ARTICLE

Mast cells disrupt the function of the esophageal epithelial barrier

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Mast cells (MCs) accumulate in the epithelium of patients with eosinophilic esophagitis (EoE), an inflammatory disorder characterized by extensive esophageal eosinophilic infiltration. Esophageal barrier dysfunction plays an important role in the pathophysiology of EoE. We hypothesized that MCs contribute to the observed impaired esophageal epithelial barrier. Herein, we demonstrate that coculture of differentiated esophageal epithelial cells with immunoglobulin E-activated MCs significanly decreased epithelial resistance by 30% and increased permeability by 22% compared with non-activated MCs. These changes were associated with decreased messenger RNA expression of barrier proteins filaggrin, desmoglein-1 and involucrin, and antiprotease serine peptidase inhibitor kazal type 7. Using targeted proteomics, we detected various cytokines in coculture supernatants, most notably granulocyte-macrophage colony-stimulating factor and oncostatin M (OSM). OSM expression was increased by 12-fold in active EoE and associated with MC marker genes. Furthermore, OSM receptor-expressing esophageal epithelial cells were found in the esophageal tissue of patients with EoE, 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. Taken together, these data suggest a role for MCs in decreasing esophageal epithelial barrier function in EoE, which may in part be mediated by OSM.

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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 the 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 (MCs) 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 MCs in active and inactive disease demonstrated that these MCs exist in subpopulations, proliferate locally, persist during disease remission, and are an important source of IL-13[11]. Interestingly, esophageal MCs 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]. MC 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 MCs contribute to esophageal epithelial barrier dysfunction in EoE by the release of MC 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 MCs and furthermore examined the effect of coculture on cytokine production.

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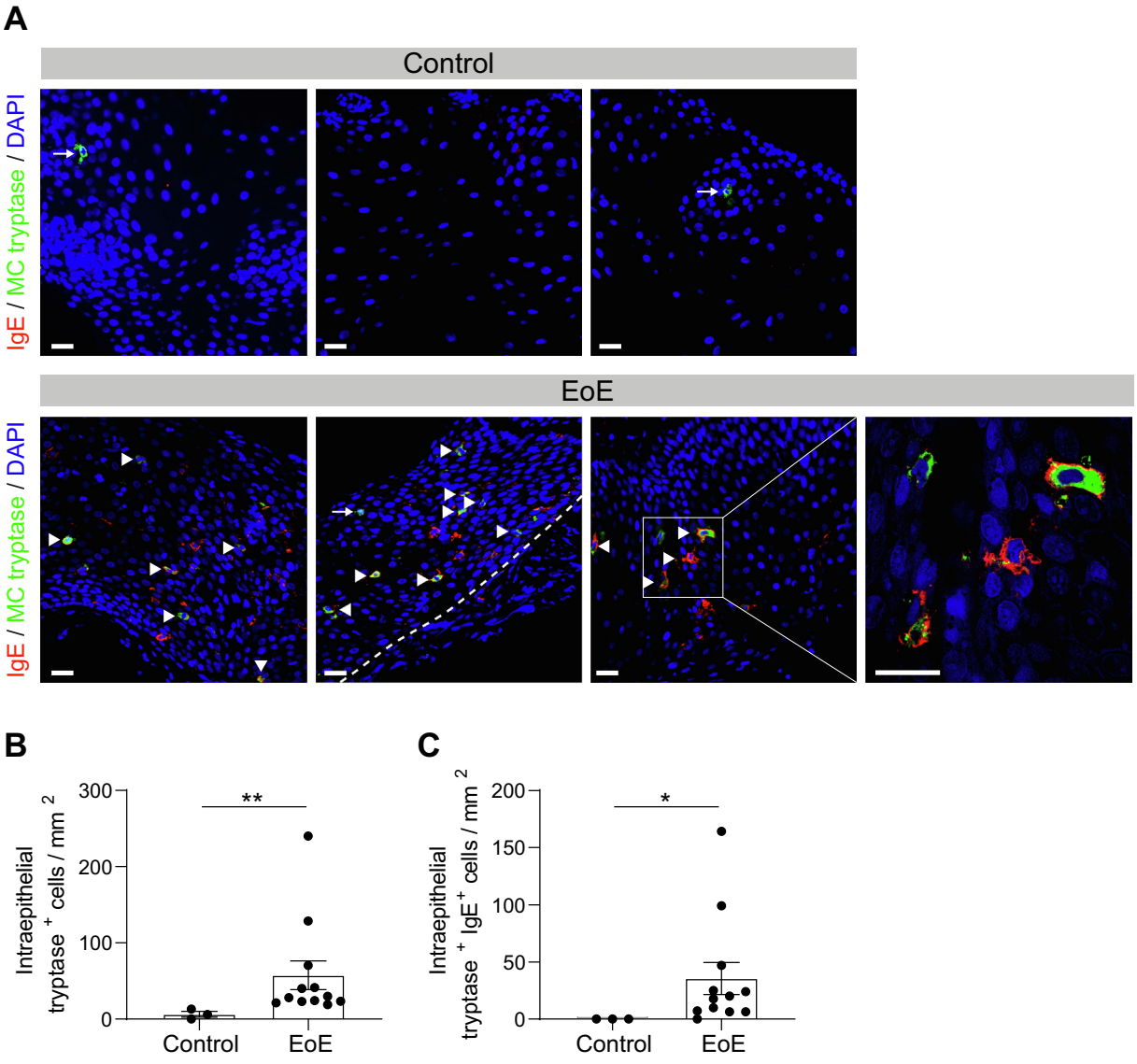


Fig. 1 Intraepithelial IgE-bearing MCs in the esophagus of patients with EoE. (A) Representative immunofluorescent staining for IgE (red) and MC tryptase (green) with a blue DAPI nuclear counterstain on esophageal biopsies from three adult non-EoE controls (top row) and three adult patients with EoE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

RESULTS

IgE-bearing intraepithelial MCs in human active EoE

Using immunofluorescence, we evaluated total and IgE-bearing MC density in the epithelium of esophageal biopsies from patients with active EoE ($n = 12$) and controls ($n = 3$). Consistent with previous reports[12,16], intraepithelial mast cell density was increased significantly in esophageal biopsies from patients with EoE compared with controls ($p = 0.0044$) (Fig. 1B). In addition, while intraepithelial MCs were detected in biopsies from controls (Fig. 1A, top row), intraepithelial IgE-bearing MCs were only found in biopsies from patients with EoE (Fig. 1A, bottom row). Similar to total MC density, IgE-bearing MC density was significantly higher in the esophageal epithelium of patients with EoE versus controls ($p = 0.0132$) (Fig. 1C). Lamina propria MCs could not be quantified due to variation in size and positioning of the biopsy.

IgE-activated MCs promote esophageal epithelial barrier dysfunction *in vitro*

Given that MCs accumulate in the esophageal epithelium of patients with EoE (Fig. 1A and 1B), bear IgE on their cell membrane (Fig. 1A and 1C), and undergo substantial degranulation[12], we aimed to determine the effect of IgE-mediated MC activation on esophageal epithelial barrier function. We used an ALI culture

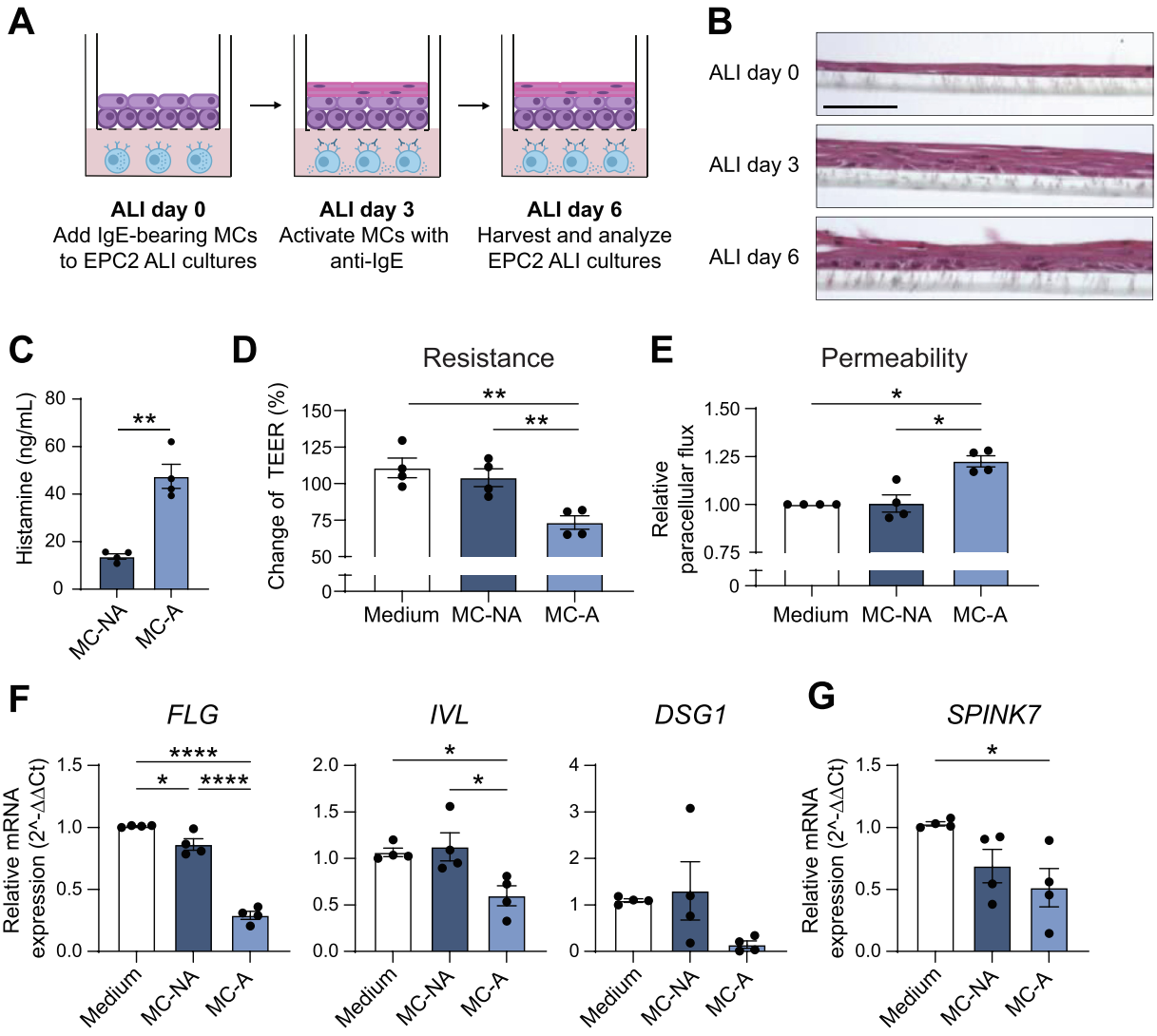


Fig. 2 IgE-mediated mast cell activation induced barrier dysfunction of the esophageal epithelium. (A) Schematic diagram of the EPC2 ALI coculture model with MCs. IgE-bearing MCs were added to the basolateral compartment of EPC2 ALI cultures at the start of ALI (day 0). Following 3 days of coculture under ALI conditions, when the EPC2 were differentiated, MCs were activated by cross-linking of membrane-bound IgE. EPC2 and MCs were cocultured for another 3 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 MC-A and MC-NA cocultured with EPC2 collected 1.5 hours after IgE-mediated MC activation. (D and E) TEER (D) and paracellular flux of FITC-Dextran (E) of EPC2 following 3 days of coculture with activated or non-activated MCs. (F and G) mRNA expression of barrier proteins FLG, IVL and DSG1 (F), and protease regulator SPINK7 (G) in EPC2 following 3 days of coculture with activated or non-activated MCs. Data are presented as mean ± standard error of mean of $n = 4$ independent experiments performed with four MC 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 analysis of variance with Bonferroni's *post hoc* test, or Kruskal-Wallis test with Dunn's *post hoc* test as appropriate, depending on data distribution. A = activated; ALI = air-liquid interface; DSG1 = desmosome desmoglein-1; FLG = filaggrin; Ig = immunoglobulin; IVL = involucrin; MC = mast cells; mRNA = messenger RNA; NA = non-activated; SPINK7 = Serine Peptidase Inhibitor Kazal Type 7; TEER = transepithelial electrical resistance.

model that resembles human differentiated esophageal epithelium to study the effects of MC activation on epithelial barrier function, as depicted schematically in Fig. 2A. On day 3 of the ALI culture when EPC2 formed a differentiated and stratified layer (Fig. 2B), MCs were activated by cross-linking of membrane-bound IgE. Analysis of histamine content in 1.5 hours supernatant confirmed MC degranulation in the coculture system (mean ± SD: 47.5 ± 10.1 ng/ml histamine for IgE-activated MCs and 13.8 ± 2.3 ng/ml histamine for non-activated MCs; $p = 0.0006$) (Fig. 2C). Coculture of EPC2 under ALI conditions with IgE-activated MCs significantly decreased EPC2 barrier resistance by 30% ($p = 0.01$) compared with non-activated MC cocultures, as measured by transepithelial electrical resistance (TEER) (Fig. 2D). Barrier permeability was evaluated by using 4 kDa

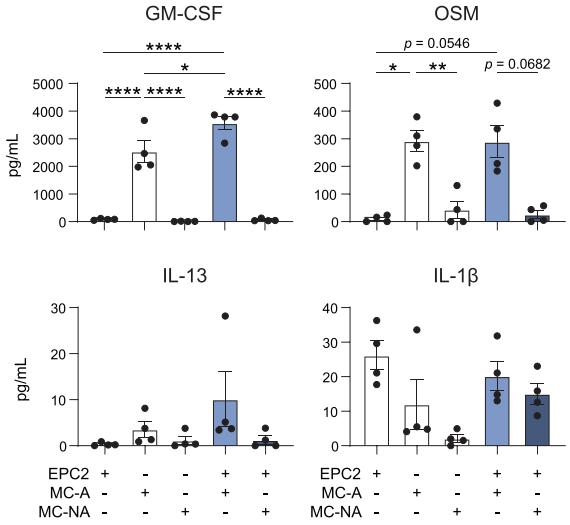


Fig. 3 Cytokines in esophageal epithelial cell and mast cell coculture. Based on a 45-cytokine array (Supplemental Fig. S3), levels of IL-13, IL-1β, GM-CSF, and OSM levels were quantified by enzyme-linked immunosorbent assay in culture supernatants from air-liquid interface day 4 of EPC2 monocultures, MC-A and MC-NA monocultures, and cocultures with EPC2 and MC-NA. Data are presented as mean ± standard error of mean of n = 4 independent experiments performed with four MC 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 analysis of variance with Bonferroni's post hoc test. A = activated; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; MC = mast cells; NA = non-activated; OSM = oncostatin M.

FITC-Dextran. Coculture of EPC2 with IgE-activated MCs, but not non-activated MCs, significantly increased epithelial permeability to FITC-Dextran by 22% ($p = 0.0079$) (Fig. 2E), confirming the TEER results. The disruptive effects of IgE-activated MCs on the barrier function of ALI-cultured EPC2 were associated with decreased messenger RNA (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, $p = 0.156$) (Fig. 2F). In addition to barrier proteins, the expression of the protease regulator serine peptidase inhibitor kazal type 7 (SPINK7) was impaired as well following coculture with IgE-activated MCs (Fig. 2G). Collectively, these results indicate that IgE-activated MCs can impair esophageal epithelial barrier function and decrease the expression of esophageal barrier proteins and antiprotease.

Secreted cytokines in esophageal epithelial cell and mast cell coculture

Since MCs 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 MC donors) collected 24 hours after IgE-mediated MC activation to identify cytokines that (i) may be responsive to intercellular crosstalk and (ii) 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 (Supplementary Fig. S2A). Cytokines that were upregulated in coculture were examined to determine if there was crosstalk between the esophageal epithelium and MCs (Supplementary Fig. 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 enzyme-linked immunosorbent assay (ELISA) on supernatants from the final experiments depicted in Fig. 2 ($n = 4$ different MC donors). Whereas GM-CSF, OSM, and IL-13 were mainly derived from IgE-activated MCs, IL-1β was mainly derived from EPC2 (Fig. 3). Two cytokines were significantly increased in IgE-activated MC monocultures compared with non-activated MC 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 MC 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 (Fig. 3). Production of OSM, IL-13, and IL-1β appeared not affected by coculture.

OSM levels are increased in the esophagus during active EoE and associate with MC 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 lungs[17,18]. 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[18–22]. One study reported increased OSM mRNA expression in esophageal biopsies from patients with EoE. 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[23], the expression of OSM was found to be increased by 12.9-fold in esophageal biopsies from patients with EoE 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 (Fig. 4A).

Next, we aimed to determine the relationship between OSM and MC levels. In active disease, OSM positively correlated with the MC marker genes CPA3 (Spearman r = 0.67, $p = 0.0390$) and TPSAB1 (Spearman r = 0.62, $p = 0.0603$) (Fig. 4C and 4D). Furthermore, we examined a publicly available single-cell RNA-sequencing dataset of whole EoE biopsies[11,24] and found OSM-expressing MCs during active EoE (Supplementary Fig. S3A), as well as OSMR-expressing (and to a lesser extent LIFR-expressing) esophageal epithelial cells (Supplementary Fig. S3B). Together, these data suggest that MCs are a potential source of esophageal OSM in active EoE, and that esophageal epithelial cells may be responsive to OSM.

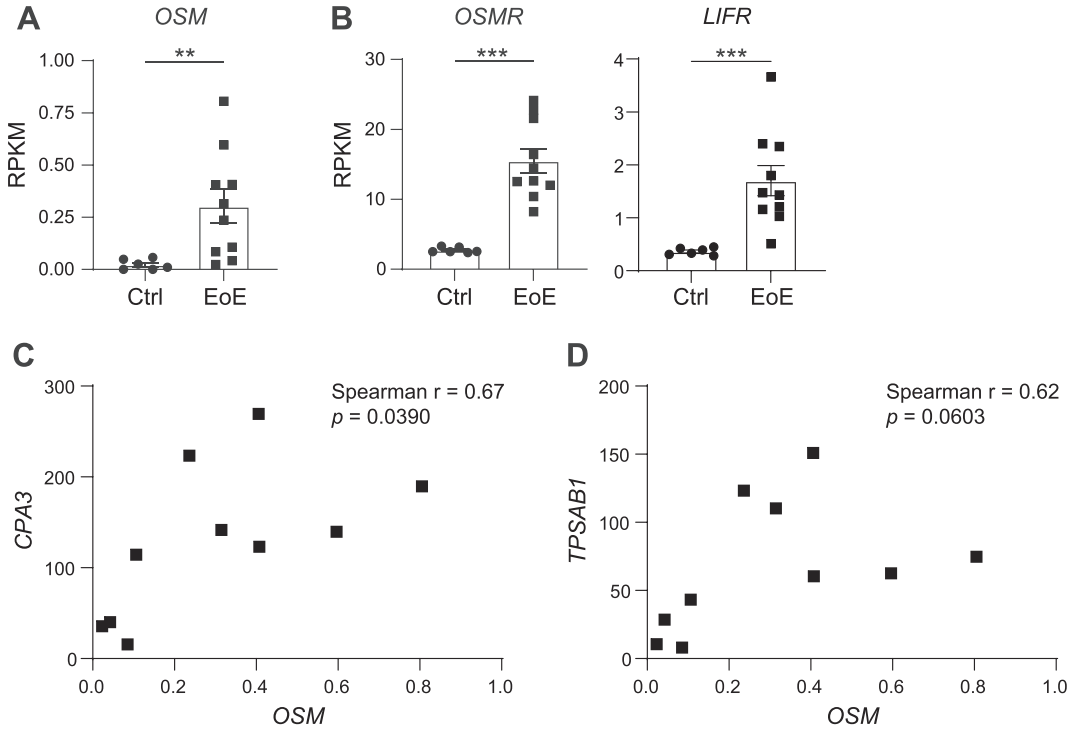


Fig. 4 Levels of OSM and its receptors in the esophagus of patients with EoE and association of OSM with MC markers. (A and B) Messenger RNA levels of OSM (A) and its receptors OSMR and LIFR (B) in esophageal biopsies from patients with EoE ($n = 10$) and healthy controls ($n = 6$). (C and D) Spearman correlation of OSM with MC markers CPA3 (C) and TPSAB1 (D) in active EoE. Spearman r values and p values are displayed in the figures. Data are derived from bulk RNA-sequencing of esophageal biopsies as reported previously[23]. Asterisks represent statistical significance: ** $p < 0.01$, *** $p < 0.001$, by Mann-Whitney test. ctrl = control; EoE = eosinophilic esophagitis; LIFR = leukemia inhibitory factor receptor; MC = mast cells; OSM = oncostatin M; OSMR = OSM receptor β-chain; RPKM =.

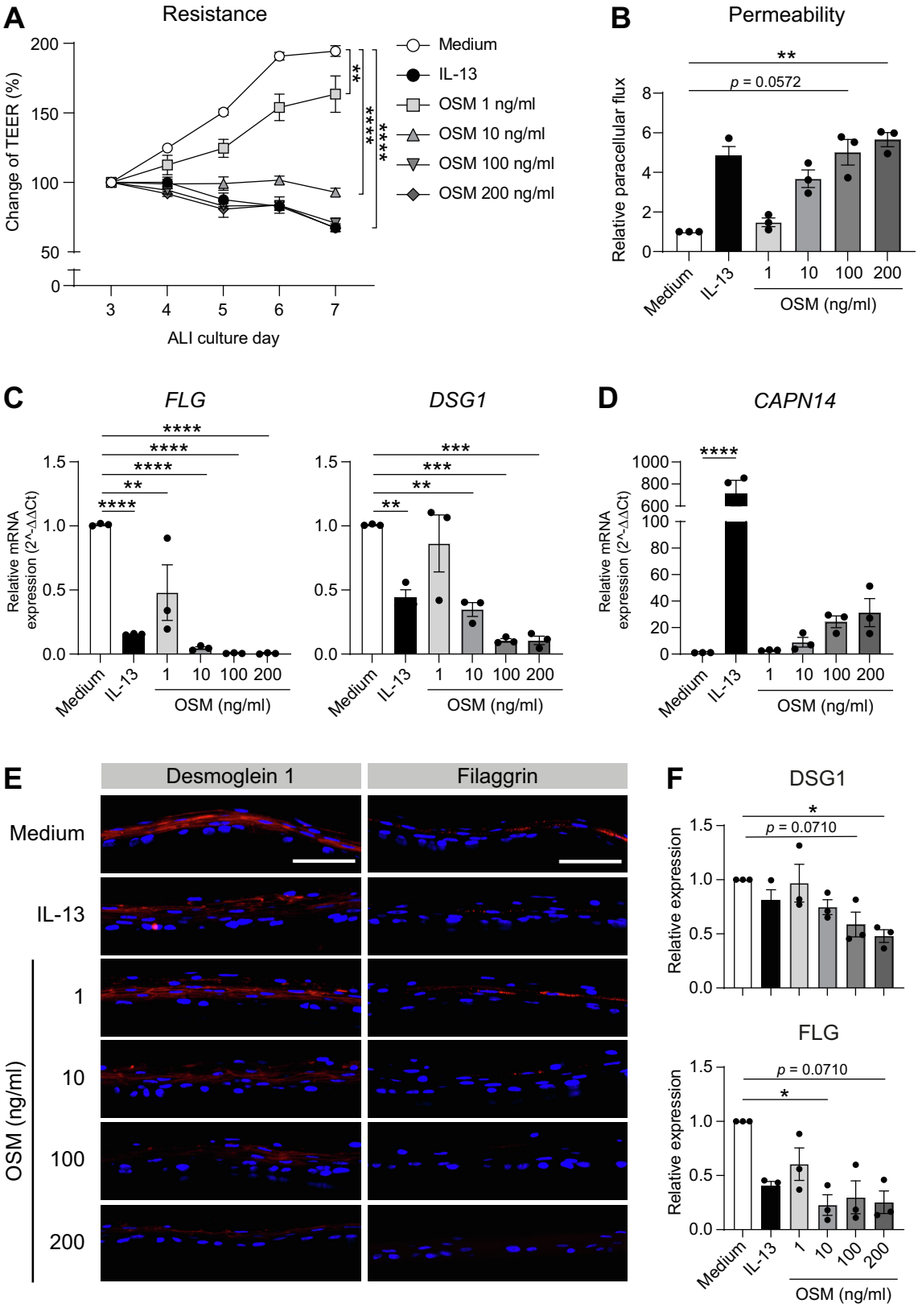
OSM disrupts esophageal epithelial barrier function *in vitro*

Given that OSM is increased in patients with EoE 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 4 days. OSM stimulation of EPC2 induced a dose-dependent decrease in barrier resistance (Fig. 5A), and an increase in barrier permeability to 4 kDa FITC-Dextran (Fig. 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 (Fig. 5C). Furthermore, immunofluorescent staining of these barrier proteins revealed that OSM dose-dependently disrupted their expression (Fig. 5E and 5F). In addition, there was a dose-dependent, though non-significant increase in the mRNA expression of CAPN14 (calpain 14; Fig. 5D), a tissue-specific protease that mediates esophageal epithelial barrier function[25]. Importantly, OSM did not decrease EPC2 viability as compared with IL-13 (Supplementary Fig. S4). Neutralization of MC-derived OSM with human anti-OSM in supernatants from IgE-activated MCs partially prevented its barrier-disruptive effects (Supplemental Fig. 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 MCs.

DISCUSSION

A defective epithelial barrier has been associated with chronic inflammatory diseases such as EoE[3]. MCs accumulate and degranulate in the esophageal epithelium of patients with EoE[12,16], but evidence of how this affects esophageal epithelial cells is lacking. Here, we demonstrated that IgE-activated MCs caused significant loss of esophageal epithelial barrier function *in vitro*, which was accompanied by decreased mRNA expression of barrier proteins and an antiprotease that is 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 MC 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 MC-derived OSM partially prevented the barrier-disruptive effects of MCs. Collectively, these findings suggest that MCs mediate esophageal epithelial barrier dysfunction and highlight a potential role for MC-derived OSM in this effect.

Accumulation of MCs in the esophageal epithelium is an important feature of EoE[12], where MCs have been suggested to contribute to fibrosis, smooth muscle contraction, and nerve signaling[9,26]. Here, we provide evidence that MCs may have an


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

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 MCs or their mediators modulate the integrity of the epithelial barrier[27–30]. Degranulation of intraepithelial esophageal MCs may result in high local concentrations of MC mediators in the epithelium, and directly affect barrier function as demonstrated in this study. The MC-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[31,32]. Besides barrier proteins, proteases, and protease inhibitors closely regulate the esophageal epithelial barrier. As we report here, IgE-activated MCs 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[33]. Epithelial barrier dysfunction induced by IgE-activated MCs 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 MCs. In a murine model of passive IgE sensitization to house dust mite allergen, the disrupted nasal epithelial barrier facilitated MC degranulation even in the absence of ongoing allergic inflammation, demonstrating that a disrupted barrier allows allergen translocation across the epithelium and consequent MC degranulation[30]. Continuous MC 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 MC sensitization or degranulation after sensitization has occurred.

Here, we have induced MC degranulation in the coculture system by cross-linking 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 trigger lack specificity[34], anti-IgE biologicals lack efficacy in clinical trials[35], and murine models for EoE do not require B cells or IgE to induce esophageal eosinophilia[36,37]. However, there is evidence of local IgE class switching of B cells and IgE production in the esophageal mucosa of patients with EoE regardless of their atopic status[38]. In line with this, we and others[16] show IgE-bearing MCs in the esophageal epithelium during active EoE, suggesting that local IgE-mediated MC 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[39,40]. 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[41–43]. Of note, there are other non-IgE stimuli that could activate MCs, including cytokines and toll-like receptor ligands[44]. The fact that EoE pathogenesis is, most likely, multifactorial could also explain why therapeutic targeting of MCs in EoE did not result in symptom improvement[45,46].

OSM was elevated in esophageal biopsies from patients with EoE 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 do not occur[47]. Human OSM signals through two heterodimeric receptors that both use glycoprotein 130 for signaling: LIFR and OSMR[48]. OSM may exert its functions through various signaling pathways, such as the JAK/STAT, ERK1/ERK2, JNK, p38, PKCd, and PI3K/Akt pathways[49]. Macrophages, neutrophils, activated T cells, and dendritic cells are potential sources of OSM[50–53]. As we report here, mast cells produced OSM *in vitro*, there was a correlation between MC marker genes and OSM expression in bulk RNA-seq, and there were OSM + MCs in singe-cell RNA-seq of whole EoE biopsies, indicating that MCs 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[18,20,21]. In line with our data on the esophageal epithelium, OSM also impaired the barrier function of airway epithelium[18]. OSM is most likely not the sole MC-derived mediator that disrupts esophageal epithelial barrier function. Activated MCs secrete a plethora of inflammatory mediators that may have barrier-disrupting effects, including histamine, tryptase, chymase, lipid mediators, and type 2 cytokines.

Fig. 5 OSM decreased the 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 4 days. (A) TEER measurements of EPC2 ALI cultures following OSM or IL-13 stimulation. B, Paracellular flux of FITC-Dextran in response to 4 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 4 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 4 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 4 days. Data are presented as mean ± standard error of mean 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 analysis of variance with Bonferroni's *post hoc* test, Kruskal-Wallis test with Dunn's *post hoc* test, or two-way repeated measures analysis of variance with Bonferroni's *post hoc* test as appropriate, depending on data relation and distribution. ALI = air-liquid interface; DSG1 = desmosome desmoglein-1; FLG = filaggrin; IL = interleukin; mRNA = messenger RNA; OSM = oncostatin M; TEER = transepithelial electrical resistance.

GM-CSF contributes to allergic inflammation by enhancing the survival, activation, and migration of eosinophils, and by regulating the function of dendritic cells[54–56]. Eosinophils and MCs abundantly coexist in the inflamed esophageal mucosa in active EoE[57]. Recently, Dunn et al. demonstrated that esophageal epithelial cell-derived GM-CSF is necessary and sufficient for a pro-survival effect on cocultured eosinophils[54]. Here, we observed GM-CSF production by both EPC2 and MCs in monoculture, which was significantly increased in coculture, suggesting intercellular crosstalk. Whether the EPC2, MCs, or both increased the production of GM-CSF upon coculture is currently unknown. The MC mediator histamine has been found to induce GM-CSF secretion from esophageal epithelial cells[58]. Conversely, soluble factors derived from the epithelial cells may fine-tune mast cell activation and inflammatory mediator production[59]. MCs and eosinophils are found in couplets in the esophageal epithelium in active EoE[57]. It is interesting to speculate that MC- 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[5,60], future studies should explore the use of primary esophageal epithelial cells from patients with EoE to mimic the environment of the inflamed esophagus more closely. Furthermore, we used MCs from healthy blood donors. In future studies, it would be interesting to compare PBMC-derived MCs from both patients with EoE and healthy controls in the coculture system.

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

METHODS

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)[61–63]. 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). EPC2s were discarded after 3 months of passages.

MCs were generated from human peripheral blood mononuclear cells (PBMCs) as previously described[64]. Briefly, PBMCs were obtained from buffy coats of healthy donors (Dutch Blood Bank, The Netherlands). Clusters of differentiation (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 4 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 8 weeks of culture, MC 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[65]. MCs were then used for experiments.

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 https://www.ncbi.nlm.nih.gov, GEO accession GSE58640)[23], and a single-cell RNA-sequencing data set of whole EoE biopsies from https://egidexpress.research.cchmc.org/[11,24]. In addition, baseline biopsy specimens from 12 adult patients (aged 18-75 years) with EoE with clinically and histologically (≥15 eos/hpf) active disease and three adult non-EoE controls were obtained by endoscopic collection, as previously described[66]. Biopsies were collected in formalin for immunofluorescent staining.

EPC2 and MC 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 4 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 6 days.

MCs were sensitized overnight with human IgE myeloma (1 µg/ml; Sigma-Aldrich; cat. AG30P) one day before the start of ALI culture. MCs 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. MCs 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 2 days. After 3 days of coculture, when the EPC2 were differentiated, MC degranulation was induced with rabbit anti-human IgE (10 µg/ml; Dako Denmark A/S, Glostrup, Denmark, cat. A0094) or MCs were left inactivated. Histamine levels in supernatant collected after 1.5 hours were quantified by ELISA (ENZO Life Sciences Inc., Farmingdale, NY, USA; cat. ENZ-KIT140) to ensure MC degranulation had occurred. Barrier function was assessed by 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[67]. 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 real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) after paracellular flux

assays. TEER results of preliminary experiments that were performed to determine the optimal concentration of MCs in the coculture system are provided in Supplementary Fig. S1.

Multiplex array and ELISA

Supernatants collected 24 h after MC activation (ALI day 4) from the preliminary coculture experiments (Supplementary Fig. S1) using 0.5×10^6 MC/ml (two different MC donors) were analyzed by Target 48 Cytokine Panel multiplex array (Olink, Uppsala, Sweden). Heatmaps were generated using Clustergrammer[68]. Then, we used ELISA on supernatants collected 24 hours after MC activation (ALI day 4) from the final experiments using 0.5×10^6 MC/ml (as described above) to confirm our findings of the multiplex array for four different MC 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, Waltham, MA, USA; cat. 88-7439-88) were measured per manufacturer's instructions.

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 4 days. IL-13 (100 ng/ml; Prospec, Rehovot, Israel; cat. CYT-446) was included as a positive inflammatory control[5,60,67]. 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 2 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.

RNA isolation, complementary DNA 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 complementary DNA 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 Bio-Rad. 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 = $2^{-\Delta\Delta Ct}$.

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 minutes 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 phosphate-buffered saline (PBS) for 90 min at room temperature. 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, Carlsbad, CA, USA; 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 room temperature for 1 hour. In between antibodies, sections were washed 3 × 5 minutes with 0.2% Tween20 (Bio-Rad) in PBS. After staining, sections were washed (3 × 5 minutes), coverslipped with ProLong Gold Antifade reagent with DAPI (Invitrogen; cat. P36931) for nuclei staining, and dried for 24 hours 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$^+$ MCs in esophageal biopsies. Images were analyzed using Fiji ImageJ version 1.53f51 (National Institutes of Heatlh, USA).

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

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 Fiji 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.

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 analysis of variance (normal distribution, equal variance), Kruskal-Wallis test (non-normal distribution), or, for paired data, two-way repeated measures analysis of variance (normal distribution, equal variance). Correlation analysis was performed using Spearman rank correlation coefficient. A p value <0.05 was considered significant. Data are reported as mean ± standard error of mean.

AUTHOR CONTRIBUTIONS

MTAK, BCAMVE, and FAR conceived the study. MTAK, MKB, YAH, BRJB, and MAPD collected the data. MTAK, MKB, BCAMVE, and FAR contributed to data analysis and interpretation. MLH and AJB provided human samples. MTAK wrote the initial and subsequent drafts of the manuscript. All authors critically reviewed the manuscript for important intellectual content. All authors approved of the final version of the manuscript.

DECLARATIONS OF COMPETING INTEREST

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APPENDIX A. SUPPLEMENTARY DATA

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