HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2014 November 01.

Published in final edited form as:

Mucosal Immunol. 2014 May ; 7(3): 718–729. doi:10.1038/mi.2013.90.

Desmoglein-1 regulates esophageal epithelial barrier function and immune responses in eosinophilic esophagitis

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Abstract

The desmosomal cadherin desmoglein-1 (DSG1) is an essential intercellular adhesion molecule that is altered in various human cutaneous disorders; however, its regulation and function in allergic disease remains unexplored. Herein, we demonstrate a specific reduction in DSG1 in esophageal biopsies from patients with eosinophilic esophagitis (EoE), an emerging allergic disorder characterized by chronic inflammation within the esophageal mucosa. Further, we show that *DSG1* gene silencing weakens esophageal epithelial integrity, and induces cell separation and impaired barrier function (IBF) despite high levels of desmoglein-3 (DSG3). Moreover, *DSG1* deficiency induces transcriptional changes that partially overlap with the transcriptome of inflamed esophageal mucosa; notably, periostin, a multipotent pro-inflammatory extracellular matrix molecule, is the top induced overlapping gene. We further demonstrate that IBF is a pathological feature in EoE, which can be partially induced through the downregulation of DSG1

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Disclosures

R.C.O. has research funding from Takeda Pharmaceuticals and Astra Zeneca. S.P.H. is a consultant with Immune Pharmaceuticals. M.E.R. has an equity interest in reslizumab (Teva Pharmaceuticals), is a consultant for Immune Pharmaceuticals, is the inventor of several eosinophilic esophagitis (EoE)-related patents owned by Cincinnati Children's Hospital Medical Center (CCHMC), is on the American Partnership for Eosinophilic Disorders (APFED) Medical Advisory Board, and is on the Steering Committees of the International Eosinophil Society (IES) and The International Gastrointestinal Eosinophil Researchers (TIGERS). The remaining authors have no disclosures.

by interleukin-13 (IL-13). Taken together, these data identify a functional role for DSG1 and its dysregulation by IL-13 in the pathophysiology of EoE and suggest that the loss of DSG1 may potentiate allergic inflammation through the induction of pro-inflammatory mediators such as periostin.

Introduction

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease that has emerged over that last decade on a worldwide scale[1]. Although symptomatically resembling gastroesophageal reflux disease (GERD), EoE is characterized by immune sensitization to a variety of foods and marked Th2-associated allergic inflammation in the esophageal mucosa that is largely refractory to acid-suppressive therapy[2]. During active disease, the histopathological changes within the inflamed esophageal mucosa include the dense accumulation of activated immune cells, including eosinophils, mast cells, and T and B lymphocytes[3]. Moreover, evidence of dilated intercellular spaces (DIS) and abnormal epithelial cell proliferation suggest that impaired barrier function (IBF) of the esophageal epithelium may potentially contribute to the pathophysiology of EoE[4–6]. While dietary modification (i.e., complete or targeted food antigen avoidance) and swallowed glucocorticoids alleviate much of the disease pathology, EoE still has one of the lowest quality-of-life indexes among other chronic pediatric diseases, including inflammatory bowel disease[7–9].

Early efforts aimed at the molecular dissection of EoE pathogenesis included gene expression profiling of esophageal mucosal biopsies from patients with active EoE, which identified a striking disease-associated transcript signature that was highly conserved across patients with EoE and largely normalized during glucocorticoid-induced disease remission[10, 11]. Several pro-inflammatory mediators, such as the chemokine (C-C-motif) ligand 26 (*CCL26*), periostin (*POSTN*), and tumor necrosis factor, alpha-induced protein 6 (*TNFAIP6*), were dramatically elevated in the inflamed esophageal mucosa in EoE[10] and in primary esophageal epithelial cells treated with the Th2 cytokine interleukin-13 (IL-13)[11]. A marked reduction in genes involved in epithelial homeostasis was also observed. In particular, the desmosomal cadherin desmoglein-1 (*DSG1*) exhibited an approximate 8-fold decrease in patients with active EoE, which only partially normalized upon disease remission[11].

DSG1 is a intercellular adhesion molecule belonging to the desmosomal cadherin family, which includes desmogleins (DSG1-4) and desmocollins (DSC1-3)[12]. Desmogleins and desmocollins are involved in maintaining epithelial homeostasis where they display spatially distinct expression patterns at various levels among different stratified epithelia[12, 13]. In particular, DSG1 is highly expressed in the epidermis and localized primarily within the suprabasal epithelial layers, where it regulates cell adhesion and supports epithelial cell differentiation[14]. In contrast, DSG3 is localized to undifferentiated, basal epithelial cells and has been suggested to promote cell proliferation[15–17].

While the function of DSG1 has been well characterized in the epidermis, its role in the esophageal epithelium under both homeostatic and inflammatory conditions remains largely unknown. DSG1 mediates intercellular adhesion by forming calcium-dependent heterotypic

and homotypic interactions between adjacent epithelial cells via its amino-terminal ectodomain[18]. However, it is becoming increasingly appreciated that DSG1 has additional roles beyond that of cell adhesion[19]. Indeed, DSG1 promotes epithelial differentiation through negatively regulating the activation of extracellular signal-regulated kinase (ERK) by epidermal growth factor receptor (EGFR) signaling[14]. DSG1 can also promote epithelial differentiation through its interactions with ERBIN, which sequesters SHOC2 to attenuate RAS-mediated ERK activation[20].

In the present study, we sought to determine the functional consequences of DSG1 dysregulation in EoE pathogenesis as DSG1 was one of the most down-regulated genes in the esophageal mucosa of EoE patients[11]. We hypothesized that the downregulation of DSG1 contributes to the pathological features of the esophageal epithelium in EoE. We demonstrate a marked reduction in the gene and protein levels of DSG1 but not DSG3 in the esophageal mucosa of patients with EoE. Suppression of *DSG1* by shRNA or IL-13 treatment induced IBF in differentiated esophageal epithelial cells grown at the air-liquid interface (ALI); notably, IBF was also observed ex vivo in biopsy samples from patients with EoE. Lastly, knockdown of *DSG1* was sufficient to induce a gene expression profile, which included periostin as the top induced gene, that substantially overlaps with the transcriptome of the inflamed esophageal mucosa of patients with EoE. These data suggest that the negative regulation of DSG1 by IL-13 can exacerbate inflammation of the esophageal mucosa in EoE by enhancing IBF and initiating a pro-inflammatory gene expression cascade.

Results

Specific dysregulation of DSG1 in EoE

We sought to establish the relative levels of all desmoglein genes (*DSG1-4*) in the esophagus and to determine whether the downregulation of *DSG1* was specific among other DSG family members. Whole-transcriptome RNA sequencing of esophageal biopsies from healthy controls (NL) (n = 6) and patients with active EoE (n = 10) showed a specific and dramatic downregulation of *DSG1* in this cohort of patients (Fig. 1A). Indeed, *DSG1* exhibited a 12.7 fold reduction in patients with active EoE (FPKM [median + interquartile range] = 19.6 + (4.6 – 24.4) in NL and 0.70 + (0.5 – 1.2) in EoE; $p = 1 \times 10^{-3}$). Notably, the most abundant DSG expressed in the esophageal mucosa was DSG3, which did not display differential expression in EoE (FPKM [median + interquartile range] = 254.4 + (239.0 – 279.5) in NL and 258.2 + (218.6 – 326.1) in EoE; $p = 0.75$) (Fig. 1C). We examined the downregulation of DSG1 in a larger cohort of NL and patients with active EoE disease (n = 25 and 39, respectively) by quantitative PCR (qPCR) and detected a 22.1-fold reduction ($p = 1 \times 10^{-4}$) in the esophageal expression of *DSG1* in active EoE (Fig. 1D). Lastly, we assessed esophageal *DSG1* levels in patients with inactive EoE (n = 10) following swallowed glucocorticoid therapy (Fig. 1E). During disease remission, expression of *DSG1* normalized to similar levels that were observed in NL (n = 11) yet was significantly different than in patients with active disease (n = 13) ($p = 3 \times 10^{-4}$).

We next performed immunofluorescent and/or immunohistochemical staining for DSG1, DSG3 and E-cadherin in the esophageal mucosa of NL and patients with active EoE to

characterize protein expression and localization. Consistent with previous reports[21], expression of DSG1 localized to the cell surface and was restricted to the suprabasal esophageal epithelium in NL, while DSG1 staining was remarkably absent in patients with active EoE (Fig. 2A, upper panel). Conversely, DSG3 was abundantly expressed throughout most of the esophageal epithelium in both NL and EoE, with more concentrated staining within the basal epithelial cell layers (Fig. 2A, lower panel). Immunohistochemical staining for DSG1 revealed a similar downregulation in EoE whereas expression of E-cadherin, a ubiquitously expressed cadherin molecule that regulates epithelial homeostasis and barrier formation[22], was unchanged between NL and patients with active EoE (Fig. 2B); these findings were supported at the gene level by qPCR analysis of esophageal biopsies from NL and active EoE patients (data not shown). These cumulative data indicate a specific downregulation of the DSG1 gene and protein in EoE, and that this is unique from the expression patterns of other desmogleins and E-cadherin.

Loss of DSG1 regulates esophageal epithelial cell integrity in vitro

In order to investigate the functional consequences of *DSG1* dysregulation on the esophageal epithelium, we developed a modified ALI culture system to induce a stratified esophageal epithelium in vitro. Similar ALI models have been used to study differentiated epithelium of both the epidermis and esophagus[23, 24]. Confluent monolayers of immortalized esophageal epithelial cells (EPC2), which have been previously shown to form stratified esophageal epithelium[25–27], were exposed to the ALI in the presence of high Ca^{2+} (1.8 mM). Following 5–7 days of differentiation at the ALI, the EPC2 cells formed a stratified epithelium as indicated by the differential H&E staining when compared to submerged cells (Fig. 3A). Gene expression analyses after ALI exposure showed significant induction of *DSG1* and keratin 10 (*KRT10*) (411 fold, $p < 1 \times 10^{-3}$ and 6,240 fold, $p < 1 \times 10^{-2}$, respectively), both of which are expressed specifically in differentiated esophageal epithelial cells[21, 28, 29] (Fig. 3B–C).

We next utilized lentiviral shRNA gene silencing to directly examine the impact of *DSG1* dysregulation on esophageal epithelial cell adhesion. EPC2 cells that were stably transduced with a *DSG1* shRNA exhibited a 92% reduction in *DSG1* expression compared to cells transduced with a non-silencing control (NSC) shRNA ($p < 5 \times 10^{-3}$) (Fig. 4A), whereas *DSG3* levels remained unaffected (Fig. 4B). Histological analyses of the NSC shRNA-transduced cells grown at the ALI showed normal stratification with a differentiated, intact esophageal epithelium (Fig. 4C, upper panel). However, while the differentiation appeared normal in the *DSG1* shRNA-transduced cells, prominent cellular separation was evident in the suprabasal layers (arrows, Fig. 4C, lower panel). Importantly, this cellular separation was consistently observed only in the *DSG1*-deficient cells (and not the NSC controls cells) using multiple *DSG1* shRNA clones, indicating that the observed phenotype was not due to sample sectioning (data not shown). A similar phenotype was observed when ALI-differentiated EPC2 cells were treated directly with recombinant wild type (WT) exfoliative toxin A (ETA), a DSG1-specific protease produced by *S. aureus* and the causative agent of epidermal blistering in staphylococcal scalded-skin syndrome (SSSS)[30] (Fig. 4D, lower panel). Notably, the inactive mutant form of ETA (S195A) did not induce cellular separation (Fig. 4D, upper panel). To directly measure whether loss of *DSG1* reduced esophageal

epithelial cell adhesion, NSC or *DSG1* shRNA-transduced cells were subjected to a dispase adhesion assay. Following mechanical disruption, a significantly greater amount of cell dissociation was observed in cells deficient in *DSG1* than in control cells ($p < 5 \times 10^{-2}$) (Fig. 4E–F).

IBF in EoE and in DSG1-deficient esophageal epithelial cells in vitro

To assess the integrity of the esophageal epithelium in EoE at the ultra-structural level, we performed electron microscopy on NL and EoE patient biopsies. While the esophageal epithelium of NL patients was composed of cohesive, intact epithelia, prominent DIS were evident throughout the esophageal epithelium of patients with EoE (arrowheads, Fig. 5A). These findings, which have been noted in previous studies of both EoE and GERD[31, 32], were consistent across multiple sections from the same patient and among different patients. We next assessed whether IBF is indeed a pathological defect in EoE by measuring transepithelial electrical resistance (TER) ex vivo in esophageal biopsies from NL and patients with EoE (Fig. 5B). We found that transcellular permeability was reduced by approximately 62% in patients with EoE compared to in NL ($R_T = 84 \pm 28$ vs. 224 ± 23 ohms × cm^2, respectively; $p < 5 \times 10^{-3}$). We also analyzed paracellular permeability in EoE by measuring macromolecular flux in esophageal biopsies and observed an approximate 46-fold increase in those from patients with EoE compared to NL ($p < 5 \times 10^{-2}$). Notably, esophageal expression of occludin (*OCLN*) and genes in the claudin (*CLDN*) and tight junction protein (*TJP*) families was not reduced in EoE, suggesting the IBF in EoE occurs in the absence of marked alteration to tight junctions (Supplementary Fig. 1A–C).

We hypothesized that the cellular separation that occurs within the esophageal epithelium following the loss of *DSG1* (Fig. 4C) may contribute to the IBF observed in EoE (Fig. 5A–C). To test this possibility, we measured TER and paracellular permeability in the ALI-differentiated NSC or *DSG1* shRNA-transduced EPC2 cells. Reduced *DSG1* expression resulted in impaired TER ($p < 5 \times 10^{-3}$, Fig. 5D) and increased FITC-dextran flux ($p < 5 \times 10^{-2}$, Fig. 5E–F) by approximately 42% and 33%, respectively. These data identify a novel mechanism whereby loss of DSG1 negatively affects esophageal epithelial integrity and is sufficient to induce IBF.

IL-13 regulates DSG1 and promotes IBF in differentiated esophageal epithelial cells

IL-13 is a critical Th2 cytokine capable of eliciting some of the transcriptional changes within the inflamed esophageal epithelium that are associated with EoE, including the downregulation of multiple epithelial cell differentiation genes[11, 33]. Therefore, we assessed the effects of IL-13 on the integrity and barrier formation of ALI-differentiated esophageal epithelial cells. Esophageal epithelial cells were left untreated or treated with 10 or 100 ng/mL IL-13 continuously throughout the ALI differentiation process. Both concentrations of IL-13 induced partial cellular separation within the suprabasal epithelial layers (arrows, Fig. 6A, middle and lower panels).

As the IL-13-induced suprabasal cell separation partially reflected the phenotype of *DSG1*-deficient cells (Fig. 4C), we next investigated the ability of IL-13 to regulate *DSG1* expression. IL-13 treatment suppressed the induction of *DSG1* in ALI-differentiated

esophageal epithelial cells (Fig. 6B), whereas induction of *KRT10* (Fig. 6C) and *DSG3* (data not shown) were unaffected. Importantly, IL-13 also promoted IBF, as a significant reduction in TER was observed at both 3 and 5 days post treatment with IL-13 (100 ng/mL) compared to untreated cells (Fig. 6D).

We also tested whether IL-13 could attenuate DSG1 expression in vivo using a transgenic murine model of EoE. Doxycycline (Dox)-inducible expression of IL-13 in the lung (using the Clara cell-specific promoter *CC10*) has been shown to induce an EoE phenotype in mice[34]. Elevated IL-13 levels in the BAL and induced esophageal eosinophilia were detected in the Dox-treated animals (Supplementary Fig. 1A–B). Overexpression of IL-13 in treated (+ Dox) mice reduced DSG1 mRNA and protein levels in the esophageal mucosa as compared to untreated (-Dox) mice (Supplementary Fig. 1C–E).

Loss of DSG1 primes for the innate inflammatory transcript signature

DSG1 has been shown to counter-regulate EGFR signaling, and more recently RAS-mediated signaling, through mechanisms that are independent of its full-length, adhesive N-terminal domain[14, 20]. Therefore, we hypothesized that DSG1 deficiency may also regulate downstream signaling responses associated with the Th2 inflammation in EoE. To test this possibility, we performed microarray analyses on NSC and *DSG1* shRNA-transduced EPC2 cells following ALI-differentiation. Interestingly, 63 transcripts coding for 53 unique genes were differentially expressed in the *DSG1*-deficient cells compared to in control cells ($p < 5 \times 10^{-2}$, fold change > 2.0) (Fig. 7A). Notably, there was a substantial overlap between this transcript profile and the EoE transcriptome identified in patient esophageal biopsies by RNA sequencing (RNA-seq); 60% of the unique DSG1-regulated transcripts were also dysregulated in esophageal biopsies of patients with EoE (Fig. 7B and Supplementary Table 1). In particular, periostin (*POSTN*) was the most highly induced gene upon *DSG1* knockdown in esophageal epithelial cells (2.5 fold) and was dramatically elevated in esophageal tissue of patients with EoE (384 fold) (Supplementary Table 1).

We next validated the significant increase in *POSTN* levels in *DSG1*-deficient esophageal epithelial cells by qPCR and observed a 17-fold increase in *POSTN* in the *DSG1* versus NSC shRNA-transduced cells ($p < 5 \times 10^{-2}$) (Fig. 8A). Furthermore, qPCR analysis demonstrated a significant and dramatic increase in *POSTN* expression in the esophageal mucosa of patients with active EoE compared to NL ($p < 1 \times 10^{-4}$, fold change = 2,047) (Fig. 8B). Notably, *POSTN* levels in EoE showed a significant inverse correlation with *DSG1* expression (same cohort as in Fig. 1D) (Spearman $r = -0.33$, $p = 0.02$) (Fig. 8C). Together, these data indicate that IL-13-induced loss of DSG1 augments the allergic inflammatory response by promoting IBF and likely elevates pro-inflammatory gene expression in EoE (Fig. 9).

Discussion

The data presented herein characterize the pathological impact of DSG1 dysregulation in EoE and define a non-redundant role for DSG1 in the regulation of the esophageal epithelial barrier function and homeostasis. In particular, we demonstrated a specific and marked downregulation of DSG1 in the esophageal epithelium of patients with EoE that was largely

reversible with patient disease status. This loss in *DSG1* expression was sufficient to induce cell separation and IBF in the esophageal epithelium. Moreover, *DSG1* deficiency led to an esophageal epithelial transcript signature that included increased gene expression of the pro-inflammatory extracellular matrix molecule periostin. These findings are particularly notable in view of the relatively high abundance of DSG3 expression in EoE and in our in vitro model, demonstrating the lack of redundancy for DSG1's regulation of esophageal responses, particularly during allergic inflammation. Interestingly, in GERD, in which DIS and IBF have been attributed to acid exposure of the esophageal epithelium, esophageal expression of *DSG1* (and *DSG3* to a lesser extent) is increased[35], further suggesting a unique, non-redundant and essential role for DSG1 in regulating disease processes specifically linked with EoE pathology.

Our data identify IL-13 as a potent regulator of *DSG1* expression in human esophageal epithelial cells cultured at the ALI and in mouse esophageal epithelial cells in a murine model of EoE. This latter finding is quite striking given the physiological differences between the human and mouse esophagus (not keratinized versus keratinized, respectively) and the existence of multiple murine *Dsg1* isoforms (α, β, and γ)[36], which were universally detected in our qPCR analysis and immunofluorescent staining (see Supplementary Materials and Methods). Previous work has demonstrated a pronounced effect of IL-13 on global gene expression in primary esophageal epithelial cells, and in particular, genes involved in epithelial differentiation[11, 33]. DSG1 was not shown to be regulated by IL-13 in these data, which, on the basis of our data in the ALI system (Fig. 3), we hypothesize was due to the low levels of *DSG1* expression in esophageal epithelial cells grown in the submerged culture conditions used in these studies. Interestingly, in skin keratinocytes, which express high baseline levels of *DSG1* even in submerged cultures, IL-4 has been shown to downregulate *DSG1* expression in vitro[37]. Notably, both IL-13 and IL-4 are significantly increased in the peripheral blood and esophageal mucosa of patients with active EoE[38, 39] and preliminary data indicate that IL-4 is also capable of negatively regulating *DSG1* expression and inducing IBF in ALI-differentiated esophageal epithelial cells in vitro (data not shown). These data, together with our findings that esophageal expression of *DSG1* normalizes in EoE patients during disease remission, indicate *DSG1* is negatively regulated by Th2 cytokines during allergic inflammation.

Within the epithelial barrier of the epidermis and intestine, the selective ability to discriminate the uptake of molecules based on size and charge is attributed to the tight junction complex[40]. For example, several tight junction genes are expressed within the epidermis including *CLDN1*, *CLDN4*, *TJP1*, and *OCLN*[41]. While only *CLDN1* has been demonstrated to have an essential role in epidermal barrier function in vivo[42], a recent study has shown *CLDN4*, *TJP1*, and *OCLN* can also independently regulate epidermal permeability in vitro[43]. Although the esophageal epithelial barrier has been studied primarily in the context of GERD, tight junction proteins have been shown to have no correlation with GERD pathogenesis[44]. Moreover, our data (Supplementary Fig. 1) demonstrate no decrease in genes encoding claudins, tight junction proteins, or occludin in EoE; none of these genes were dramatically altered in *DSG1*-deficient cells following shRNA transduction or IL-13

treatment (Supplementary Table 3). Together, these data suggest physiologically distinct mechanisms regulate the epithelial barrier properties of the esophagus and the epidermis.

Despite our evidence substantiating IBF as a bona fide pathological feature in EoE, little is known regarding the role of the esophageal barrier in regulating allergic inflammation. Within the epidermis, Langerhans cells continually sample external antigens by inducing transient reorganization of tight junctions within the stratum corneum without affecting barrier integrity; this homeostatic surveillance is further amplified in activated Langerhans cells[45]. These data suggest a potential mechanism by which IBF could lead to increased antigen sensitization, which is dependent on tissue resident antigen presenting cells. It is important to note that Langerhans cells[46, 47] and high baseline expression of the high affinity IgE receptor FcεRI[48] have been observed in both healthy and inflamed esophageal epithelium. Moreover, data suggesting esophageal epithelial cells can present antigen and MHC class II expression is increased in EoE[49] further suggest that the esophagus is not a unifunctional, static organ involved solely in food transport, but rather has an active role in immunosurveillance during allergic inflammation.

Our finding of elevated periostin expression in *DSG1*-deficient cells is particularly notable as periostin has been implicated in multiple allergic inflammatory disease including asthma, atopic dermatitis, and EoE[50–52]. Periostin can directly enhance eosinophil adhesion[50, 53], as well as increase keratinocyte production of thymic stromal lymphopoetin (TSLP)[52], a potent Th2-skewing cytokine that has been genetically linked to EoE susceptibility[54, 55]. Although periostin is expressed primarily in fibroblasts, treatment of both bronchial epithelial cells[56] and esophageal epithelial cells[50] with IL-13 induces periostin expression in these cell types. Interestingly, periostin has been shown to enhance signaling through EGFR and integrin $\alpha_v\beta_5$ to induce epithelial-mesenchymal transition (EMT)[57], a process associated with the loss of epithelial cell markers as epithelial cells adopt a fibroblast-like phenotype and increased migratory properties (e.g., loss of cell adhesion)[58]. While a previous report demonstrated that DSG1 supports epithelial differentiation and reduces proliferative capacity through negative regulation of EGFR signaling[14], a similar suppressive effect of DSG1 on EMT induction has not been addressed. As it has been recently proposed that EMT is actively occurring in EoE[59, 60], perhaps the loss of DSG1-dependent adhesion and the upregulation of periostin synergistically disrupt the homeostatic interactions between epithelial cells and fibroblasts during allergic inflammation.

The clinical importance of DSG1 and its role in maintaining epithelial integrity has been reported in several cutaneous diseases of autoimmune, infectious, and genetic origin[30]. In pemphigus foliaceous, autoantibodies against the ectodomain of DSG1 induce severe epidermal acantholysis[30]; interestingly, heightened Th2 inflammation, including elevated Th2 cytokine expression[61] and the presence of activated eosinophilic infiltrates[62], are observed in some instances. In Netherton syndrome, an epidermal inflammatory disease involving IBF with marked eosinophilia and elevated IgE levels[63], the primary etiology has been attributed to the genetic loss of the epithelial-derived serine protease inhibitor, Kazal type, 5 (*SPINK5*)[64]. In this disease, the uncontrolled activity of endogenous trypsin-like proteases leads to aberrant cleavage of epithelial barrier proteins within the stratum corneum (including DSG1). Mice deficient in *Spink5* display increased DSG1 degradation and

significant epidermal pathology, including epidermal acantholysis and IBF[65]. However, it has been unclear whether the loss of DSG1 alone is sufficient to induce IBF as several proteases (e.g., kallikriens 5, 7, and 14 and elastase 2) and stratum corneum proteins are involved in barrier formation (e.g., filaggrin, involucrin, and loricrin) and are also altered in Netherton syndrome[65–67].

Recently, genetic variants in DSG1 were identified in a new clinical syndrome manifesting with severe atopic dermatitis, multiple allergies, and metabolic wasting (SAM syndrome) in two consanguineous families[68]. Homozygous loss-of-function variants led to reduced expression of DSG1 and loss of cell adhesion[68]. Notably, one of three patients with SAM syndrome was reported to have EoE as well as elevated keratinocyte expression of *IL5* and *TSLP*[68]. Interestingly, in a genome-wide association study, we identified a non-coding variant in intron 1 of *DSG1* with suggestive association with EoE risk (meta $p = 6.57 \times 10^{-6}$)[54], the function of which has yet to be determined. Nonetheless, these supportive findings in SAM coincide with our data as they provide additional evidence that links *DSG1* deficiency as an initiating factor to IBF in allergic inflammation.

In summary, the observations presented herein support a pathophysiological role for DSG1 dysregulation in EoE. We propose a pathogenic cycle in which the localized increase in select Th2 cytokines (e.g. IL-13) within the inflamed esophagus decreases expression of DSG1, which is sufficient to weaken esophageal epithelial integrity and induce IBF. This loss of DSG1 initiates a pro-allergic transcriptional response (e.g., increased periostin expression) that potentiates the inflammatory response and may have far-reaching implications beyond the initial IBF insult, such as promoting EMT (Fig. 9).

Methods

Human subjects

NL (healthy control patients) were defined as having no history of EoE diagnosis with 0 eosinophils per high-power field (HPF) and no evidence of esophagitis within distal esophageal biopsies obtained during the same endoscopy procedure as the analyzed samples. Patients with EoE had clinician-diagnosed EoE and as having active disease in concomitant distal esophageal biopsies with greater than 15 eosinophils per HPF. EoE patients in disease remission (inactive) had clinician-diagnosed EoE and distal esophageal biopsies with less than 15 eosinophils per HPF (range = 0–1) following swallowed glucocorticoid therapy.

RNA sequencing (RNA-seq) and bioinformatic analyses

RNA isolated from esophageal biopsies of 6 NL and 10 patients with active EoE (mean eosinophils/HPF = 164 ± 29 SEM) was subjected to RNA sequencing at the CCHMC Gene Discovery and Genetic Variation Core as previously described[69]. The paired-end sequencing reads were aligned against the GRCh37 genome model using TopHat 2.04 with Bowtie 2.03[70, 71]. The separate alignments were then merged using Cuffmerge with RefSeq gene models as a reference. The aligned reads were then quantified for differential expression analysis using Cuffdiff[72]. Statistical significance was determined using a t-test with a

threshold of $p < 5 \times 10^{-2}$ and a 2.0-fold cut-off filter in GeneSpringR 11.5 (Agilent Technologies Incorporated, Clara, CA, USA).

qPCR analysis

RNA samples were prepared as previously described[34]. Briefly, total RNA (250–500 ng) was DNAase treated, and cDNA was generated using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). qPCR was performed, and SYBRR Green incorporation was analyzed using iQ5 software (Bio-Rad Laboratories, Hercules, CA, USA). Specific primer sequences are listed in Supplementary Table 2.

Immunohistochemical and immunofluorescent staining. For immunohistochemical staining, formalin-fixed, paraffin-embedded distal esophageal biopsies were serially sectioned and de-paraffinized using xylene followed by graded ethanol washes. Heat-induced epitope retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, p.H. 6.0) was used, and endogenous peroxidase activity was quenched in 2% H_2O_2. Slides were blocked in PBS with 3% goat serum for 1 hr followed by overnight incubation at 4°C in the following primary antibodies (2 μg/mL): anti-DSG1 (sc-2011) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-E-cadherin (#3195, Cell Signaling Technology, Inc., Danvers, MA, USA). Slides were then washed, incubated for 1 hr at room temperature in biotin-conjugated anti-rabbit IgG (1:250), and developed using the Vectastain ABC System according to manufacturer's protocol (Vector Laboratories, Burlingame, CA, USA). Lastly, developed slides were counterstained with Harris hematoxylin. Immunofluorescent staining was performed as previously described[50] using the following primary antibodies (2 μg/mL): anti-DSG1 (sc-2011) or anti-DSG3 (sc-23912) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Nuclei were stained with DAPI. Patients with active EoE that were analyzed by staining had eosinophil levels ranging from 98 to 265 eosinophils per HPF.

ALI culture system

The esophageal epithelial cell line (human telomerase reverse transcriptase [hTERT]-immortalized EPC2 cell line) was a kind gift from Dr. Anil Rustgi (University of Pennsylvania, Philadelphia, PA, USA) and has been extensively characterized in previous studies[25, 26, 73]. For the ALI culture system, EPC2 cells were grown to confluence while fully submerged in low-calcium ($[Ca^{2+}] = 0.09$ μM) keratinocyte serum-free media (K-SFM) (Life Technologies, Grand Island, NY, USA) on 0.4-μm pore-size permeable supports (Corning Incorporated, Corning, NY, USA). Confluent monolayers were then switched to high-calcium ($[Ca^{2+}] = 1.8$ μM) K-SFM for an additional 3–5 days. To induce epithelial stratification and differentiation, the culture medium was removed from the inner chamber of the permeable support in order to expose the cell monolayer to the air interface. Differentiated esophageal epithelial equivalents were analyzed 5–7 days post exposure.

DSG1 knockdown

EPC2 cells were transduced with shRNA targeting the last exon of *DSG1* or a NSC shRNA using the GIPZ lentiviral system (Thermo Fisher Scientific, Rockford, IL, USA). Lentiviral particles were prepared at the CCHMC Viral Vector Core facility. Forty-eight hours post transduction, cells were selected for stable integration using puromycin (1 μg/mL), which

was maintained throughout all subsequent experiments. Transduction efficiency was assessed by GFP fluorescence, and knockdown efficiency as compared to NSC shRNA-transduced cells was assessed by qPCR and immunofluorescence staining as described herein.

Expression and purification of recombinant ETA

The plasmids encoding WT ETA or the inactive ETA mutant S195A[74] with a 5X histidine epitope tag[75] were kindly provided by Dr. John Stanley (University of Pennsylvania, Philadelphia, PA, USA). The cDNAs were subcloned into the pT7-7 vector, validated by Sanger sequencing, and purified from isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced BL21 (DE3) pLysS *E. coli* cell lysates using Ni^{+}-NTA agarose column chromatography (QIAGEN Incorporated, Germantown, MD, USA). Purified ETA WT and S195A proteins were analyzed by SDS-PAGE and Coomassie staining or western blot using anti-histidine antibodies; in transiently transfected HEK293T cells, cleavage of DSG1 by WT ETA protein and non-cleavage of DSG1 by S195A ETA protein were confirmed by western blot (data not shown). Final protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL, USA).

Dispase adhesion assays

Dispase adhesion assays were performed as previously described[76]. Briefly, confluent monolayers of NSC or *DSG1* shRNA-transduced EPC2 cells were grown in 1.8 mM Ca^{2+} for 24 h, washed twice with PBS, and then detached from tissue culture wells by incubation in 2.4 U/mL dispase (Life Technologies Corporation, Grand Island, NY, USA) for 15–20 min at 37°C. Detached monolayers were then subjected to mechanical stress by pipetting with a 1-mL pipet 5 times. Aliquots were then cyto-centrifuged and stained with Hema 3R (Thermo Fisher Scientific, Rockford, IL, USA). One-, two-, and three-cell clusters were counted from the entire field under 10X magnification.

Microarray analyses

RNA was isolated using the miRNeasy kit (QIAGEN Incorporated, Germantown, MD) according to the manufacturer's protocol. RNA quality assessment, library preparation, hybridization to the GeneChipR Human Gene 2.0 ST exon array (Affymetrix, Santa Clara, CA, USA), and analysis were performed at the CCHMC Gene Expression Microarray Core. Expression profiles were analyzed using GeneSpringR 11.5 (Agilent Technologies Incorporated, Clara, CA, USA), and statistical significance was determined using a t-test with a threshold of $p < 5 \times 10^{-2}$ and a 2.0-fold cut-off filter.

Electron microscopy

Transmission electron microscopy was performed at the Pathology Research Core at Cincinnati Children's Hospital Medical Center (CCHMC). Biopsy specimens used in this analysis were from NL (n = 3) and from patients with active EoE (n = 3) who had a previous EoE diagnosis and an esophageal biopsy with greater than 15 eosinophils per HPF (range = 60–100 eosinophils/HPF). The representative images shown were taken at 10,000X magnification.

TER and paracellular flux assays

For ex vivo studies, esophageal biopsies from NL or patients with active EoE were mounted into mini-Ussing chambers, and TER measurements and paracellular flux assays using FITC-dextran (average molecular weight = 4 kDa) were performed as previously described[77]. A total of 6 NL biopsies were analyzed for TER and paracellular permeability. A total of 9 and 4 biopsies from patients with EoE were assessed for TER and paracellular permeability, respectively. To account for potential differences in biopsy thickness across the different patient groups, an arbitrary cut-off of 2% FITC-dextran flux was used, resulting in 5 of the 9 biopsies from patients with EoE being excluded. The data shown represent the mean ± SEM. In vitro measurements for TER following IL-13 treatment were assessed using an EVOM (World Precision Instruments, Inc., Sarasota, FL, USA), whereas TER and paracellular flux assays in DSG1-deficient cells were performed as previously described[78].

Statistical analyses

Statistical significance was determined using a t-test (two-tailed). Non-normally distributed data from patient biopsy samples were analyzed using a Mann-Whitney test, and the Spearman correlation was used to test for correlated gene expression. All statistical analyses were performed using GraphPad PrismR (GraphPad Software Incorporated, La Jolla, CA, USA).

Study approvals

For human subjects, written informed consent was obtained prior to a patient's enrollment in the studies, and all human studies were approved by the CCHMC Institutional Review Board (IRB protocol 2008-0090). All experiments involving mice were approved by the CCHMC IACUC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIH U19 AI070235, NIH R01 DK076893, the PHS Grant P30 DK0789392, Food Allergy Research & Education (FARE, formerly Food Allergy Project [FAP] / Food Allergy Initiative [FAI] and Food Allergy & Anaphylaxis Network [FAAN]), the Buckeye Foundation, the Campaign Urging Research for Eosinophilic Diseases (CURED) Foundation, and the Angels for Eosinophilic Research Foundation. J.D.S. was supported by a T32 NIH training grant (HL091805) and the Thrasher Research Fund Early Career Award (NR-0171). We would like to Dr. John Stanley (University of Pennsylvania) for the ETA constructs and Dr. Anil Rustgi (University of Pennsylvania) for the EPC2-hTERT cell line. We would also like to thank Shawna Hottinger for editorial assistance, all of the participating families and the Cincinnati Center for Eosinophilic Disorders, and members of the Division of Allergy and Immunology for improving the health of children with allergic and immune conditions through innovative research, outstanding clinical care, and education of the current and next generation of leaders in healthcare and research.

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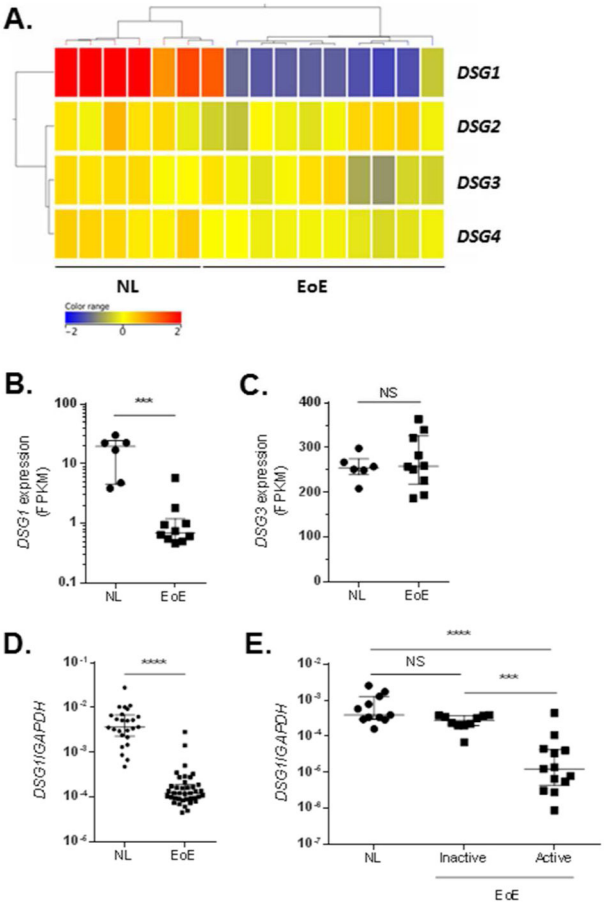

Figure 1. Specific reduction in desmoglein-1 (DSG1) gene expression in EoE

Heatmap depicting expression levels of desmogleins 1–4 (*DSG1–4*) (A) and individual FPKM values for *DSG1* and *DSG3* (B-C) from RNA sequencing of esophageal biopsies from 10 patients with active EoE versus 6 healthy controls (NL). Quantitative PCR (qPCR) analysis of *DSG1* expression in esophageal biopsies from NL (n = 25) and patients with active EoE (n = 39) (D). qPCR analysis of *DSG1* expression in esophageal biopsies from NL (n = 11) and patients with inactive (n = 10) or active (n = 13) EoE (E). Data are represented as the median + interquartile range: NS (not significant), *** $p < 5 \times 10^{-4}$, and **** $p < 1 \times 10^{-4}$.

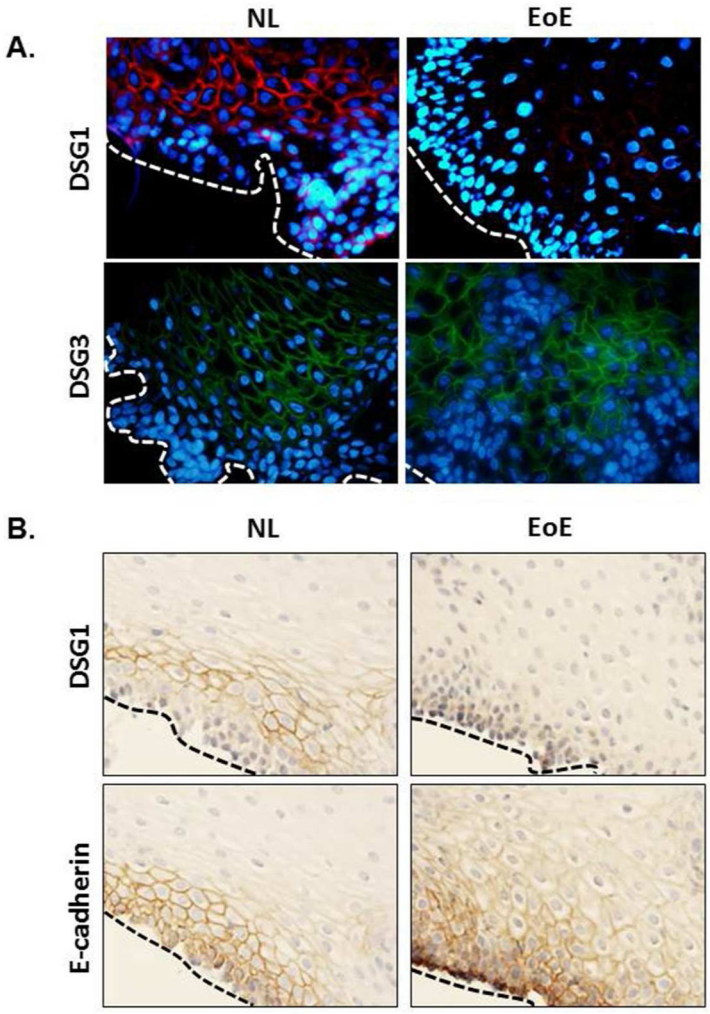

Figure 2. Loss of DSG1 protein expression in EoE

Immunofluorescence (A) or immunohistochemical staining (B) of esophageal biopsy sections from controls (NL) and patients with active EoE. In (A), DSG1 (upper panel, in red) and DSG3 (lower panel, in green) are shown. Nuclei are stained with DAPI (blue). In (B), DSG1 (upper panel, in brown) and E-cadherin (lower panel, in brown) are shown. Dashed lines in (A) and (B) indicate the basal epithelial layer. Images in are representative of 4 patients per group.

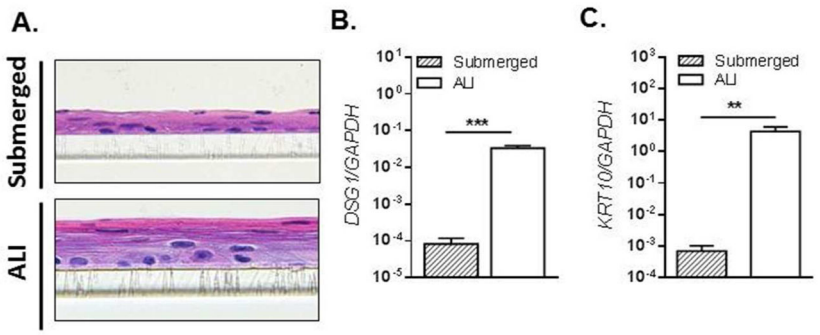

Figure 3. Differentiation of esophageal epithelial cells at the air-liquid interface (ALI)

H&E-stained sections of EPC2 cells grown in submerged cultures or differentiated at the ALI (A). qPCR analysis of desmoglein-1 (*DSG1*) (B) and keratin 10 (*KRT10*) (C) expression in submerged or ALI-differentiated EPC2 cells. Data are representative of 4 experiments performed in duplicate and are represented as the mean + SEM: ** $p < 5 \times 10^{-3}$ and *** $p < 5 \times 10^{-4}$.

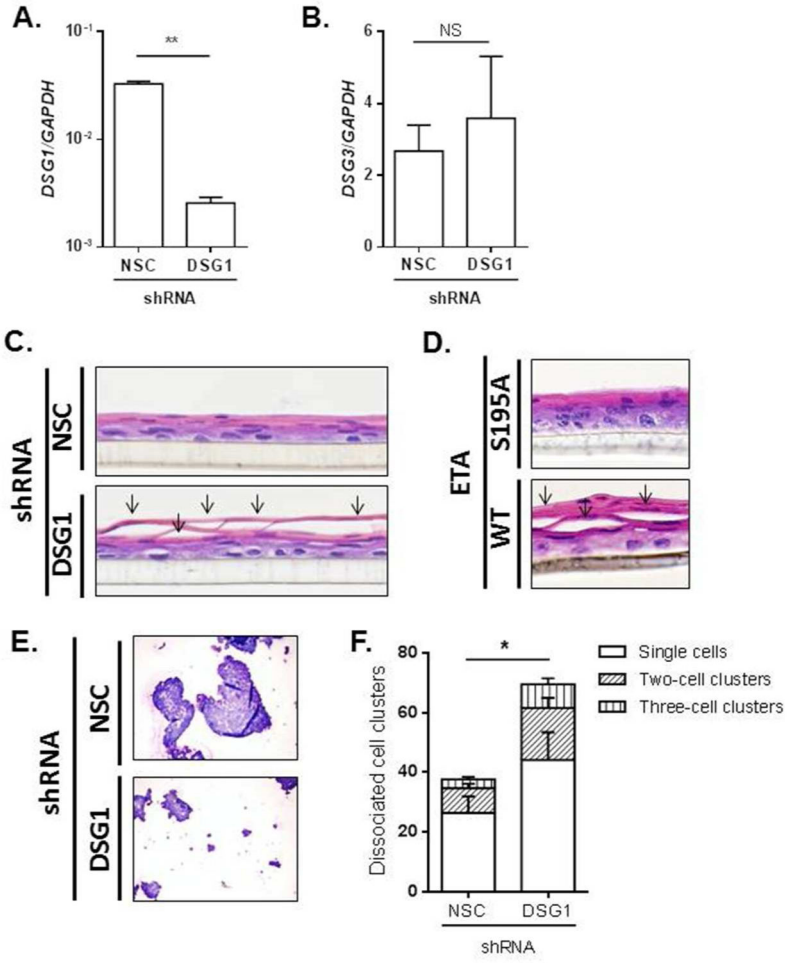

Figure 4. Loss of *DSG1* reduces esophageal epithelial cell adhesion

qPCR analysis of *DSG1* (A) and *DSG3* (B) in ALI-differentiated EPC2 cells stably transduced with non-silencing control (NSC) or *DSG1* shRNA. H&E-stained sections from stably transduced cells differentiated at the ALI (C). H&E-stained sections from EPC2 cells exposed to the air interface and treated with 10 μg/mL ETA (WT) or the S195A inactive mutant for 24 h (D). Arrows (C–D) indicate cell separation within the suprabasal epithelium. Cytospins from NSC or *DSG1* shRNA-transduced EPC2 cells following dispase adhesion assays (E) and quantification of dissociated cell clusters are shown (F). Images in (C–E) are representative of 4–5 experiments performed in duplicate. Data in (A–B) and (E) are from 3 experiments performed in duplicate and are represented as the mean + SEM: NS (not significant), * $p < 5 \times 10^{-2}$ and ** $p < 5 \times 10^{-3}$.

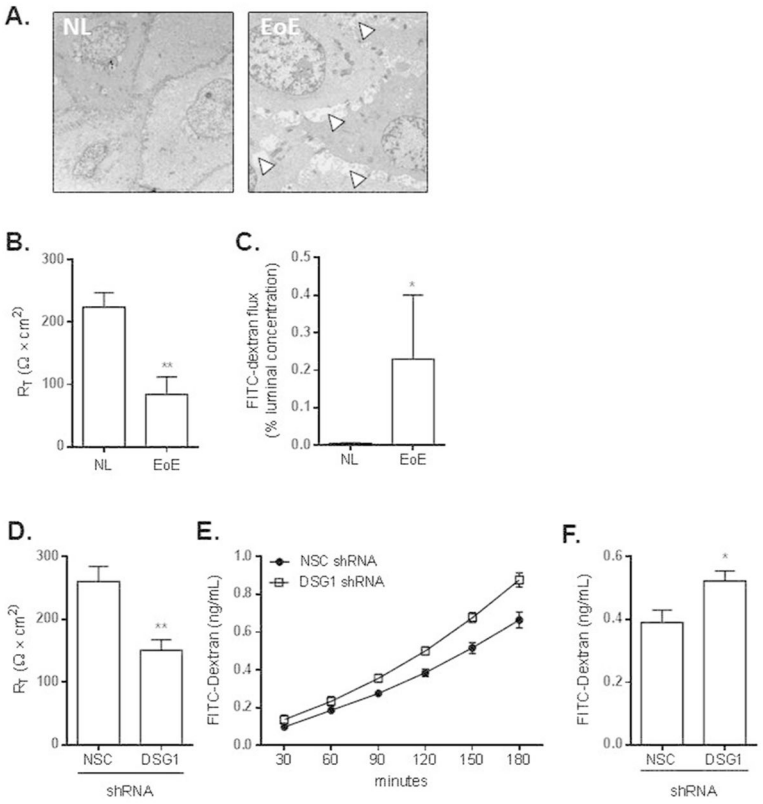

Figure 5. Impaired barrier function (IBF) in EoE can be replicated in *DSG1* deficient esophageal epithelial cells

Representative electron micrographs of esophageal biopsies from healthy (NL) controls (n = 3) and patients with active EoE (n = 3). Arrowheads indicate the presence of dilated intercellular spaces (DIS) in EoE (A). TER (R_T) measurements from esophageal biopsies from healthy (NL) control and patients with active EoE (n = 6 and 9, respectively) (B). FITC-dextran flux assays from NL and active EoE esophageal biopsies (n = 6 and 4, respectively) (C). TER (R_T) measurements from NSC and *DSG1* shRNA-transduced EPC2 cells following ALI differentiation (D). Kinetic analysis of FITC-dextran flux was also performed (E). Total FITC-dextran flux following 180 minutes are depicted in (F). Data in (D–F) are from two independent experiments performed in quadruplicate. All data are represented as the mean + SEM: *, $p < 5 \times 10^{-2}$; **, $p < 5 \times 10^{-3}$.

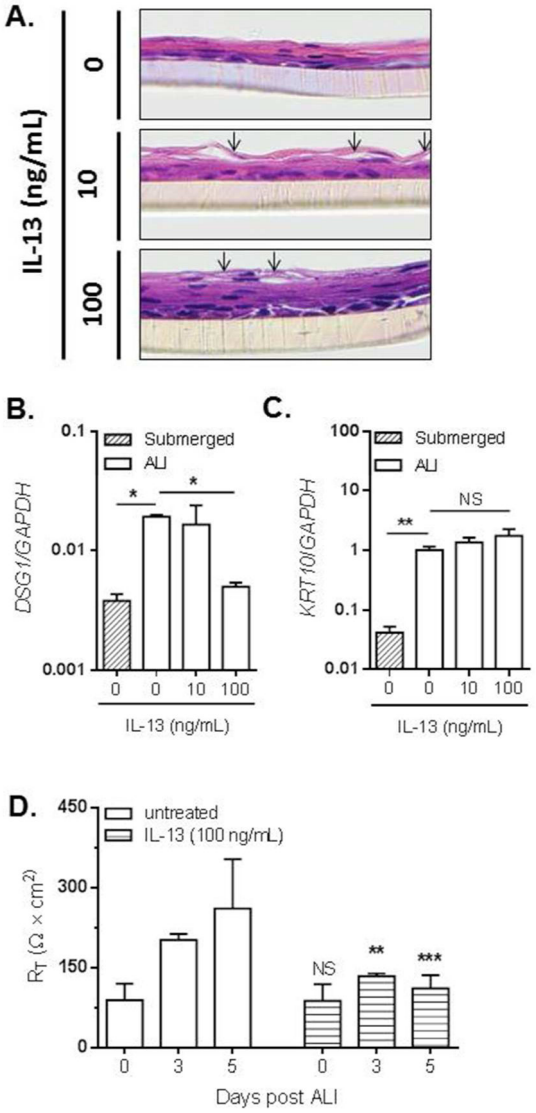

Figure 6. IL-13 downregulates *DSG1* and promotes IBF in esophageal epithelial cells

H&E-stained sections of EPC2 cells differentiated at the ALI in the presence of 0 (untreated), 10, or 100 ng/mL IL-13 (A). Arrows indicate a cell separation within to the suprabasal epithelium. Images are representative of 3 experiments performed in duplicate. Expression levels of *DSG1* (B) and *KRT10* (C) were measured by qPCR in submerged or ALI-differentiated EPC2 cells in the absence (0 ng/mL) or presence of IL-13 (10 or 100 ng/mL). TER (R_T) measurements on EPC2 cells at 0, 3, and 5 days following differentiation at the ALI in the absence (untreated) or presence of IL-13 (100 ng/mL) (D). Data are from 3 experiments performed in duplicate and are represented as the mean + SEM: NS (not significant), ** $p < 5 \times 10^{-3}$ and *** $p < 1 \times 10^{-4}$ as compared to the untreated cells at the same days post-ALI differentiation.

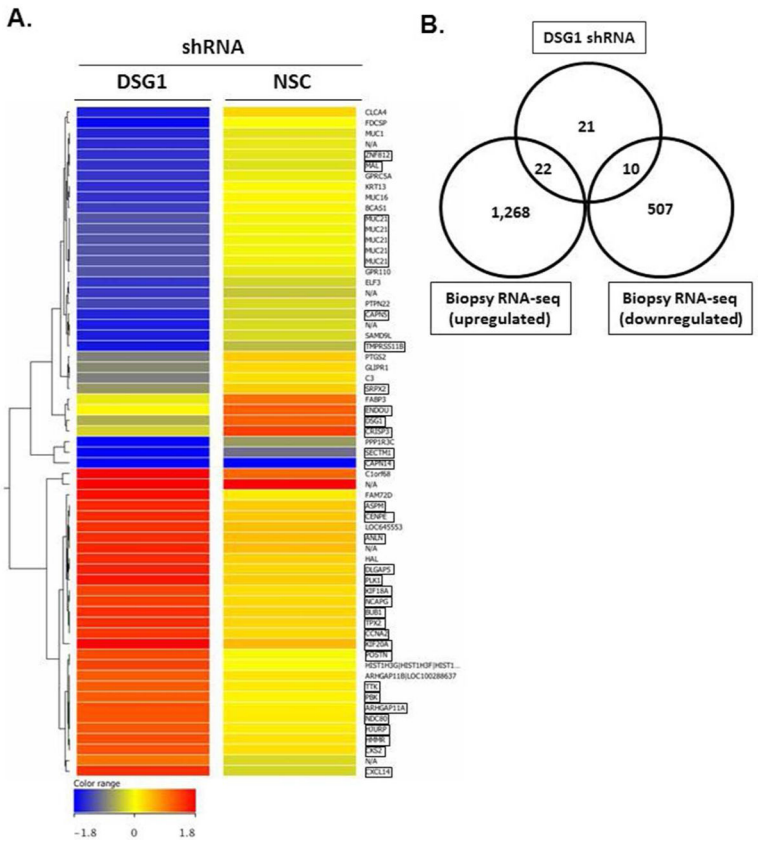

Figure 7. Loss of *DSG1* promotes epithelial pro-inflammatory transcriptional responses

Heatmap of 63 transcripts with differential expression ($p < 5 \times 10^{-2}$, fold change > 2.0) in *DSG1*-deficient EPC2 cells compared to non-silencing control (NSC) cells following ALI-differentiation (A). Venn diagram depicting the number of genes (n = 32) (boxed transcripts in [A]) dysregulated following *DSG1* knockdown that overlap with differentially expressed genes in esophageal mucosa of EoE patients (n = 10) compared to healthy control patients (NL) (n = 6) identified by RNA sequencing (B).

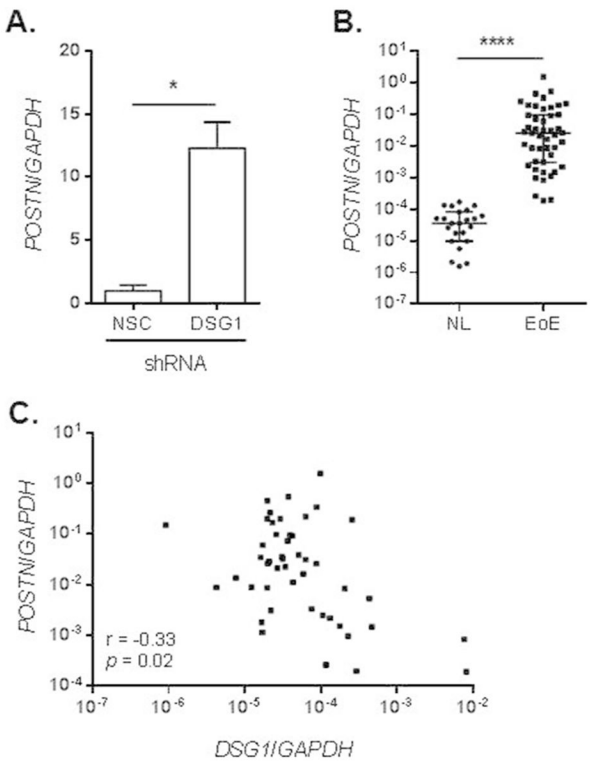

Figure 8. *DSG1* deficiency increases periostin (*POSTN*) expression

qPCR analysis of *POSTN* expression in non-silencing control (NSC) and *DSG1* shRNA-transduced EPC2 cells following ALI differentiation (A) and in patient biopsies (same cohort as in Fig. 1D) (B). Spearman correlation between esophageal expression of *POSTN* and *DSG1* (from Fig. 1D) in patients with active EoE (C). Data in (A) are representative of three independent experiments performed in duplicate and represented as the mean + SEM: * $p < 5 \times 10^{-2}$ and **** $p < 1 \times 10^{-4}$.

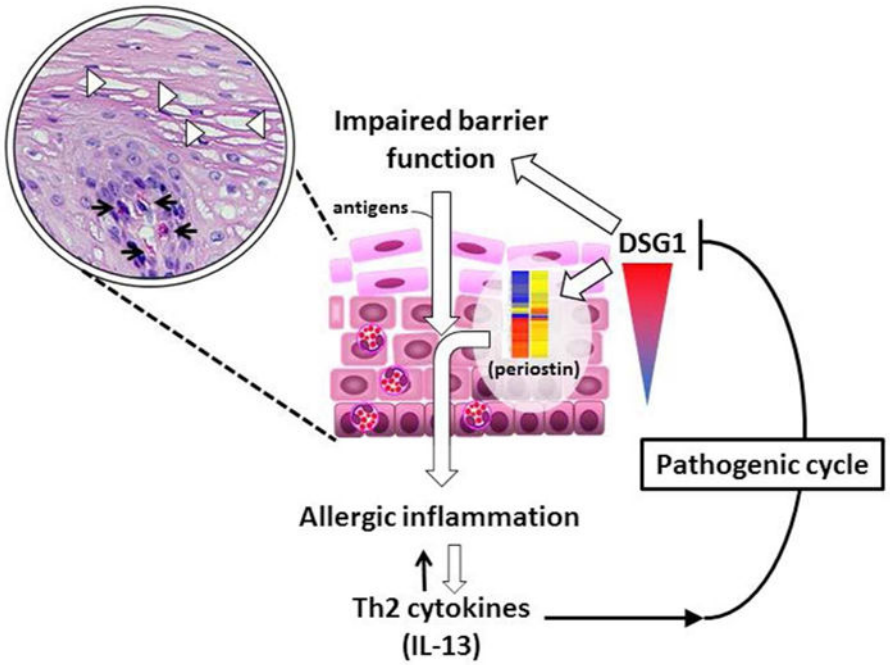

Figure 9. Model of *DSG1* dysregulation in EoE pathogenesis

Downregulation of *DSG1* by select Th2 cytokines (e.g. IL-13) results in impaired barrier function (IBF) and increased antigen exposure as well as the expression of pro-allergic mediators including periostin (*POSTN*), forming a pathogenic cycle to further exacerbate allergic inflammation. Inset shows H&E staining of inflamed esophageal mucosa with dilated intercellular spaces (DIS) (arrowheads) and eosinophilic infiltration (arrows).