 0.5×10^6 cells/ml. Mast cells and EPC2 were cocultured and monocultured in a 1:1 mixture of IMDM Glutamax I and high-calcium KSFM ($[Ca^{2+}] = 1.89$ mM), and half of the media was refreshed every two days. After three days of coculture, when the EPC2 were differentiated, mast cell degranulation was induced with rabbit anti-human IgE (10 µg/ml; Dako Denmark A/S, Glostrup, Denmark, cat. A0094) or mast cells were left inactivated. Histamine levels in supernatant collected after 1.5 h were quantified by ELISA (ENZO Life Sciences Inc., Farmingdale, NY, USA; cat. ENZ-KIT140) to ensure mast cell degranulation had occurred. Barrier function was assessed by transepithelial electrical resistance (TEER) using a Millicell ERS-2 Volt-ohm meter (Merck Millipore, Burlington, MA, USA) and paracellular flux assays using 4-kDa FITC-Dextran (Sigma-Aldrich; cat. 46944) as previously described.[24] TEER was measured over time and the change in TEER relative to baseline (ALI day 3) was calculated. Paracellular flux assays were performed after the final TEER measurement. EPC2 ALI cultures were harvested for further analysis by RT-qPCR after paracellular flux assays. TEER results of preliminary experiments that were performed to determine the optimal concentration of mast cells in the coculture system are provided in Figure S1.

5.2.4 Multiplex array and ELISA

Supernatants collected 24 h after mast cell activation (ALI day 4) from the preliminary coculture experiments (Figure S1) using 0.5×10^6 mast cells per ml (2 different mast cell donors) were analyzed by Target 48 Cytokine Panel multiplex array (Olink, Uppsala, Sweden). Heatmaps were generated using Clustergrammer.[25] Then, we used ELISA on supernatants collected 24 h after mast cell activation (ALI day 4) from the final experiments using 0.5×10^6 mast cells per ml (as described above) to confirm our findings of the multiplex array for 4 different mast cell donors. Levels of IL-1β (cat. DY201), GM-CSF (cat. DY215), OSM (cat. DY295) (all from R&D Systems, Minneapolis, MN, USA), and IL-13 (ThermoFisher Scientific; cat. 88-7439-88) were measured per manufacturer's instructions.

5.2.5 Oncostatin M stimulation of EPC2

At day 3 of ALI culture, differentiated EPC2 were stimulated with recombinant human oncostatin M (OSM; R&D Systems; cat. 8475-OM) at 1 – 200 ng/ml for four days. IL-13 (100 ng/ml; Prospec, Rehovot, Israel; cat. CYT-446) was included as a positive inflammatory control.[5,24,26] Cytotoxicity of OSM was measured using the Cytotoxicity Detection Kit (Roche, Basel, Switzerland; cat. 11644793001) per manufacturer's instructions. Media plus OSM and IL-13 were refreshed every two days. Barrier function was measured by TEER at various time points. After the final TEER measurement on ALI day 6, paracellular flux assays were performed, and EPC2 ALI cultures were harvested for further analysis by RT-qPCR and immunofluorescent staining of barrier proteins.

5.2.6 RNA isolation, cDNA synthesis and real-time qPCR

RNA from EPC2 ALI cultures was treated with RNase-free DNase I (Qiagen, Hilden, Germany) and isolated using the RNeasy Mini Kit (Qiagen). 500 ng RNA was reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). PrimePCR SYBR Green assays for *DSG1* (Unique Assay ID: qHsaCED0044569), *FLG* (qHsaCED0036604), *IVL* (qHsaCED0046054), *SPINK7* (qHsaCID0038075), *CAPN14* (qHsaCID0017001) and *RPS13* (qHsaCID0038672) were purchased from BioRad. Results were normalized to ribosomal protein S13 (*RPS13*). mRNA expression levels were calculated by subtracting *RPS13* cycle threshold (Ct) from the gene of interest Ct to obtain ΔCt. Then, the medium control ΔCt was subtracted from the treatment condition ΔCt to obtain ΔΔCt. Fold change = $2^{-\Delta\Delta Ct}$.

5.2.7 Immunofluorescent and histological staining

Formalin-fixed, paraffin-embedded esophageal biopsy and EPC2 sections were deparaffinized and rehydrated. For immunofluorescent staining, antigen retrieval was induced by boiling the deparaffinized sections in sodium citrate buffer (10 mM trisodium citrate dihydrate in deionized water, set to pH=6.0 with 0.1 M citric acid) for 12 min in a microwave. After cooling down, sections were blocked in 3% bovine serum albumin (BSA; Sigma-Aldrich; cat. A9647-506) + 5% normal goat serum (Dako Denmark A/S; cat. X0907) in PBS for 90 min at RT. Then, biopsy sections were stained with a mixture of rabbit anti-IgE (10 µg/ml; Dako Denmark A/S; cat. A0094) (secondary antibody goat anti-rabbit AF594) and mouse anti-mast cell tryptase (0.1 µg/ml; Abcam, Cambridge, UK; cat. ab2378), followed by secondary antibody goat anti-mouse AF488 (10 µg/ml; Invitrogen; cat. A11001). EPC2 sections were stained with rabbit anti-DSG1 (1 ug/ml; Abcam; cat. ab209490) or rabbit anti-FLG (1 ug/ml; Abcam; cat. ab234406), followed by secondary antibody goat anti-rabbit AF594 (10 µg/ml; Invitrogen; cat. A11072). The primary antibodies were diluted in 3% BSA-PBS and were incubated overnight at 4°C. The secondary antibodies were diluted in 3% BSA-PBS and were incubated at RT for 1 h. In between antibodies, sections were washed 3 × 5 min with 0.2% Tween20 (BioRad) in PBS. After staining, sections were washed (3 × 5 min), coverslipped with ProLong Gold Antifade reagent with DAPI (Invitrogen; cat. P36931) for nuclei staining, and dried for 24 h before images were taken with a Keyence Fluorescence Microscope BZ-9000. A Leica TCS SP8 X confocal microscope (Leica Biosystems, Amsterdam, The Netherlands) was used for close-up images of tryptase$^+$ IgE$^+$ mast cells in esophageal biopsies. Images were analyzed using ImageJ software.

For histological staining of EPC2 sections, deparaffinized sections were stained in Mayer's Hematoxylin Solution (5 min; Avantor, Radnor, PA, USA), rinsed in running tap water (5 min), and stained in Eosin Y solution (2 min; Sigma-Aldrich). Stained sections were dehydrated, coverslipped with Pertex-xylene (1:1), and dried for 24 h before images were taken with an Olympus BX50 microscope (Olympus Life Science, Waltham, MA, USA).

5.2.8 Immunostained cell density analysis

Images of esophageal biopsies for the quantification of immunostained cells were taken at 20× magnification, and 1-2 biopsies were analyzed per patient. The boundaries of the epithelial area were defined manually using ImageJ software, obtaining the area of quantification (in mm^2).

Cell density per mm^2 was calculated by counting the immunostained cells in the epithelium and dividing by the area. Only stained cells in the epithelium were counted because not all paraffin-embedded biopsies included lamina propria.

5.2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.4.1 (GraphPad Software Incorporated, San Diego, CA, USA). Distribution (normality) of the data was determined with Shapiro-Wilk test, and equality of group variances was assessed using F test. Statistical significance between two groups was tested with Welch's t test (normal distribution, unequal variance) or Mann-Whitney test (non-normal distribution). Statistical significance between three or more groups was tested with one-way ANOVA (normal distribution, equal variance), Kruskal Wallis test (non-normal distribution), or, for paired data, two-way RM ANOVA (normal distribution, equal variance). Correlation analysis was performed using Spearman rank correlation.

5.3 RESULTS

5.3.1 IgE-bearing intraepithelial mast cells in human active EoE

Using immunofluorescence, we evaluated total and IgE-bearing mast cell density in the epithelium of esophageal biopsies from patients with active EoE (n = 12) and controls (n = 3). Consistent with previous reports,[12,27] intraepithelial mast cell density was increased significantly in esophageal biopsies from EoE patients compared with controls (p = 0.0044) (Figure 1B). In addition, while intraepithelial mast cells were detected in biopsies from controls (Figure 1A, top row), intraepithelial IgE-bearing mast cells were only found in biopsies from EoE patients (Figure 1A, bottom row). Similar to total mast cell density, IgE-bearing mast cell density was significantly higher in the esophageal epithelium of patients with EoE versus controls (p = 0.0132) (Figure 1C). Lamina propria mast cells could not be quantified due to variation in size and positioning of the biopsy.

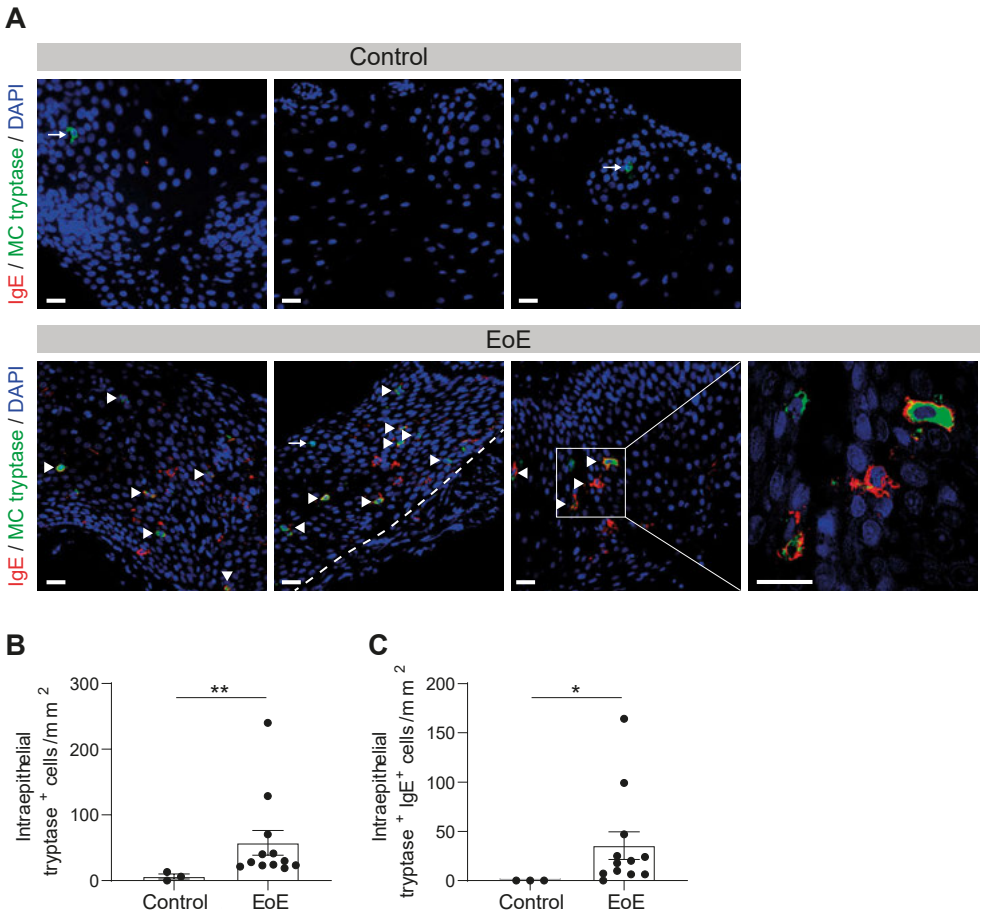


Figure 1. Intraepithelial IgE-bearing mast cells in the esophagus of EoE patients. A, Representative immunofluorescent staining for IgE (red) and mast cell tryptase (green) with a blue DAPI nuclear counterstain on esophageal biopsies from three adult non-EoE controls (top row) and three adult EoE patients (bottom row). Arrows indicate tryptase$^+$ IgE$^-$ mast cells, and arrow heads indicate tryptase$^+$ IgE$^+$ mast cells. Dashed line separates epithelium (above line) from lamina propria (below line). Scale bar = 20 µm. **B** and **C**, Comparison of intraepithelial total mast cell density (tryptase$^+$ cells) (**B**) and IgE+ mast cell density (tryptase$^+$ IgE$^+$ cells) (**C**) in esophageal biopsies from EoE patients and controls. Asterisks represent statistical significance: *$p < 0.05$, **$p < 0.01$, by Mann-Whitney test.

5.3.2 IgE-activated mast cells promote esophageal epithelial barrier dysfunction *in vitro*

Given that mast cells accumulate in the esophageal epithelium of EoE patients (Figure 1A, B), bear IgE on their cell membrane (Figure 1A, C), and undergo substantial degranulation,[12] we aimed to determine the effect of IgE-mediated mast cell activation on esophageal epithelial barrier function. We used an ALI culture model that resembles human differentiated esophageal epithelium to study the effects of mast cell activation on epithelial barrier function, as depicted schematically in Figure 2A. On day 3 of the ALI culture, when EPC2 formed a differentiated and stratified layer (Figure 2B), mast cells were activated by cross-linking of membrane-bound IgE. Analysis of histamine content in 1.5 h-supernatant confirmed mast cell degranulation in the coculture system (mean ± SD: 47.5 ± 10.1 ng/ml histamine for IgE-activated mast cells and 13.8 ± 2.3 ng/ml histamine for non-activated mast cells; $p = 0.0006$) (Figure 2C). Coculture of EPC2 under ALI conditions with IgE-activated mast cells significantly decreased EPC2 barrier resistance by 30% ($p = 0.01$) compared with non-activated mast cell cocultures, as measured by TEER (Figure 2D). Barrier permeability was evaluated by using 4kDa FITC-Dextran. Coculture of EPC2 with IgE-activated mast cells, but not non-activated mast cells, significantly increased epithelial permeability to FITC-Dextran by 22% ($p = 0.0079$) (Figure 2E), confirming the TEER results. The disruptive effects of IgE-activated mast cells on the barrier function of ALI-cultured EPC2 were associated with decreased mRNA expression of the barrier proteins FLG (3.0-fold, $p < 0.0001$), IVL (1.9-fold, $p = 0.016$) and, though not significant, DSG1 (10.6-fold, $p = 0.156$) (Figure 2F). In addition to barrier proteins, the expression of the protease regulator SPINK7 (serine peptidase inhibitor kazal type 7) was impaired as well following coculture with IgE-activated mast cels (Figure 2G). Collectively, these results indicate that IgE-activated mast cells can impair esophageal epithelial barrier function and decrease the expression of esophageal barrier proteins and antiprotease.

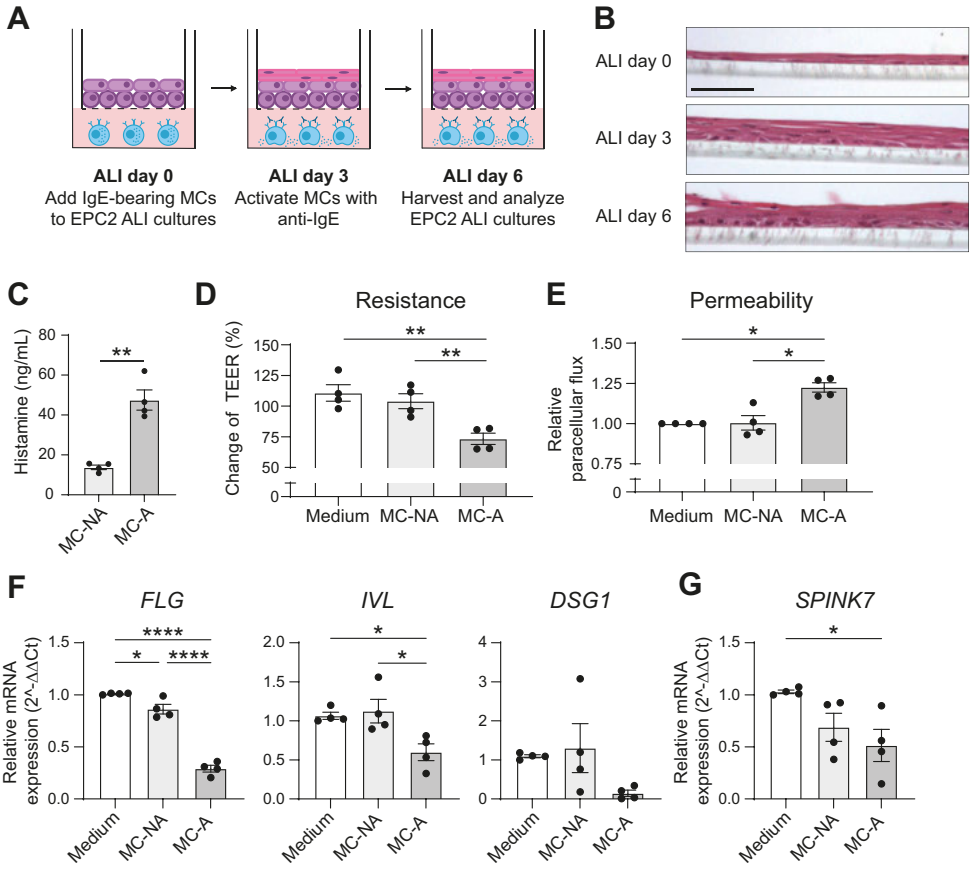


Figure 2. IgE-mediated mast cell activation induced barrier dysfunction of esophageal epithelium. A, Schematic diagram of the EPC2 ALI coculture model with mast cells. IgE-bearing mast cells were added to the basolateral compartment of EPC2 ALI cultures at the start of ALI (day 0). Following three days of coculture under ALI conditions, when the EPC2 were differentiated, mast cells were activated by cross-linking of membrane-bound IgE. EPC2 and mast cells were cocultured for another three days. **B,** Representative hematoxylin and eosin staining of EPC2 cultures at various time points and stages during the ALI protocol. **C,** Histamine content measured in the supernatant of activated mast cells (MC-A) and non-activated mast cells (MC-NA) cocultured with EPC2 collected 1.5 h after IgE-mediated mast cell activation. **D** and **E,** TEER (**D**) and paracellular flux of FITC-Dextran (**E**) of EPC2 following three days of coculture with activated or non-activated mast cells. **F** and **G,** mRNA expression of barrier proteins FLG, IVL and DSG1 (**F**), and protease regulator SPINK7 (**G**) in EPC2 following three days of coculture with activated or non-activated mast cells. Data are presented as mean ± SEM of n = 4 independent experiments performed with four mast cell donors and two technical replicates per condition. Individual symbols represent independent experiments. Asterisks represent statistical significance: *p < 0.05, **p < 0.01, ****p <0.0001, by Welch's t test, one-way ANOVA with Bonferroni's post hoc test, or Kruskal Wallis test with Dunn's post hoc test as appropriate, depending on data distribution.

5.3.3 Secreted cytokines in esophageal epithelial cell and mast cell coculture

Since mast cells and esophageal epithelial cells are in close proximity in active EoE, it is interesting to speculate that there may be intercellular crosstalk that promotes inflammation. First, we used a 45-analyte multiplex cytokine array on mono- and coculture supernatants (n = 2 different mast cell donors) collected 24 h after IgE-mediated mast cell activation to identify cytokines that a) may be responsive to intercellular crosstalk and b) may contribute to esophageal epithelial barrier dysfunction. From the 45 cytokines measured in the supernatant, 23 (51%) were within the detection limit and had at least one condition with a concentration of ≥ 1 pg/ml (Figure S2A). Cytokines that were upregulated in coculture were examined to determine if there was crosstalk between the esophageal epithelium and mast cells (Figure S2B). Next, we verified a selection of these cytokines (granulocyte-macrophage colony-stimulating factor (GM-CSF), oncostatin M (OSM), IL-13 and IL-1β) using ELISA on supernatants from the final experiments depicted in Figure 2 (n = 4 different mast cell donors). Whereas GM-CSF, OSM and IL-13 were mainly derived from IgE-activated mast cells, IL-1β was mainly derived from EPC2 (Figure 3). Two cytokines were significantly increased in IgE-activated mast cell monocultures compared with non-activated mast cell monocultures: GM-CSF (mean ± SD: 2541 ± 779 vs. 7.0 ± 6.2 pg/ml; $p < 0.0001$) and OSM (mean ± SD: 292 ± 74 vs. 44 ± 62 pg/ml; $p = 0.0074$). Interestingly, of these two cytokines, GM-CSF was robustly detected in IgE-activated mast cell monocultures and upregulated in coculture with EPC2 (mean ± SD: 2541 ± 779 vs. 3567 ± 779 pg/ml; $p = 0.0121$), suggesting that its secretion is responsive to intercellular crosstalk (Figure 3). Production of OSM, IL-13 and IL-1β appeared not affected by coculture.

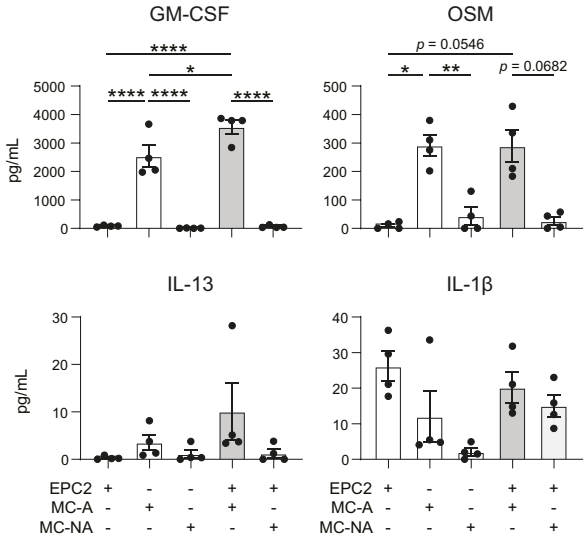


Figure 3. Cytokines in esophageal epithelial cell and mast cell coculture. Based on a 45-cytokine array (Figure S3), levels of IL-13, IL-1β, GM-CSF and OSM levels were quantified by ELISA in culture supernatants from ALI day 4 of EPC2 monocultures, activated (MC-A) and non-activated mast cell (MC-NA) monocultures, and cocultures with EPC2 and (non) activated mast cells. Data are presented as mean ± SEM of n = 4 independent experiments performed with four mast cell donors and two technical replicates per condition. Individual symbols represent independent experiments. Asterisks represent statistical significance: *p < 0.05, **p < 0.01, ****p < 0.0001, by one-way ANOVA with Bonferroni's post hoc test.

5.3.4 OSM levels are increased in the esophagus during active EoE and associate with mast cell marker genes

OSM is a member of the IL-6 cytokine family and has been shown to contribute to barrier dysfunction in the skin and lung.[28,29] Previous studies have reported increased OSM levels in psoriatic skin, sinus tissue from patients with allergic rhinitis, sputum of asthmatic patients, and in nasal polyps and fluid from patients with polypoid chronic rhinosinusitis.[29-33] One study reported increased OSM mRNA expression in esophageal biopsies from EoE patients.[29] We further studied if expression of OSM in esophageal biopsies from patients with EoE was increased. In addition, we evaluated the expression of the OSM receptors OSM receptor β-chain (OSMR) and leukemia inhibitory factor receptor (LIFR) to determine whether the esophageal mucosa also contains OSM-responsive cells. Using data from a previously published RNA-sequencing study,[21] the expression of OSM was found to be increased by 12.9-fold in esophageal biopsies from EoE patients compared with controls (mean RPKM ± SD: 0.30 ± 0.24 vs. 0.02 ± 0.02; $p = 0.0075$). Also, the expression of both OSM receptors OSMR and LIFR was increased by 5.7-fold (mean RPKM + SD: 15.22 ± 5.23 vs. 2.74 ± 0.40; $p < 0.0001$) and 4.7-fold (mean RPKM + SD: 1.70 ± 0.85 vs. 0.36 ± 0.07; $p = 0.0011$) in EoE, respectively (Figure 4A).

Next, we aimed to determine the relationship between OSM and mast cell levels. In active disease, OSM positively correlated with the mast cell marker genes CPA3 (Spearman r = 0.67, p = 0.0390) and TPSAB1 (Spearman r = 0.62, p = 0.0603) (Figure 4C, D). Furthermore, we examined a publicly available single-cell RNA-sequencing dataset of whole EoE biopsies[11,22] and found OSM-expressing mast cells during active EoE (Figure S3A), as well as OSMR-expressing (and to a lesser extent LIFR-expressing) esophageal epithelial cells (Figure S3B). Together, these data suggest that mast cells are a potential source of esophageal OSM in active EoE, and that esophageal epithelial cells may be responsive to OSM.

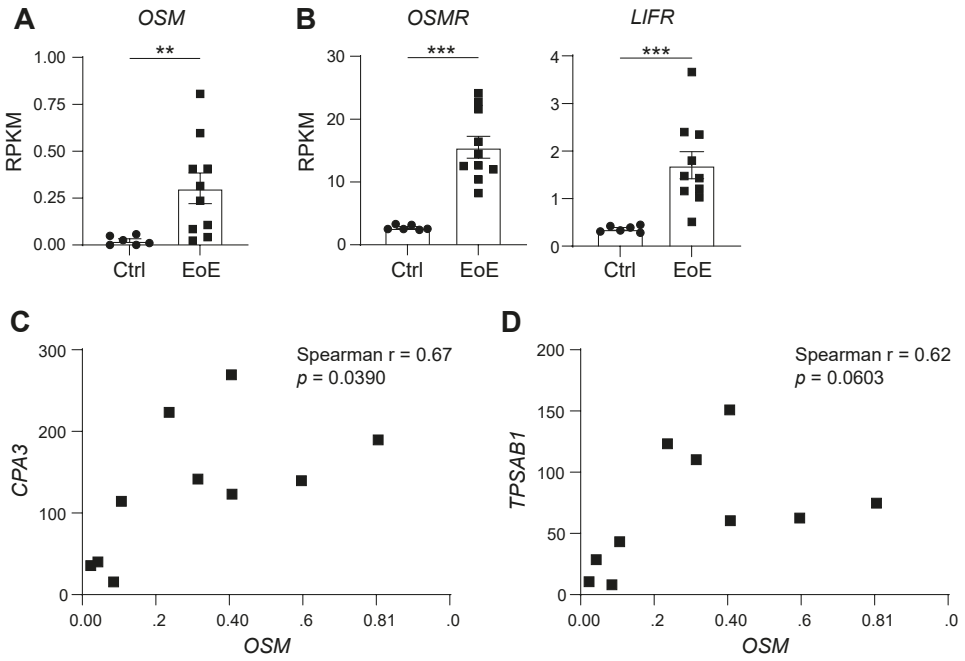
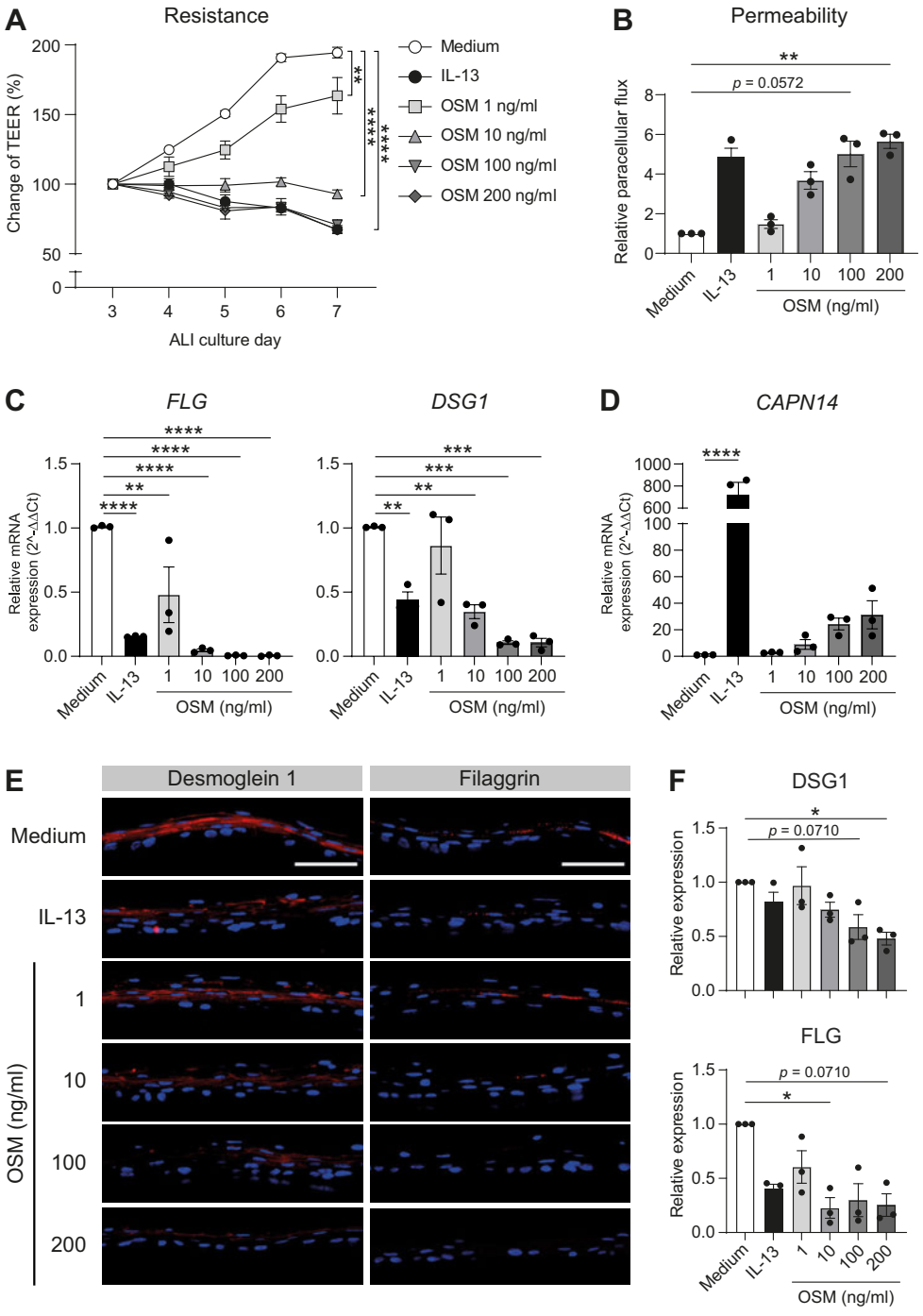


Figure 4. Levels of OSM and its receptors in the esophagus of EoE patients and association of OSM with mast cell markers. A and **B,** mRNA levels of OSM (**A**) and its receptors OSMR and LIFR (**B**) in esophageal biopsies from EoE patients (n = 10) and healthy controls (n = 6). **C** and **D**, Spearman correlation of OSM with mast cell markers CPA3 (**C**) and TPSAB1 (**D**) in active EoE. Spearman r values and P values are displayed on the figures. Data are derived from bulk RNA-sequencing of esophageal biopsies as reported previously.[21] Asterisks represent statistical significance: **p < 0.01, ***p < 0.001, by Mann-Whitney test.

5.3.5 OSM disrupts esophageal epithelial barrier function *in vitro*

Given that OSM is increased in EoE patients and esophageal epithelial cells express receptors for OSM, we next evaluated the effect of OSM stimulation on the esophageal epithelial barrier. Differentiated EPC2 ALI cultures were stimulated with concentrations of OSM ranging from 1-200 ng/ml or 100 ng/ml IL-13 as a positive control for four days. OSM stimulation of EPC2 induced a dose-dependent decrease in barrier resistance (Figure 5A), and increase in barrier permeability to 4 kDa FITC-Dextran (Figure 5B) to a similar degree as IL-13 from 100 ng/ml OSM onwards. The barrier-disrupting effects of OSM were associated with a dose-dependent decrease in the mRNA expression of the barrier proteins FLG and DSG1 (Figure 5C). Furthermore, immunofluorescent staining of these barrier proteins revealed that OSM dose-dependently disrupted their expression (Figure 5E, F). In addition, there was a dose-dependent, though non-significant increase in the mRNA expression of CAPN14 (calpain-14; Figure 5D), a tissue-specific protease that mediates esophageal epithelial barrier function.[34] Importantly, OSM did not decrease EPC2 viability as compared with IL-13 (Figure S4). Neutralization of mast cell-derived OSM with human anti-OSM in supernatants from IgE-activated mast cells partially prevented its barrier-disruptive effects (Figure S5). Collectively, these data indicate that OSM directly impairs barrier function via the downregulation of specific barrier proteins, and contributes to the barrier-disruptive effects of IgE-activated mast cells.

Figure 5. OSM decreased barrier function of the esophageal epithelium and disrupted the integrity of epithelial barrier proteins. EPC2 were grown until differentiated at ALI day 3, and then the cells were left untreated (medium) or were stimulated with OSM (1-200 ng/ml) or IL-13 (100 ng/ml) for four days. **A**, TEER measurements of EPC2 ALI cultures following OSM or IL-13 stimulation. **B**, Paracellular flux of FITC-Dextran in response to four days of OSM or IL-13 stimulation. **C** and **D,** mRNA expression in EPC2 of barrier proteins FLG and DSG1 (**C**), and protease CAPN14 (**D**) in response to four days of OSM or IL-13 stimulation. **E,** Representative immunofluorescent staining of the barrier proteins DSG1 and FLG in red with a blue DAPI nuclear counterstain in EPC2 ALI cultures stimulated with OSM or IL-13 for four days. Scale bar = 50 μm. **F**, Quantification of DSG1 and FLG protein expression from stained sections of EPC2 ALI cultures stimulated with OSM or IL-13 for four days. Data are presented as mean ± SEM of n = 3 independent experiments performed with two technical replicates per condition. Individual symbols represent independent experiments. Asterisks represent statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, by one-way ANOVA with Bonferroni's post hoc test, Kruskal Wallis test with Dunn's post hoc test, or repeated measures two-way ANOVA with Bonferroni's post hoc test as appropriate, depending on data relation and distribution.


A
Resistance
Change of TEER (%)
ALI culture day
Medium
IL-13
OSM 1 ng/ml
OSM 10 ng/ml
OSM 100 ng/ml
OSM 200 ng/ml
B
Permeability
Relative paracellular flux
p = 0.0572
OSM (ng/ml)
C
FLG
DSG1
Relative mRNA expression (2^-ΔΔCt)
D
CAPN14
E
Desmoglein 1
Filaggrin
OSM (ng/ml)
F
DSG1
FLG
Relative expression
p = 0.0710

5.4 DISCUSSION

A defective epithelial barrier has been associated with chronic inflammatory diseases such as EoE.[3] Mast cells accumulate and degranulate in the esophageal epithelium of patients with EoE,[12,27] but evidence of how this affects esophageal epithelial cells is lacking. Here, we demonstrated that IgE-activated mast cells caused significant loss of esophageal epithelial barrier function *in vitro*, which was accompanied with decreased mRNA expression of barrier proteins and an antiprotease that are commonly dysregulated in EoE. In addition, we detected various cytokines in coculture supernatants, most notably GM-CSF, which was increased in coculture, and OSM, a member of the IL-6 cytokine family. Interestingly, the expression of OSM was increased in EoE esophageal biopsies and associated with mast cell marker genes. In addition, esophageal epithelial cells express receptors for OSM in active EoE. Stimulation of esophageal epithelial cells *in vitro* with OSM resulted in a dose-dependent decrease in barrier function and expression of DSG1 and FLG, and neutralization of mast cell-derived OSM partially prevented the barrier-disruptive effects of mast cells. Collectively, these findings suggest that mast cells mediate esophageal epithelial barrier dysfunction and highlight a potential role for mast cell-derived OSM in this effect.

Accumulation of mast cells in the esophageal epithelium is an important feature of EoE,[12] where mast cells have been suggested to contribute to fibrosis, smooth muscle contraction and nerve signaling.[9,35] Here, we provide evidence that mast cells may have an additional role in the pathophysiology of EoE by decreasing barrier function upon activation by interfering with the expression of barrier proteins and antiprotease. This builds on previous reports showing that mast cells or their mediators modulate the integrity of the epithelial barrier.[36-39] Degranulation of intraepithelial esophageal mast cells may result in high local concentrations of mast cell mediators in the epithelium, and directly affect barrier function as demonstrated in this study. The mast cell-induced epithelial barrier dysfunction was accompanied by decreased expression of the epithelial differentiation proteins FLG and IVL, and desmosome DSG1. These barrier proteins are essential for maintaining an intact barrier and are downregulated in active EoE.[40,41] Besides barrier proteins, proteases and protease inhibitors closely regulate the esophageal epithelial barrier. As we report here, IgE-activated mast cells disrupted the expression of protease regulator SPINK7 in esophageal epithelial cells. In active EoE, loss of SPINK7 leads to increased proteolytic activity, epithelial barrier dysfunction, and production of proinflammatory and proallergic cytokines and chemokines by epithelial cells.[42] Epithelial barrier dysfunction induced by IgE-activated mast cells as shown in this study may also be relevant to other barrier organs that are potential sites for type 2 inflammation such as the skin, lungs and gut.

The impaired esophageal epithelial barrier as observed in active EoE could have a direct effect on mast cells. In a murine model of passive IgE sensitization to house dust mite allergen, the disrupted nasal epithelial barrier facilitated mast cell degranulation even in the absence of ongoing allergic inflammation, demonstrating that a disrupted barrier allows allergen translocation across the epithelium and consequent mast cell degranulation.[39] Continuous mast cell degranulation within the epithelium could exacerbate local inflammation by maintaining barrier dysfunction as demonstrated here, instigating a vicious cycle of leaky barriers and chronic

inflammation. Collectively, this emphasizes the importance of maintaining an intact barrier to prevent mast cell sensitization or degranulation after sensitization has occurred.

Here, we have induced mast cell degranulation in the coculture system by crosslinking membrane-bound IgE. There is debate on the role of IgE in EoE because allergen-specific serum IgE and skin prick/patch testing for EoE food triggers lack specificity,[43] anti-IgE biologicals lack efficacy in clinical trials,[44] and murine models for EoE do not require B cells or IgE to induce esophageal eosinophilia.[45,46] However, there is evidence of local IgE class switching of B cells and IgE production in the esophageal mucosa of EoE patients regardless of their atopic status.[47] In line with this, we and others[27] show IgE-bearing mast cells in the esophageal epithelium during active EoE, suggesting that local IgE-mediated mast cell activation, triggered e.g. by food antigens that translocate across the disrupted epithelial barrier, may occur. Interestingly, a role for local IgE has been demonstrated in the colon of patients with irritable bowel syndrome and in the nasal mucosa of patients with seasonal idiopathic rhinitis in the absence of systemic IgE.[48,49] Whether this concept of localized mucosal allergy in the absence of atopy also applies to EoE remains to be determined but is of great interest. While IgE sensitization is common in EoE, it is not merely an IgE-mediated food allergy and may well implicate delayed cell-mediated immune mechanisms as well.[50-52] Of note, there are other non-IgE stimuli that could activate mast cells, including cytokines and toll-like receptor ligands.[53] The fact that EoE pathogenesis is, most likely, multifactorial could also explain why therapeutic targeting of mast cells in EoE did not result in symptom improvement.[54,55]

OSM was elevated in esophageal biopsies from EoE patients and directly disrupted esophageal epithelial barrier function *in vitro*. The mechanism of OSM-mediated barrier dysfunction is currently unknown, but it is thought to involve dysregulation of the normal epithelial repair process in which epithelial differentiation and the establishment of a proper barrier does not occur.[56] Human OSM signals though two heterodimeric receptors that both use glycoprotein 130 for signaling: LIFR and OSMR.[57] OSM may exert its functions through various signaling pathways, such as the JAK/STAT, ERK1/ERK2, JNK, p38, PKCd, and PI3K/Akt pathways.[58] Macrophages, neutrophils, activated T cells, and dendritic cells are potential sources of OSM.[59-62] As we report here, mast cells produced OSM *in vitro*, there was a correlation between mast cell marker genes and OSM expression in bulk RNA-seq, and there were OSM+ mast cells in singe-cell RNA-seq of whole EoE biopsies, indicating that mast cells may be an important source of local OSM in active EoE. Increased expression of OSM has been reported in other allergic disorders such as severe asthma, allergic rhinitis and chronic rhinosinusitis.[29,31,32] In line with our data on esophageal epithelium, OSM also impaired barrier function of airway epithelium.[29] OSM is most likely not the sole mast cell-derived mediator that disrupts esophageal epithelial barrier function. Activated mast cells secrete a plethora of inflammatory mediators that may have barrier-disrupting effects, including histamine, tryptase, chymase, lipid mediators, and type 2 cytokines.

GM-CSF contributes to allergic inflammation by enhancing the survival, activation and migration of eosinophils, and by regulating the function of dendritic cells.[63-65] Eosinophils and mast cells abundantly coexist in the inflamed esophageal mucosa in active EoE.[66] Recently, Dunn *et*

al.[63]demonstrated that esophageal epithelial cell-derived GM-CSF is necessary and sufficient for a pro-survival effect on cocultured eosinophils. Here, we observed GM-CSF production by both EPC2 and mast cells in monoculture, which was significantly increased in coculture, suggesting intercellular crosstalk. Whether the EPC2, mast cells or both increased the production of GM-CSF upon coculture is currently unknown. The mast cell mediator histamine has been found to induce GM-CSF secretion from esophageal epithelial cells.[67] Conversely, soluble factors derived from the epithelial cells may fine-tune mast cell activation and inflammatory mediator production.[68] Mast cells and eosinophils are found in couplets in the esophageal epithelium in active EoE.[66] It is interesting to speculate that mast cell- or epithelial cell-derived factors, such as GM-CSF, may contribute to local inflammation in EoE by promoting eosinophil survival, activation and migration to the esophagus.

This study has some limitations. We used an immortalized human esophageal epithelial cell line as a model of differentiated human esophageal epithelium. Although there are marked transcriptional and morphologic similarities between the human esophageal epithelium and differentiated EPC2 cultured under ALI conditions,[5,26] future studies should explore the use of primary esophageal epithelial cells from EoE patients to mimic the environment of the inflamed esophagus more closely. Furthermore, we used mast cells from healthy blood donors. In future studies, it would be interesting to compare PBMC-derived mast cells from both EoE patients and healthy controls in the coculture system.

In conclusion, we demonstrated that IgE-activated mast cells induce esophageal epithelial barrier dysfunction via the downregulation of barrier proteins and antiprotease expression, which may in part be mediated by the production of OSM among other pro-inflammatory mediators. Our study suggests that mast cells may contribute to the pathophysiology of EoE by impairing the function of the esophageal barrier.

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SUPPLEMENTARY FIGURES

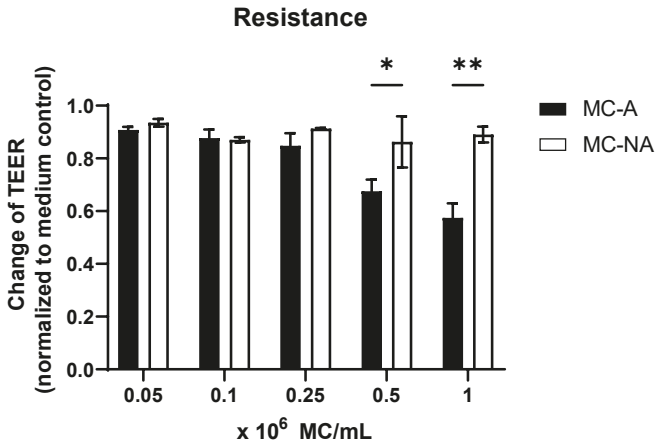

Figure S1. Effect of graded mast cell concentrations on TEER of EPC2 ALI cultures. One day prior to the start of ALI culture, mast cells were primed overnight with trinitrophenol-specific murine IgE mAb (clone 26.28). The following day, at the start of ALI, mast cells were washed and added to the basolateral compartment in graded concentrations. After three days of coculture, mast cells were activated with rat anti-mouse IgE (2 ng/ml). TEER was measured over time and the change in TEER at ALI day 6 vs. ALI day 3 was calculated. Asterisks represent statistical significance: *$p < 0.05$, **$p < 0.01$, by two- way ANOVA with Bonferroni's post hoc test.

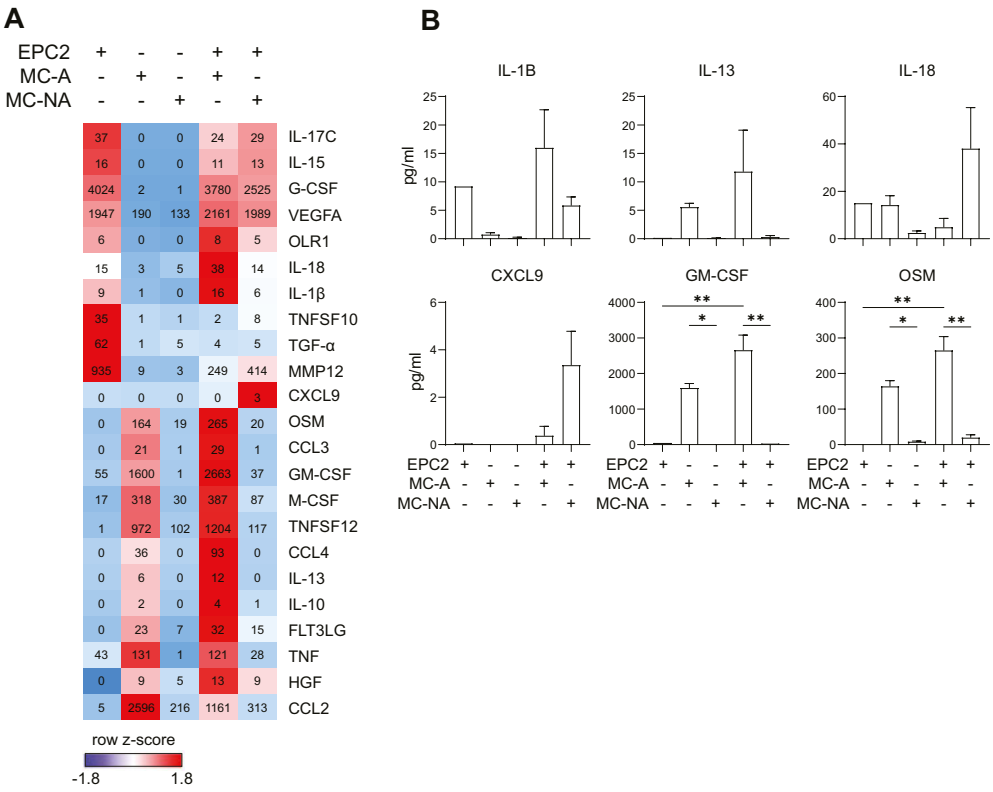

Figure S2. Cytokine and chemokine production in coculture. A 45-cytokine array (Olink, Uppsala, Sweden) was used on supernatants from our preliminary experiments as described in Figure S1. Supernatants of EPC2 monocultures, activated (MC-A) and non-activated mast cell (MC-NA) monocultures, and cocultures with EPC2 and (non)activated mast cells were collected on ALI day 4. Supernatants from 0.5 x 10^6 mast cells per ml were analyzed. **A,** Heatmap shows z-scores for the 23 cytokines that were within the detection limit and with concentrations > 1 pg/ml in at least one group. **B**, Levels of coculture-responsive cytokines as determined by the cytokine array. Data are from n = 1 (EPC2 monoculture) or n = 2 (all other conditions) performed with two mast cell donors and two technical replicates per condition, and are presented as mean ± SEM. Asterisks represent statistical significance: *p < 0.05, ***p < 0.001, by one-way ANOVA with Bonferroni's post hoc test.

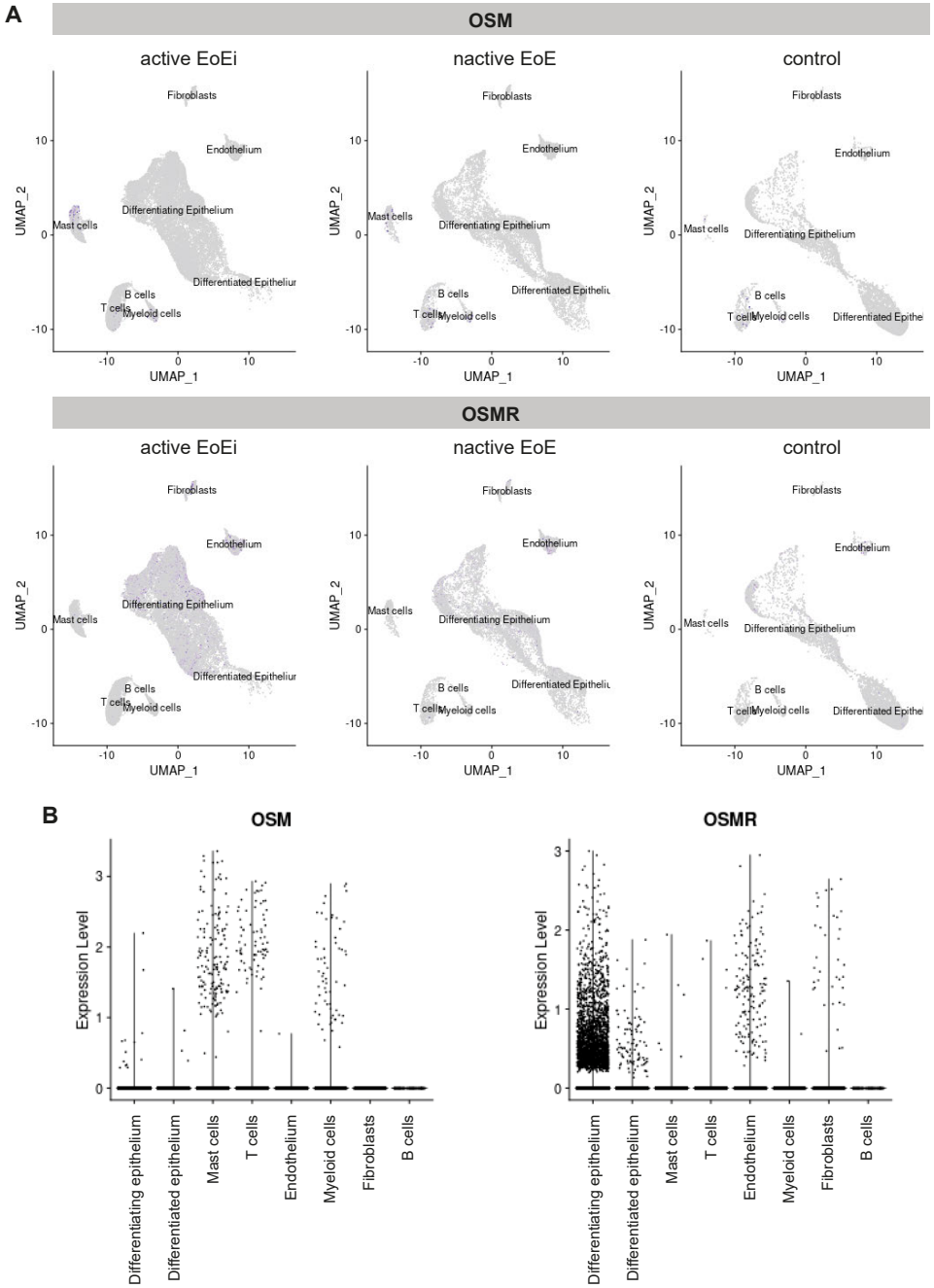

Figure S3. OSM and OSMR mRNA expression in esophageal biopsies. A and B, Feature plots (A) and violin plots (B) of OSM and OSMR expression in biopsies obtained from patients with active EoE (n = 5), inactive EoE (n = 3) and non-EoE controls (n = 2). Purple dots in A indicate positive expression. Data are derived from publicly available single-cell RNA-sequencing dataset of esophageal biopsies.[11,12]

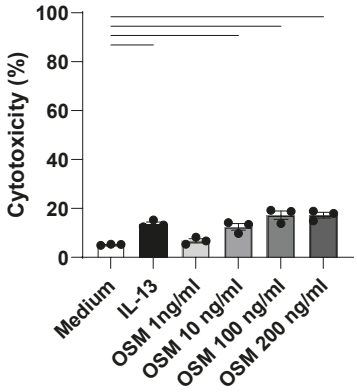

Figure S4. Cytotoxicity of OSM on EPC2 ALI cultures. LDH release by EPC2 ALI cultures following four days of OSM or IL-13 stimulation. Triton X100 was included as a positive control for maximum LDH release (100% lysis of cells). Lines indicate $p < 0.05$, by one-way ANOVA with Bonferroni's post hoc test.

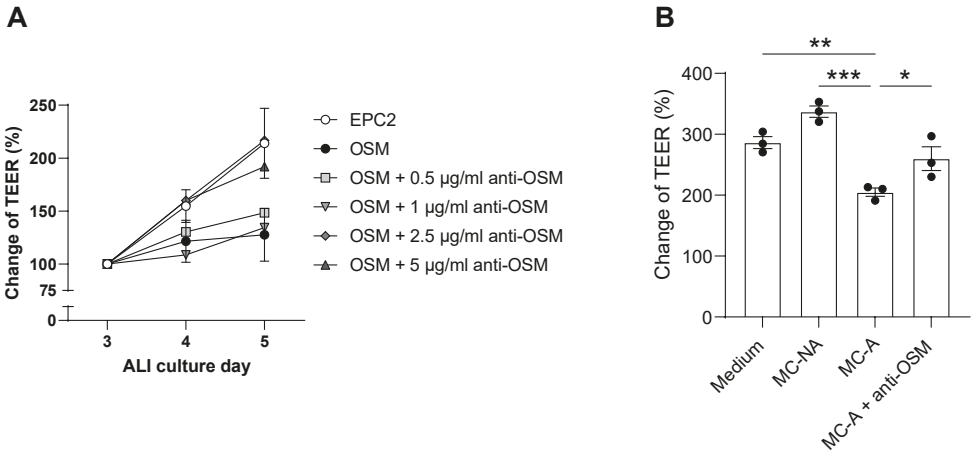

Figure S5. Neutralization of mast cell-derived OSM partially prevented the barrier-disruptive effects of mast cells on esophageal epithelial cells. A, An OSM-neutralizing antibody (R&D Systems; cat MAB295) abolished the disruptive effect of OSM (10 ng/ml) on EPC2 barrier function as measured by TEER. **B**, To evaluate the effect of mast cell-derived OSM on EPC2 barrier function, conditioned media were obtained from 1×10^6 mast cells per ml 24 h after IgE-mediated activation. Conditioned media from non-activated (MC-NA) and IgE-activated mast cells (MC-A) were added to differentiated EPC2 at ALI day 3 with or without an OSM-neutralizing antibody (12.5 µg/ml) for two days. TEER of EPC2 at ALI day 5 vs. ALI day 3 is shown. Data are representative of n = 2 independent experiments performed with three technical replicates per condition. Individual symbols represent technical replicates. Asterisks represent statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by one-way ANOVA with Bonferroni's post hoc test.

CHAPTER 6

General discussion and future directions

6.1 WHEN EOE GOES WEST

The relatively recent onset of the epidemic of allergic disorders like EoE, particularly in Western countries, leads to the question as to what may underlie its development. Multiple population-based studies have reported a rapid increase in the incidence and prevalence of EoE over the past two to three decades, but for unclear reasons.[1-4] Importantly, this drastic increase outpaces any expansion of upper endoscopies with biopsy sampling.[1,3,5,6] While inherited genetic variations can predispose an individual to develop EoE, it cannot be the only cause since not everyone with a genetic predisposition develops EoE, as evidenced by twin studies.[7,8] This underscores the involvement of environmental factors in increasing the risk of EoE. Indeed, over the last years, it has become evident that lifestyle changes due to urbanization and modernization (e.g., exposure to detergent residues from household products such as toothpaste),[9] a westernized diet (i.e., low in fruits and vegetables, and high in sugar, salt, and saturated fat),[10,11] and early-life environmental factors (e.g., birth by cesarean section and antibiotic use in infancy)[12] likely contribute to the development and/or progression of EoE.

Early infancy is a period of unique susceptibility for immune development, in which colonization of the gut microbiota is essential to establish the mucosal barrier, to develop tolerogenic immune functions, and to promote immune maturation.[13] The hygiene hypothesis proposes that the loss of certain microbes that protect against inflammatory diseases, due to modern hygiene measures, results in increased susceptibility to allergic disorders.[14] In addition, specific early-life environmental factors are believed to lead to microbial dysbiosis and subsequent dysregulation in immunity. Birth by cesarean section, and antibiotic or acid suppressant use affect the gut microbiome and are associated with an increased risk of pediatric-onset EoE.[12] The role of the esophageal microbiome in EoE development and progression is currently unknown. Independent of microbial dysbiosis, environmental factors can cause epigenetic changes that may control the initiation or maintenance of a disease.[15,16] These include changes in DNA methylation, histone modification, and microRNA levels. Environment-induced epigenetic modifications have not been explored in EoE patients, but have been suggested to contribute to the development of other allergic disorders such as asthma.[17,18]

Esophageal epithelial barrier dysfunction is a hallmark pathogenic feature of EoE.[19] Recently, Pothoven and Schleimer[20] proposed the barrier hypothesis, which postulates that epithelial barrier dysfunction precedes the development of allergic sensitization. Later, Akdis[21] extended this hypothesis by suggesting that environmental changes caused by industrialization, urbanization, and a westernized lifestyle negatively affect epithelial barriers and that this underlies the development of allergy. Examples of such environmental changes include increased daily exposure to detergents and air pollutants, and consumption of processed foods containing emulsifiers. In line with this hypothesis, Doyle *et al*.[9] demonstrated that the common household detergent sodium dodecyl sulfate (SDS) disrupted the barrier function of human esophageal epithelial cells, and elicited type 2 inflammatory signals and eosinophilia in the esophagus of mice. The potential of toothpaste containing sodium lauryl sulfate (SLS, also known as SDS) to disrupt the esophageal barrier and cause allergic inflammation is currently under investigation

in an open-label clinical trial with healthy volunteers (clinicaltrials.gov identifier: NCT05482256). Such studies are of high interest, as a disrupted esophageal epithelial barrier may promote sensitization and a type 2 immune response. If the epithelium cannot fully repair and close the barrier due to repeated exposure to allergens or environmental triggers such as SLS/SDS, a vicious circle of leaky barriers and chronic inflammation is instigated. Novel therapeutic strategies should aim at breaking this circle by restoring barrier function to prevent translocation of allergens and ensuing inflammation. In **Chapter 2**, we propose that short-chain fatty acids (SCFAs) butyrate and propionate may be of therapeutic benefit in the management of EoE due to their barrier-restorative capacities, which is further discussed later in this chapter.

6.2 THE ESOPHAGEAL EPITHELIAL BARRIER AS A THERAPEUTIC TARGET FOR THE TREATMENT OF EOE

A closed esophageal epithelial barrier serves as a protective wall against environmental factors including microbes, toxins, and antigens.[21] On the other hand, an impaired esophageal epithelial barrier allows translocation of food and aeroantigens through the epithelium, facilitating contact with the immune system to elicit a local type 2 immune response in an environmentally or genetically predisposed individual. Esophageal epithelial barrier dysfunction is an important feature of EoE pathogenesis,[19] and can be genetically inherited (e.g. genetic variations in calpain-14),[22] or acquired by exposure to environmental toxins (e.g. SLS/SDS)[9] or inflammation (e.g. IL-13).[23] Preventing the loss of esophageal epithelial barrier integrity or restoring its function once impaired could be a potential therapeutic strategy for EoE.

6.2.1 Nutraceuticals and their signaling mechanisms to target the epithelial barrier

In **Chapter 2**, we proposed that the SCFAs butyrate and propionate may have therapeutic potential for the treatment of EoE. Here, we used IL-13 – a key type 2 cytokine in EoE[24] – to disrupt the barrier function of esophageal epithelial cells, which was counteracted by butyrate and propionate. While peripheral levels of SCFAs, particularly of butyrate and propionate, may be difficult to influence due to metabolism by colonocytes and liver cells,[25] exposure from the luminal side of the epithelium through oral administration could also support the restoration of the esophageal epithelial barrier. Importantly, since esophageal transit time is rather short (1-2 seconds for liquids, 4-8 seconds for (semi)solid foods),[26] a viscous liquid that flows slower and coats the full length of the esophagus is preferred to increase contact time between SCFAs and the esophageal epithelium. The topical corticosteroid oral viscous budesonide is an example of such viscous liquid that provides full esophageal coverage, and was shown to induce and maintain remission of disease activity in children with EoE.[27,28] It would therefore be interesting to investigate whether an intervention with oral viscous SCFA formulations, either alone or in combination with other therapies, could therapeutically alleviate esophageal epithelial barrier dysfunction and ensuing inflammation. The success of this approach would require that IL-13 is the main inducer of barrier dysfunction in EoE. It is however tempting to speculate that SCFAs may provide protection against

a wider range of proinflammatory cytokines and perhaps environmental toxins, but this remains to be elucidated. Interestingly, the epithelial barrier has been therapeutically targeted with butyrate in a murine model of rheumatoid arthritis, which attenuated symptoms and partially prevented disease onset.[29]

In addition, the barrier-restorative effects of SCFAs butyrate and propionate were at least in part mediated via the inhibition of histone deacetylase (HDAC) activity (**Chapter 2**). Histone acetylation induced by histone acetyl transferases is associated with gene transcription, while HDACs remove acetyl groups from hyperacetylated histones, thereby suppressing gene transcription. In nasal epithelial cells of patients with allergic rhinitis,[30] and bronchial epithelial cells from asthmatic patients,[31] endogenous HDAC activity was increased and contributed to the development of an impaired barrier, while inhibition of HDAC activity restored barrier function and tight junction expression. It would therefore be interesting to evaluate whether altered HDAC activity contributes to barrier dysfunction in EoE, as it may represent a novel target for therapeutic intervention in EoE patients.

Interestingly, vitamin D was recently identified as a key regulator of esophageal epithelial barrier function, where vitamin D supplementation in *in vitro* and preclinical models of vitamin D deficiency attenuated histological markers of barrier dysfunction, esophageal eosinophilia, epithelial tissue remodeling and fibrosis.[32] While there has been no clinical trial conducted yet that tests the benefit of vitamin D supplementation in EoE, the preclinical data are supportive of the therapeutic potential of barrier-directed compounds.

6.2.2 Oncostatin M and epithelial barrier function

Oncostatin M (OSM), a member of the IL-6 cytokine family, has been identified as a potent inducer of barrier dysfunction in type 2 inflammatory disorders.[33] OSM levels have been found increased in several allergic disorders, including allergic rhinitis,[34] asthma,[33] and atopic dermatitis.[35] In **Chapter 5**, we demonstrated that OSM levels are increased in esophageal biopsies of EoE patients, that mast cells are a potential source of esophageal OSM in active EoE, and that OSM dose-dependently impaired esophageal epithelial barrier function. Notably, neutralizing OSM in supernatants from IgE-activated mast cells in part prevented the barrier-disruptive effects of mast cells on esophageal epithelial cells, suggesting that therapeutic targeting of OSM might be beneficial in the treatment of EoE through restoration of the esophageal epithelial barrier.

A humanized monoclonal antibody that blocks OSM has been developed for the treatment of inflammatory and fibrotic diseases, such as Crohn's disease, systemic sclerosis, and rheumatoid arthritis. However, while the anti-OSM monoclonal antibody was well-tolerated in healthy subjects, and demonstrated sufficient affinity to block OSM in the systemic circulation and target tissue,[36] phase II clinical trials in systemic sclerosis[37] and rheumatoid arthritis[38] have failed to demonstrate a benefit. Currently, there is no (pre)clinical data available on anti-OSM efficacy in allergic disorders associated with loss of barrier function. In addition, it is questionable whether therapeutic targeting of OSM alone is sufficient to restore esophageal barrier function,

considering the fact that there are many other proinflammatory mediators upregulated in EoE that have barrier-disruptive capacities, such as IL-13, IL-9, and TGF-β.[23,39,40]

6.2.3 Therapeutic challenges

Currently, there are no Food and Drug Administration-approved agents available that specifically target the epithelial barrier. Although it is without question that esophageal epithelial barrier dysfunction is an important pathogenic feature of EoE, most current clinical data are correlative, making it difficult to separate cause from effect in interpreting the importance of loss of epithelial barrier function. For example, topical corticosteroids, which are successful therapies for EoE, affect the underlying immune response while also improving esophageal mucosal integrity and barrier function.[41] Similarly, targeted dual inhibition of IL-4 and IL-13 signaling through anti–IL-4Rα blockade with dupilumab treatment suppressed cellular and molecular skin and systemic markers of type 2 inflammation, and improved markers of skin barrier function in patients with atopic dermatitis.[42,43] Whether inflammation is the primary cause of epithelial barrier dysfunction or whether it is barrier dysfunction that leads to allergen sensitization and subsequent inflammatory responses is a long-standing chicken-and-egg dilemma that well illustrates the complexity of the pathogenesis of many allergic diseases, including EoE. In any case, it surely maintains and contributes to the chronic nature of EoE by facilitating transport of allergens, environmental stimuli, and microbes. While preservation or reconstitution of the esophageal barrier function is an interesting novel domain, a deeper understanding of the mechanisms of barrier regulation and disruption in EoE is warranted for the development of such new therapeutic strategies.

6.3 IS EOE CAUSED BY A RESTRICTED LOCAL IMMUNE RESPONSE TO FOOD ALLERGENS?

Although food allergens have been recognized as a trigger of EoE, the mechanisms by which they initiate or maintain EoE remain ill-defined. Understanding the pathogenic role of food allergens in EoE is a prerequisite for the development of therapeutic strategies.

6.3.1 Local antigen deposition: implications for sensitization

One of the possible mechanisms by which food antigens may initiate inflammation is by penetration of the impaired esophageal epithelial barrier, uptake and presentation by an antigen presenting cell (APC), and activation of disease by cellular, humoral, and cytokine-mediated pathways. This mechanism is strongly suggested by documenting the presence of food[44,45] and aeroantigens[46] within the esophageal epithelium of active but -less so- of inactive EoE patients, likely facilitated by impaired barrier function. This may allow local antigen presentation by dendritic cells and other APCs, such as epithelial cells, promoting Th2 cell skewing in an (often) already atopic individual.[47] When coupled with the observation that food[48] and aeroallergens[48,49] can drive EoE-like inflammation in mice, and the fact that patients respond well to elemental and empirical food elimination diets,[50] it is tempting to speculate that local antigen exposure could

be integral to the instigation and/or progression of EoE. However, it is currently unknown, but of high interest, whether the antigens that are found within the esophageal epithelium represent EoE triggers.

In the face of epithelial damage or activation, the epithelial alarmins thymic stromal lymphopoietin (TSLP) and IL-33 are produced and secreted, promoting chemotaxis and activation of dendritic cells, mast cells, and group 2 innate lymphoid cells (ILC2s).[51-54] Chronic antigen exposure due to a disrupted barrier drives pathogenic effector Th2 (peTh2) cell differentiation. peTh2 cells may respond to local antigens that are being presented by dendritic cells or other APCs, and promote eosinophil recruitment and barrier dysfunction by the production of IL-5 and IL-13.[55] Although (pe)Th2 cells are an important source of type 2 cytokines (IL-4, IL-5, IL-9, and IL-13), it is now also clear that ILC2s that lack antigen-specific receptors are an important and much earlier source of type 2 cytokines.[56,57] IL-4 drives B cell class switching to IgE,[58] resulting in the production of allergen-specific IgE antibodies, which then bind to their high-affinity receptor on the surface of esophageal mast cells (**Chapter 5**) and basophils. Mast cells and basophils degranulate in response to IgE cross-linking by local antigen and release preformed mediators and newly synthesized cytokines with ensuing barrier disruption (**Chapter 5**), smooth muscle contraction, fibrosis, and nerve signaling.[59,60] IL-5 promotes eosinophil recruitment and activation.[61] IL-9 promotes mast cell expansion and function, and causes ILC2 activation.[62] IL-13 can directly cause esophageal epithelial barrier dysfunction by disrupting barrier protein expression and dysregulating the protease/antiprotease response, and induce eotaxin-3 production by esophageal epithelial cells to promote eosinophil chemotaxis to the esophagus.[23,24,63] If left untreated, the local inflammatory milieu promotes barrier dysfunction, enhancing chronic antigen exposure and type 2 inflammation, which ultimately leads to esophageal remodeling.

6.3.2 Mast cell activation and a potential pathogenic role for local IgE

Since the first report on mast cell accumulation in the esophagus of patients with EoE in 2001,[64] subsequent studies have proposed a pathogenic role for mast cells in EoE related to fibrosis, smooth muscle contraction, and nerve signaling.[59,60] In **Chapter 5**, we propose an additional role for mast cells in the pathophysiology of EoE by decreasing barrier function upon activation. We demonstrated that IgE-activated mast cells caused significant loss of esophageal epithelial barrier function *in vitro*, which was accompanied with decreased mRNA expression of barrier proteins and an antiprotease that are commonly dysregulated in EoE. There is compelling evidence of local mast cell activation in EoE,[65] but how these mast cells are activated remains elusive. Local IgE antibodies bound to mast cells have been found in esophageal biopsies from both children[66] and adults[67] (**Chapter 5**) with EoE, regardless of the atopic status. This suggests that cross-linking of IgE by food antigens may occur. However, it is currently unknown if the IgE on mast cells is specific to triggers of EoE. In any case, cross-linking of membrane-bound IgE by antigen seems plausible considering the presence of food and aeroantigens in the esophageal epithelium of EoE patients.[44-46] Other triggers of mast cell activation include granule proteins from eosinophils (e.g. major basic protein), cytokines, and pathogen-associated molecular patterns.

Despite the ineffectiveness of anti–IgE biologics to improve symptoms,[68,69] and the inability of the skin prick test (SPT) and serum IgE measurements to reliably identify EoE trigger foods,[70] a pathogenic role for IgE in EoE should not (yet) be refuted. Local B cells appear to be generating IgE,[66] which is substantiated by our data demonstrating *de novo* IgE synthesis by biopsy tissue from EoE patients but not controls (**Chapter 3**). In **Chapter 3**, we observed acute mucosal responses to food injections in EoE patients characterized by edema, erythema, and smooth muscle contraction (determined by the formation of a muscular ring). Some patients had symptoms following mucosal injections that are comparable with the recently described FIRE (food-induced immediate responses of the esophagus) concept.[71] These responses are characterized by an intense, painful sensation occurring within 5 min after ingesting certain foods or beverages, such as fruits, vegetables, and wine. Upon activation, mast cell mediators can induce smooth muscle contraction, vascular permeability (which can result in esophageal edema) and pain senstation.[59,60,72] When coupled with the observation that IgE-bearing mast cell numbers are increased in the esophageal epithelium of EoE patients (**Chapter 5**),[67] there may be a role for allergen-specific IgE-mediated mast cell degranulation in the acute esophageal responses to food injections described in **Chapter 3**. Nevertheless, the definitive establishment of a pathogenic role for IgE-mediated mast cell activation in acute esophageal responses to food allergens would require evidence of mast cell activation just after challenging esophageal tissue with a confirmed food trigger of EoE, as well as evidence of allergen co-localization to IgE^+ mast cells, and this remains to be demonstrated.

Nonetheless, murine models of EoE do not require B cells or IgE to induce esophageal eosinophilia, suggesting that IgE may not be involved in the initiation of EoE.[73,74] It is likely that EoE is a mixed IgE/non-IgE-mediated food allergy, in which some acute symptoms – as those seen after local injection of food antigens in **Chapter 3** – may be associated with IgE but the chronic eosinophilic inflammation may not be as dependent on IgE. This is supported by a study by Warners *et al.*[75] that demonstrated acute as well as delayed mucosal responses to food antigen injections in EoE patients but not controls. Considering the observation that there was no overlap between the foods that induced an acute response and the foods that induced a delayed response, it becomes plausible that different food allergens can trigger different (i.e., immediate and delayed) immune mechanisms in the esophagus of EoE patients.

Regardless of the multiple independent lines of evidence that underscore a pathogenic role for mast cells in EoE, clinical trials testing mast cell-directed therapeutics for EoE have been disappointing in terms of symptom improvement.[76,77] It is therefore possible that targeting mast cells alone is ineffective at inducing clinical and histological remission, or is only effective for specific endotypes of EoE. Furthermore, this implies that there are other cellular players involved in the pathogenesis of EoE.

6.3.3 Local food allergen exposure to study immune activation

Exposure of the esophageal mucosa to food allergens can cause acute visible mucosal responses in EoE patients, such as edema, erythema, and smooth muscle contraction (**Chapter 3**). The fact that these food-induced acute esophageal responses could be responsible for painful esophageal symptoms and potentially exacerbate esophageal inflammation, stresses the need for better understanding of the cellular and molecular processes mediating such reactions. Therefore, in **Chapter 4**, we studied the early transcriptional response of the esophageal mucosa to food allergen stimulation. We identified 40 genes with a potential role in the early immune response to food allergens (most notably *IL1B*, *CEBPB*, *TNFSF18*, *SLC15A3*, and *PHLDA2*), which were enriched in processes related to immune activation, such as the acute phase response, the cellular response to external stimuli, and cell population proliferation. *TNFSF18* (also known as GITRL) was most prominently upregulated during an acute response to food injection: 12-fold compared with baseline, 18-fold compared with negative visible mucosal response. Interestingly, TNFSF18 appears to play a role in other atopic conditions such as asthma[78] and atopic dermatitis.[79,80] It is tempting to speculate that TNFSF18 may mediate interactions between TNFSF18-expressing cells including esophageal epithelial cells, and TNFRSF18/GITR-expressing cells including T cells and ILC2s during an acute mucosal response to food in EoE patients to promote inflammation. Further in-depth analysis of how TNFSF18 potentiates acute mucosal response to food and contributes to EoE pathogenesis in needed. In the future, the TNFSF18-TNFRSF18 pathway may be of therapeutic interest for EoE, as it may become in asthma.[78]

Another, perhaps more clinically feasible way of studying the food-induced local immune response in EoE is by exposing esophageal biopsies to food in culture, as described in **Chapter 3**. By doing so, an immunological response is triggered that may reflect the inflammatory cascade seen in EoE patients, as these biopsies contain all different cell types involved in the pathophysiology of EoE.[81] This allowed us to study the food-induced immune response as measured by cytokine levels. Several studies have explored the utility of gut and lung biopsy-based models to study allergen-induced immune responses in short term *ex vivo* cultures.[82-84] Interestingly, levels of IL-5 and IL-9 were significantly increased in biopsy culture supernatants from EoE patients upon exposure to milk and apple, respectively (**Chapter 3**). This raises the question of the cellular source(s) of IL-5 and IL-9 as well as their contribution to the pathophysiology of EoE. IL-5 is produced by eosinophils, mast cells, and Th2 cells. It mainly affects eosinophils by regulating their expansion, survival, and migration, and by priming them to respond to specific signals.[61] IL-9 exacerbates allergic responses by promoting mast cell expansion and function,[62] and has been shown to directly disrupt the barrier function of stratified primary esophageal epithelial cells.[39] Of interest, eosinophil and non-eosinophil inflammatory cells that are adjacent to mast cells were found to produce IL-9 in active EoE esophageal tissue.[85] To our knowledge, we are the first to report food-specific induction of IL-5 and IL-9 in the inflamed esophagus of EoE patients, highlighting a potential role for both cytokines in the allergen-specific immune response in EoE. In terms of future directions, the effect of food allergen stimulation on the local immune response in esophageal biopsies should be further examined by immunohistochemistry, flow cytometric analysis of cellular activation markers, and (single-cell) RNA-sequencing. Ultimately, this biopsy-based model

may be useful for evaluating the inflammatory status of esophagus in response to treatment, or for preclinical testing of medication or nutraceuticals.

The insights obtained from the transcriptome analysis of the acute visible mucosal response to food injections, and the *ex vivo* food allergen stimulation of esophageal biopsies together provide convincing evidence that food allergens can indeed induce local immune responses in EoE. A major strength and advantage of the esophageal allergen injection and *ex vivo* stimulation methods is that they provide the opportunity to characterize the immune response just after stimulation with an EoE trigger food. Such kind of studies will provide new insight into the immune mechanisms that underly EoE, as most studies thus far have mainly focused on the chronic immune response by comparing active vs. inactive disease.[86-90] This could potentially lead to the identification of new therapeutic targets for EoE.

6.3.4 Lessons learnt from hypersensitivity reactions of the nasal mucosa and skin

EoE might be a form of localized allergy. This concept of localized allergy in the absence of systemic atopy was first described in 1975 for allergic rhinitis patients with negative skin prick tests (SPT),[91] which is now considered a distinct variant of allergic rhinitis, termed local allergic rhinitis (LAR).[92,93] Subsequent studies have confirmed the presence of allergen-specific IgE in nasal secretions of LAR patients after natural exposure to aeroallergens[93,94] and nasal allergen challenge[94-98] in the absence of systemic IgE to the same allergen. Similarly, there is evidence of local IgE class switching of B cells and local IgE production in EoE patients irrespective of their atopic status.[66] Yet, the specificity of local IgE in EoE remains to be elucidated. In any case, similar to the nasal epithelium in LAR patients, the esophageal epithelium in EoE patients may be a site for the initiation and development of humoral responses, which provides an explanation for the dissociation between SPT results and serum IgE measurements, and confirmed triggers of EoE.[66]

Furthermore, EoE shares similarities with dermatoses that are due to T cell responses of the skin independent of IgE.[99] It is therefore logical to consider allergen-induced T cell-mediated mechanisms for the pathogenesis of EoE as well. In patients with food-induced exacerbations of atopic dermatitis (AD), relevant food allergen-specific T cells in the peripheral blood as well as the skin have been detected.[100,101] Furthermore, positive atopy patch tests to aero- and food allergens can be detected in the absence of corresponding IgE-mediated responses.[102] However, the atopy patch tests is of limited value in the search for trigger foods of EoE,[70] perhaps due the fact that here the skin and not the esophagus is tested. To date, the presence of local food allergen-specific T cells in EoE has not been demonstrated, although there are some first indications of a potential food-specific T cell receptor repertoire.[103]

6.3.5 Evidence of peripheral markers of allergen-specific immune activation in EoE

There are several independent lines of evidence of peripheral markers of allergen-specific immune activation in EoE, which are all T cell-related.[55,104-106] Morgan *et al*.[55] demonstrated that peripheral GPR15$^+$ peTh2 cells were enriched among milk-reactive CD4$^+$ T cells in patients with

milk-triggered EoE, suggesting that these cells are an expanded, food allergen-specific population with enhanced esophagus homing potential. Furthermore, Dellon *et al*.[105] and Cianferoni *et al*.[104] demonstrated an increase in the proliferation of peripheral allergen-specific Th2 cells upon stimulation with a confirmed food trigger in adult and pediatric EoE patients, respectively. Dilollo *et al*.[106] demonstrated that milk-induced IL-4 production by peripheral memory Th cells most accurately predicts milk-triggered EoE. While it is currently not fully understood how and where allergic sensitization occurs in EoE, these results raise the possibility that a more systemic process of sensitization might be involved. Nonetheless, from these studies it cannot be excluded that EoE is a local disorder in which specific immune factors 'leak' to the periphery.

6.4 TOWARDS PERSONALIZED DIETARY TREATMENT

Long-term maintenance therapy targeted at symptom relief and histologic resolution of eosinophilic inflammation reduces the risk of food impactions, and is associated with improved quality of life.[107-109] Dietary therapy is an attractive choice to attain medication-free disease control for many patients, as it allows them to tackle the root cause of their disease.[110] It is beyond doubt that the identification and subsequent elimination of only the trigger food (groups) is the preferred treatment option for EoE, as strict empiric elimination diets with systematic reintroduction substantially impact the patient's quality of life.[111] However, the inability to identify food triggers before elimination is currently a major limitation of dietary therapy of EoE, and new methods to identify these triggers are urgently needed.

Since EoE might be a form of local mucosal allergy, food challenge tests that focus on the true location of the immune response – that is the esophageal mucosa – and not the skin or serum may be the way to go to develop reliable tests for the identification of trigger foods for targeted elimination diets. This was first addressed in a study by Warners *et al*.[75] that demonstrated immediate mucosal blanching and/or total luminal obstruction or a delayed wheal or flare reaction after mucosal injection of specific foods in EoE patients. Interestingly, the sensitizations identified through these local food injections poorly corresponded with sensitizations identified through SPT and serum IgE,[75] suggesting that esophageal challenge may indeed be needed for a better prediction of the causative foods. In **Chapter 3**, we confirmed the observations of Warners *et al*.[75] that esophageal challenge by food injections can trigger immediate esophageal responses in adult EoE patients. Since these injections are rather invasive for the patient, we also tested whether flushing the esophagus with a highly allergenic mixture of fresh foods could induce esophageal responses to a similar degree, but responses were barely notable. Given the moderate responsiveness to esophageal challenge by flush, the clinical challenges associated with injections, and the invasiveness for patients as endoscopic challenge can induce short-lasting but severe symptoms, both challenge tests will not likely become a useful test in clinical practice.

Therefore, we also explored the usefulness of a less-invasive *ex vivo* food challenge method using esophageal biopsies to identify trigger foods of EoE (**Chapter 3**), as these biopsies could

be collected during routine endoscopy for disease monitoring. The accuracy rate for the biopsy-based approach was 64%, and although this rate is not perfect, it is higher than the 50% accuracy rate reported for SPT and serum IgE, suggesting that there may be clinical utility in this approach. Our results should be considered as a first step in the development of a local assay to aid in the identification of EoE trigger foods. Future studies should use clinically proven EoE trigger foods to increase the accuracy of the assay, and standardize the assay by normalizing for cellular composition.

As also noted earlier in this chapter, there are multiple studies that aimed to determine peripheral markers of allergen-specific immune activation in EoE, which would be considered a minimally invasive approach as it solely uses peripheral blood samples.[104-106] Both studies by Cianferoni *et al*.[104] and Dilollo *et al*.[106] demonstrated milk-reactive peripheral T cells in pediatric and adult patients with milk-induced EoE, respectively, but further studies are needed to determine whether this type of T cell assay also applies to other EoE trigger foods. Interestingly, Dellon *et al*.[105] tested the applicability of multiple allergens (milk, wheat, egg, soy, and peanut) in a T cell proliferation assay combined with food-specific esophageal IgG4 levels. Accuracy rates were between 53-75%, which is higher than previously reported for SPT alone,[112] but false-positive and/or negative results remain. In the subsequent prospective pilot trial, an individualized assay-based elimination diet improved eosinophil counts, endoscopic severity, and symptoms of dysphagia. However, a smaller than expected number of patients (~20%) achieved histologic remission of <15 eos/hpf.[105] This suggests that remission will only occur if all EoE trigger foods are eliminated from the diet, and underscores the importance of a high sensitivity rate (few false-negative results) for testing modalities that aim to identify EoE trigger foods to guide elimination diets. Importantly, when searching for minimally invasive peripheral biomarkers of EoE, one should keep in mind that EoE patients often suffer from other atopic disorders, which greatly complicates the identification of suitable biomarkers.

Without question, a patient-centered and individualized approach is highly desirable for the (dietary) treatment of EoE. Although there are some promising novel testing modalities for trigger foods of EoE in the pipeline, their usefulness to guide dietary therapy needs further evaluation before they can be used routinely.

6.5 CONCLUDING REMARKS

Eosinophilic esophagitis is an emerging, immune-mediated chronic disease that has a substantial impact on the quality of life of patients. In this thesis, we conducted *in vitro* experiments with esophageal epithelial cells, *ex vivo* experiments with esophageal biopsy tissue, and a clinical study to uncover the local immune responses that underly EoE to increase our understanding of its pathophysiology and to improve (dietary) management. Briefly, the scientific advances described in this thesis include:

- The SCFAs butyrate and propionate can counteract IL-13-induced esophageal epithelial barrier dysfunction and may be of therapeutic benefit in the management of EoE (**Chapter 2**).

- Results of food challenge using esophageal biopsies provide distinct results from SPT and serum IgE measurements, and may better reflect clinical response to food exposure. Upon further development and validation, this biopsy-based *ex vivo* model of local food challenge may be used for studying the food-induced local immune response in EoE and identifying EoE trigger foods to guide elimination diets (**Chapter 3**).

- Genes that are associated with a food-induced acute visible mucosal response are enriched in biological processes related to activation of the immune system. Among the associated genes, TNFSF18/GITRL was most highly upregulated (**Chapter 4**).

- IgE-activated mast cells can disrupt the function of the esophageal epithelial barrier, which may in part be mediated by the proinflammatory cytokine OSM (**Chapter 5**).

Overall, with this thesis we provide unprecedented insight into the local (food-induced) immune responses in EoE. In addition, our findings are a step towards the dietary management of the disease. Future studies should:

- study the potential of barrier-targeting compounds, such as SCFAs, to therapeutically alleviate esophageal barrier dysfunction and ensuing inflammation.

- continue to unravel the local food-induced immune responses in EoE as it may lead to the identification of novel therapeutic targets.

- further refine the biopsy-based ex vivo food allergen stimulation method to evaluate its usefulness to a) aid in the identification of EoE trigger foods and b) study the local food-induced immune response.

- identify the role of TNFSF18/GITRL in the food-induced immune response in EoE to evaluate whether the TNFSF18-TNFRSF18 pathway may provide a new therapeutic target for EoE.

- explore in detail whether there is a role for local IgE and IgE-mediated mast cell activation in the pathophysiology of EoE.

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ADDENDUM

Nederlandse Samenvatting

Lekensamenvatting

List of Publications

About the Author

Dankwoord - Acknowledgements

NEDERLANDSE SAMENVATTING

Eosinofiele oesofagitis (EoE) is een steeds vaker voorkomende chronische ontstekingsziekte van de slokdarm die wordt veroorzaakt door een allergische reactie op specifieke voedsel- en (in mindere mate) inhalatieallergenen. Klinisch karakteriseert EoE zich door symptomen van slokdarmdysfunctie, zoals problemen met slikken (dysfagie) en voedsel dat vast blijft steken in de slokdarm (voedselimpacties). Histologisch kenmerkt EoE zich door een sterke infiltratie van eosinofielen in het slokdarmepitheel (>15 eosinofielen per high power field (± 0,3 mm^2)). Nadat het eerste geval van EoE in 1978 werd beschreven, werd het begin jaren negentig erkend als een unieke ziekte-entiteit. Sindsdien wordt er met name in welvarende, westerse landen een sterke toename van het aantal nieuwe EoE-patiënten per jaar gezien. Inmiddels is EoE uitgegroeid tot een belangrijke hoofdoorzaak van klachten in de slokdarm bij kinderen en volwassenen.

De afgelopen 25-30 jaar is er veel inzicht verkregen in het ziektebeloop en de pathofysiologie van EoE. Desalniettemin zijn er nog talloze belangrijke vraagstukken gerelateerd aan de preventie, fenotypering, diagnostiek en behandeling van EoE die onderzocht en verbeterd moeten worden. Een beter begrip van de onderliggende ziektemechanismen ligt hierbij vaak ten grondslag. In dit proefschrift zijn daarom de onderliggende allergeen-geïnduceerde mechanismen van EoE onderzocht. Daarnaast hebben we onderzocht of slokdarmprovocatietesten kunnen helpen bij het identificeren van triggervoedingsmiddelen en hebben we het therapeutisch potentieel van korte-keten vetzuren (KKVZ) op de epitheelbarrière van de slokdarm getest, gezien de gestegen interesse in dieetbehandelingen voor EoE.

Behandeling van EoE

Barrièredysfunctie van het slokdarmepitheel is een belangrijk kenmerk van EoE. Het opnieuw sluiten van de slokdarmbarrière kan therapeutisch interessant zijn omdat het de doordringing van allergenen en de daaruit voortvloeiende ontstekingsreacties in de slokdarm potentieel kan voorkomen. Nutraceutische middelen, zoals KKVZ die dit soort barrière-sluitende capaciteiten bezitten, kunnen een veelbelovend hulpmiddel zijn in de behandeling van EoE. In het eerste experimentele hoofdstuk, **hoofdstuk 2**, besteden we daarom aandacht aan de effecten van KKVZ, met name acetaat, propionaat en butyraat, op een barrière van slokdarmepitheelcellen. In dit hoofdstuk gebruikten we een *in vitro* 'air-liquid interface' kweekmodel van gedifferentieerd slokdarmepitheel om te bestuderen of deze KKVZ de barrièrefunctie kunnen herstellen na ontregeling door IL-13, een cytokine met een belangrijke rol in EoE. Onze resultaten lieten zien dat butyraat en propionaat, in tegenstelling tot acetaat, de barrièrefunctie herstelden na de inflammatoire trigger IL-13. Deze effecten waren hoogstwaarschijnlijk onafhankelijk van de KKVZ-receptoren GPR41, GPR43 en GPR109a, maar ten minste voor een deel afhankelijk van het remmen van de activiteit van histondeacetylase (HDAC) door butyraat en propionaat. Hiermee laten we zien dat KKVZ wellicht van therapeutische interesse kunnen zijn in het voorkomen of behandelen van EoE.

De identificatie en eliminatie van alleen de triggervoedingsmiddelen is de voorkeursbehandeling van EoE. Verschillende studies hebben laten zien dat het elimineren van

de triggervoedingsmiddelen inderdaad effectief is in het verminderen van symptomen. Echter is het nog steeds heel moeilijk voor patiënten en hun behandelende artsen om erachter te komen welke voedingsmiddelen een trigger van EoE-klachten zijn. Allergietesten zoals de huidpriktest en IgE metingen in het serum zijn namelijk niet voorspellend genoeg, mogelijk omdat de allergische reactie zich alleen in de slokdarm afspeelt. In **hoofdstuk 3** beschrijven we daarom drie verschillende slokdarmprovocatietesten die zouden kunnen helpen bij het identificeren van triggers en hierdoor een persoonlijk dieet mogelijk maken. Daarnaast vergeleken we de uitkomsten met die van de huidpriktest en serum IgE metingen. De eerste test was een zogenaamde slokdarmpriktest, waarbij zes verschillende voedselextracten in de mucosa van de slokdarm werden geïnjecteerd tijdens een endoscopie. Dit gaf in 8 van de 11 patiënten een acute visuele reactie van de slokdarmmucosa die werd gekarakteriseerd door oedeem, emfyseem of spiercontracties op de plaats van injectie. Om de natuurlijke manier van blootstelling aan voedingsmiddelen beter na te bootsen, werd ook getest of een mix van voedingsmiddelen, die als een soort douche langs de slokdarmwand werd gespoten, mucosale reacties kan uitlokken. Echter was de respons matig in vergelijking met de intramucosale injecties.

Er was geen duidelijke relatie tussen de voedingsmiddelen die een reactie in de slokdarm uitlokten en de voedingsmiddelen die een positieve huidpriktest of serum IgE veroorzaakten. Daarnaast zijn de huidpriktest en serum IgE niet goed voorspellend van EoE triggers. Samen suggereert dit dat provocatie van de slokdarm inderdaad nodig zou kunnen zijn voor de betere voorspelling van triggers. Desalniettemin zullen de bovengenoemde slokdarmtesten zeer waarschijnlijk in de praktijk niet toegepast kunnen worden omdat ze vrij invasief zijn en kortdurende maar pijnlijke klachten kunnen veroorzaken in patiënten.

Daarom hebben we als laatste in **hoofdstuk 3** ook een relatief minder invasieve methode getest waarbij biopten *ex vivo* werden blootgesteld aan voedselextracten en cytokines in het supernatant werden gemeten. Interessant was dat de proinflammatoire cytokines IL-5 en IL-9 duidelijk meer werden geproduceerd na stimulatie van biopten met respectievelijk koemelk en appel. Multivariabele analyse van de cytokineniveaus in het supernatant door middel van 'machine learning' onthulde dat de *ex vivo* test potentiële triggervoedingsmiddelen beter kan onderscheiden van potentieel 'veilige' voedingsmiddelen dan de huidpriktest en serum IgE metingen. De resultaten beschreven in dit hoofdstuk suggereren dat provocatie van de slokdarm de klinische respons op voedingsmiddelen in mensen met EoE beter reflecteert dan de huidpriktest en serum IgE. Daarnaast benadrukken ze de potentie van de *ex vivo* provocatiemethode om gebruikt te kunnen worden om allergeen-geïnduceerde immuunreacties te onderzoeken en triggers van EoE te identificeren.

Nieuwe inzichten in de pathofysiologie van EoE

Zoals in hoofdstuk 3 beschreven staat, kan blootstelling van de slokdarm aan voedselallergenen acute visuele reacties veroorzaken in EoE patiënten. Het feit dat deze acute reacties verantwoordelijk kunnen zijn voor pijnlijke slokdarmklachten en potentieel de ontsteking van de slokdarm kunnen verergeren, benadrukt de behoefte aan het beter begrijpen van de onderliggende cellulaire en moleculaire processen. Daarom hebben we in **hoofdstuk 4** het genexpressielandschap van de

acute visuele reactie op lokale allergeeninjecties in kaart gebracht. Hiervoor hebben we RNA-sequencing gedaan op biopten die waren afgenomen voor en 20 minuten na de slokdarmpriktest. Onze resultaten lieten zien dat lokale allergeeninjecties de expressie van 40 'vroege EoE genen' (met name *CEBPB*, *IL1B*, *TNFSF18*, *PHLDA2*, and *SLC15A3*) triggeren die gerelateerd zijn aan de activatie van het immuunsysteem, zoals de acute fasereactie en cellulaire reactie op externe stimuli. Van deze 40 vroege EoE genen was de genexpressie van TNFSF18 (ook GITRL genoemd) het meest verhoogd na een positieve visuele reactie. TNFSF18 behoort tot de TNF superfamilie en is het best bestudeerd voor zijn costimulatoire effect op T cellen met een bekende rol in astma en atopische dermatitis. Daarnaast laten we zien dat slokdarmepitheelcellen een potentiële bron zijn van TNFSF18. Om te bepalen of TNFSF18 een nieuw potentieel therapeutisch doelwit van EoE is, is verder onderzoek naar de precieze rol van TNFSF18 in de acute allergeen-geïnduceerde immuunreactie nodig.

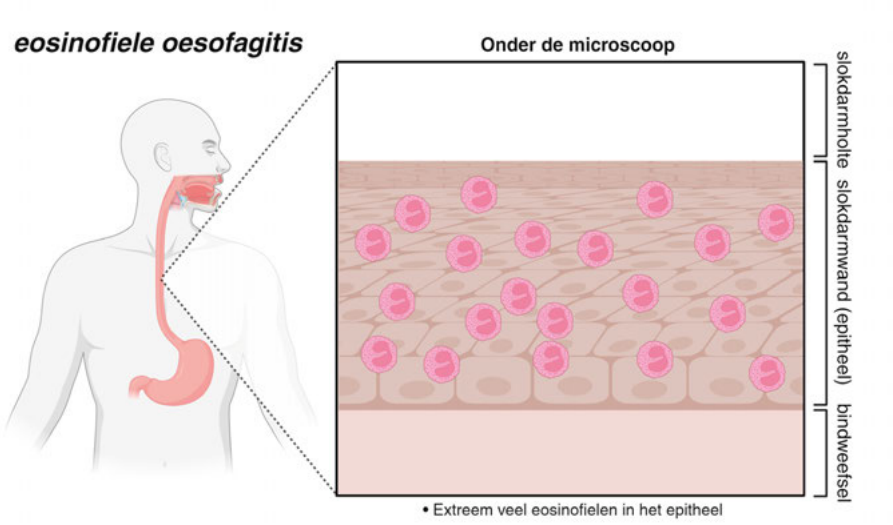
De ophoping van grote aantallen mestcellen in het slokdarmepitheel is een belangrijk kenmerk van EoE. Deze mestcellen kunnen degranuleren en bevinden zich tussen de epitheelcellen, maar het is nog onbekend wat voor effect dit heeft op de barrièrefunctie van het epitheel. In het laatste experimentele hoofdstuk van dit proefschrift, **hoofdstuk 5**, hebben we daarom een cokweeksysteem met primaire humane mestcellen en gedifferentieerde slokdarmepitheelcellen opgezet om het effect van mestcellen en hun mediatoren op de barrièrefunctie van het slokdarmepitheel te onderzoeken. Onze resultaten lieten zien dat IgE-geactiveerde mestcellen, in tegenstelling tot niet-geactiveerde mestcellen, de barrièrefunctie van slokdarmepitheelcellen verstoorden. Dit ging gepaard met verminderde genexpressie van barrière-eiwitten en een anti-protease. Om meer inzicht te krijgen in welke mestcelmediatoren (deels) verantwoordelijk zouden kunnen zijn voor de verminderde barrièrefunctie van het slokdarmepitheel, hebben we een geselecteerde groep van 45 cytokines in het cokweeksupernatant gemeten. Het proinflammatoire cytokine oncostatin M (OSM) werd veel geproduceerd door IgE-geactiveerde mestcellen en staat bekend om zijn barrièreverslechterende capaciteiten in de long. Interessant was dat OSM tot 12 keer meer in de slokdarm van EoE patiënten voorkomt dan in die van gezonde controles, associeerde met genmarkers voor mestcellen en direct de barrièrefunctie van slokdarmepitheel verslechterde. Al met al laten onze data zien dat IgE-geactiveerde mestcellen de barrièrefunctie van het slokdarmepitheel verstoren, wat wellicht voor een deel wordt gemedieerd door OSM naast nog andere proinflammatoire mediatoren. Onze studie suggereert dat mestcellen aan de pathofysiologie van EoE zouden kunnen bijdragen door de barrièrefunctie van het slokdarmepitheel te verslechteren.

Tot slot

In **hoofdstuk 6** bespreek ik de interpretaties en implicaties van onze belangrijkste bevindingen. Verder doe ik suggesties voor toekomstige onderzoeksrichtingen. Al met al hebben we met dit proefschrift inzicht verkregen in de lokale immuunreacties van EoE die worden geïnduceerd door voedselallergenen. Daarnaast zijn onze bevindingen een eerste stap in de richting van betere dieetmanagement van de aandoening.

LEKENSAMENVATTING

Eosinofiele oesofagitis (EoE) is een chronische ontsteking van de slokdarm. EoE wordt veroorzaakt door een allergische reactie, een ontspoorde reactie van het afweersysteem op onschuldige stoffen uit voedingsmiddelen. Stoffen die een dergelijke reactie kunnen uitlokken, worden allergenen genoemd. Kenmerkend voor EoE is dat er extreem veel eosinofielen in de slokdarmwand aanwezig zijn (**Figuur 1**). Dit zijn afweercellen die bij EoE de slokdarmwand binnendringen en er (samen met andere aanwezige afweercellen) een ontsteking veroorzaken. Zonder behandeling zal deze ontsteking verergeren waardoor littekenvorming kan ontstaan in de slokdarm en deze stugger en nauwer wordt. De gevolgen hiervan zijn dat het doorslikken van eten moeizamer gaat en dat, in ernstige gevallen, het eten in de slokdarm kan blijven hangen. Dit kan het gevoel geven dat eten niet goed zakt en het kan ook pijn veroorzaken. Ongeveer 1 op de 3000 mensen heeft EoE en het komt zowel bij kinderen als volwassenen voor.



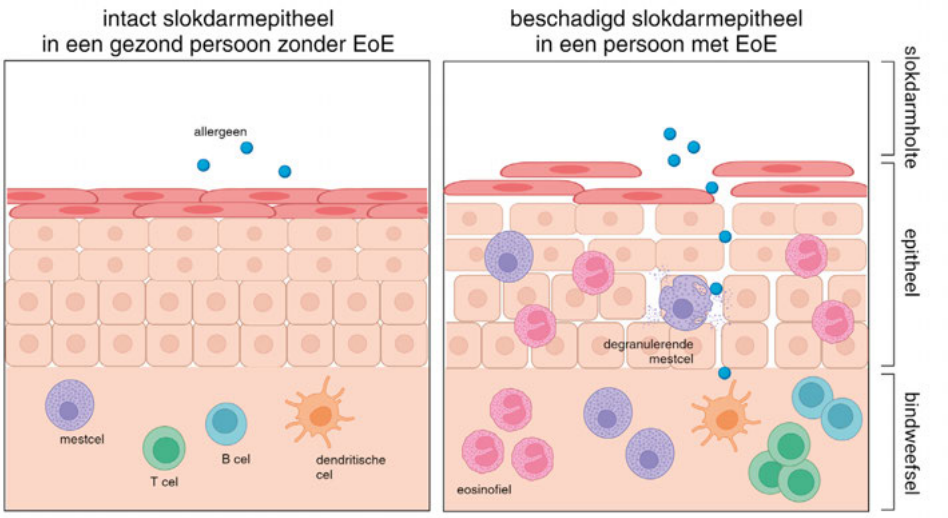
Figuur 1. Eosinofiele oesofagitis onder de microscoop. Gemaakt met BioRender.com.

Omdat EoE pas in de jaren '90 voor het eerst werd erkend, zijn er nog een hoop open vragen over het ontstaan en de behandeling ervan. Het beter begrijpen van de cellulaire en moleculaire werkingsmechanismen van EoE is belangrijk om deze vragen te kunnen beantwoorden. In dit proefschrift zijn daarom deze werkingsmechanismen onderzocht. Wij waren met name geïnteresseerd in wat er gebeurt met het afweersysteem in de slokdarm van EoE patiënten zodra het in contact komt met allergenen uit voedsel. Dit hebben we onderzocht in samenwerking met het Amsterdam UMC met een klinische studie. Tijdens een kijkonderzoek (endoscopie) werd een flexibele holle slang (endoscoop) met daarop een kleine camera via de mond in de slokdarm van EoE patiënten gebracht. Middels een injectienaald die door de endoscoop paste, konden

we voedingsmiddelen in de slokdarmwand injecteren en de reactie van de slokdarm op deze voedingsmiddelen bestuderen (**hoofdstuk 3**). Na 20 minuten werden stukjes slokdarmweefsel (biopten) afgenomen van de injectieplekken om geanalyseerd te worden met 'RNA sequencing'. Met deze geavanceerde techniek konden we bestuderen welke genen actief zijn in de biopten. Dit hielp ons begrijpen hoe de slokdarm reageert op bepaalde voedselallergenen (**hoofdstuk 4**). Uit de analyse bleek dat 40 genen actief waren. Het merendeel hiervan speelde een rol bij de activatie van het afweersysteem. Sommige van deze genen zouden wellicht een nieuw therapeutisch target kunnen zijn voor de behandeling van EoE.

Naast een klinische studie hebben we ook laboratoriumstudies gedaan om meer inzicht te krijgen in het werkingsmechanisme van EoE. In het lab kunnen we namelijk een miniatuurversie van de slokdarmwand namaken, bestaande uit zogenaamde epitheelcellen. Deze slokdarmwand van epitheelcellen (het slokdarmepitheel) beschermt het onderliggende weefsel van indringers, zoals bacteriën en voedselallergenen. Echter, in mensen met EoE lekt het slokdarmepitheel. Dit lekkende epitheel kan genetisch aangeboren zijn, verworven door veel blootstelling aan stoffen die schadelijk zijn voor epitheelcellen (bijv. emulgatoren in voeding) of verworven door ontstekingen. Voedselallergenen kunnen dan makkelijk door het lekkende epitheel dringen en verschillende soorten afweercellen activeren die er tussen of er net onder liggen.

Naast eosinofielen komen mestcellen in grote aantallen in en onder het epitheel van EoE patiënten voor. Mestcellen zijn een soort afweercellen die als bewakers op de uitkijk staan voor ziektekiemen die het lichaam proberen binnen te dringen. Aan de andere kant kunnen ze ook overgevoelig worden en reageren op allergenen. Als mestcellen een allergeen tegenkomen, worden ze geactiveerd en scheiden ze heel snel chemische stoffen uit die een allergische reactie kunnen opwekken. Deze vorm van activatie wordt degranulatie genoemd. In **hoofdstuk 5** hebben we laten zien dat mestcellen die degranuleren het slokdarmepitheel verder kunnen afbreken, wat ervoor zorgt dat allergenen door het epitheel kunnen blijven dringen (**Figuur 2**). Op deze manier wordt het lekkende slokdarmepitheel in stand gehouden. Het opnieuw sluiten van het slokdarmepitheel zou in theorie dus kunnen voorkomen dat er een ontsteking ontstaat. Allergenen kunnen dan namelijk niet meer door het epitheel dringen en mestcellen en andere afweercellen activeren. In **hoofdstuk 2** hebben we gevonden dat bepaalde voedingsstoffen die ontstaan door de fermentatie van voedingsvezels door darmbacteriën, de zogenaamde korte-keten vetzuren, dit soort epitheel-sluitende capaciteiten bezitten. Het zou interessant zijn om verder te onderzoeken of een dieet met korte-keten vetzuursupplementen de ontstekingen in de slokdarm zou kunnen remmen en symptomen zou kunnen verlichten.



Figuur 2. Het slokdarmepitheel van mensen met EoE is beschadigd en lek. Links: het intacte slokdarmepitheel vormt een sterke beschermende wand voor alles wat wordt doorgeslikt. In het bindweefsel onder het slokdarmepitheel zitten verschillende afweercellen die belangrijk zijn voor de afweerreactie tegen bijvoorbeeld bacteriën en virussen. Allergenen kunnen deze afweercellen niet bereiken omdat het epitheel dicht is. Eosinofielen komen niet voor in een gezonde slokdarm. Rechts: in mensen met EoE is het slokdarmepitheel beschadigd en lek. Er zijn heel afweercellen aanwezig waarvan een deel zich ook tussen de epitheelcellen bevinden. Allergenen kunnen door het epitheel dringen en er een ontsteking veroorzaken waarbij verschillende afweercellen (o.a. mestcellen, eosinofielen, T cellen, B cellen en dendritische cellen) een complexe rol spelen. Als er niet wordt ingegrepen met medicijnen of een dieet, zal de ontsteking voortzetten. Dit zal leiden tot littekenvorming en uiteindelijk het stugger en nauwer worden van de slokdarm, wat gepaard gaat met slikproblemen en eten dat vast kan blijven steken. Gemaakt met BioRender.com.

Desalniettemin heeft een dieetbehandeling waarbij alleen de voedingsmiddelen worden gemeden die klachten veroorzaken de voorkeur van vele patiënten. Deze zogenaamde 'triggervoedingsmiddelen' verschillen van persoon tot persoon, maar er zijn geen betrouwbare tests beschikbaar die deze triggers van EoE kunnen identificeren. Omdat patiënten niet altijd meteen na het eten van triggervoedingsmiddelen klachten krijgen (soms zelfs uren later), is het ook moeilijk voor ze om er zelf achter te komen welke voedingsmiddelen ze voortaan beter kunnen mijden. In **hoofdstuk 3** hebben we daarom drie nieuwe allergietests in de slokdarm van 12 EoE patiënten uitgevoerd om te onderzoeken of deze tests triggervoedingsmiddelen van 'veilige' voedingsmiddelen kunnen onderscheiden. Dit deden we door a) voedingsmiddelen te injecteren in de slokdarmwand tijdens endoscopie, b) voedingsmiddelen langs de slokdarmwand te sprayen tijdens endoscopie en c) voedingsmiddelen bij biopten in een 'reageerbuis' te doen. Onze resultaten lieten zien dat met name de test waarbij biopten blootgesteld werden aan voedingsmiddelen de meeste potentie heeft om triggervoedingsmiddelen te identificeren. Verder onderzoek naar deze reageerbuistest is echter nodig voordat het uiteindelijk gebruikt kan worden in de kliniek en een persoonlijk dieet mogelijk kan maken.

Alles bij elkaar hebben we met het onderzoek in dit proefschrift meer inzicht gekregen in het werkingsmechanisme van EoE. Daarnaast zijn onze resultaten een belangrijke eerste stap naar het mogelijk maken van een persoonlijk dieet gebaseerd op allergietests in de slokdarm.

LIST OF PUBLICATIONS

IN THIS THESIS

Kleuskens MTA, Haasnoot ML, Garssen J, Bredenoord AJ, Van Esch BCAM, Redegeld FA. Transcriptomic profiling of the acute mucosal response to local food injections in adults with eosinophilic esophagitis. *J Allergy Clin Immunol*. In press.

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Głobińska A, Boonpiyathad T, Satitsuksanoa P, **Kleuskens M**, van de Veen W, Sokolowska M, Akdis M. Mechanisms of allergen-specific immunotherapy: Diverse mechanisms of immune tolerance to allergens. *Ann Allergy Asthma Immunol*. 2018 Sep;121(3):306-312. doi: 10.1016/j.anai.2018.06.026. Epub 2018 Jun 30. PMID: 29966703.

ABOUT THE AUTHOR

Mirelle Kleuskens was born on 21 February 1995 in Venray, the Netherlands. She grew up with her younger sister Sophie and parents Johan and Bernie in Melderslo, a small village in the province of Limburg. Mirelle obtained her Atheneum degree in 2013 from het Dendron College in Horst, and then moved to Wageningen to study Animal Sciences at Wageningen University & Research. In 2016, she obtained her bachelor's degree after completing a minor in immunology and a literature thesis on the autoimmune disorder multiple sclerosis. She then pursued her interest in immunology in the Master's program of Animal Sciences with a specialization in molecule, cell, and organ functioning. As part of the Master's program, Mirelle conducted a seven-month research internship at the Cell Biology and Immunology group of Wageningen University & Research under the supervision of prof. dr. Geert Wiegertjes, dr. Maria Forlenza, and dr. Annelieke Wentzel, where she studied macrophage polarization in common carp. In April 2018, Mirelle moved to Davos, a beautiful city in the Swiss Alps, to join the Swiss Institute of Allergy and Asthma Research to conduct a six-month research internship. Here, she studied the role of B cells in tolerance to allergens under the supervision of dr. Willem van de Veen in the Immune Regulation group led by prof. dr. Mübeccel Akdis.

After graduation, Mirelle started as a PhD candidate at the division of Pharmacology within the Utrecht Institute for Pharmaceutical Sciences (UIPS) under the supervision of dr. Betty van Esch, dr. Frank Redegeld, and prof. dr. Johan Garssen. Here, she worked on the 'LOIRE' project on local immune responses in eosinophilic esophagitis. This project was part of the public-private partnership on new concepts for specialized nutrition that influence immune fitness, in which partners from industry, academia, and the government collaborated. The LOIRE project was conducted in close collaboration with prof. dr. Arjan Bredenoord and Laura Haasnoot from the Amsterdam UMC.

During her PhD trajectory, Mirelle was trained in the PhD program of Infection & Immunity of the Graduate School of Life Sciences, and completed courses on immunology, programming, and transferable skills. She received travel grants from the European Academy of Allergy and Clinical Immunology (EAACI) to attend the annual congress in Prague, Czech Republic in 2022, and to attend the EAACI Winter School in Davos, Switzerland in 2023. In addition, Mirelle was awarded with the best poster prize at the UIPS biennial symposium in 2020, and the EAACI Digital Winter School in 2022. As of July 2023, Mirelle is appointed as a postdoctoral researcher in the same group to continue her research on eosinophilic esophagitis.

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