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REVIEW

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Epithelial cell dysfunction, a major driver of asthma development

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Abstract

Airway epithelial barrier dysfunction is frequently observed in asthma and may have important implications. The physical barrier function of the airway epithelium is tightly interwoven with its immunomodulatory actions, while abnormal epithelial repair responses may contribute to remodelling of the airway wall. We propose that abnormalities in the airway epithelial barrier play a crucial role in the sensitization to allergens and pathogenesis of asthma. Many of the identified susceptibility genes for asthma are expressed in the airway epithelium, supporting the notion that events at the airway epithelial surface are critical for the development of the disease. However, the exact mechanisms by which the expression of epithelial susceptibility genes translates into a functionally altered response to environmental risk factors of asthma are still unknown. Interactions between genetic factors and epigenetic regulatory mechanisms may be crucial for asthma susceptibility. Understanding these mechanisms may lead to identification of novel targets for asthma intervention by targeting the airway epithelium. Moreover, exciting new insights have come from recent studies using single-cell RNA sequencing (scRNA-Seq) to study the airway epithelium in asthma. This review focuses on the role of airway epithelial barrier function in the susceptibility to develop asthma and novel insights in the modulation of epithelial cell dysfunction in asthma.

KEYWORDS

airway remodelling, asthma, (epi)genetics, epithelial barrier, type 2 responses

1 | INTRODUCTION

Asthma is a chronic inflammatory airway disease characterized by coughing, wheezing, chest tightness, variable airflow limitation and airway hyper-responsiveness (AHR)[1] to environmental specific (allergens such as house dust mite (HDM), pollen and animal dander) and nonspecific (eg tobacco smoke, air pollution) stimuli. Asthma is a heterogeneous disease with a complex aetiology. Allergen-induced asthma is the most common form, with atopy and allergic sensitization being identified as major risk factors.[2] Other risk factors include increased viral infections during early childhood, exposure to tobacco smoke and air pollution.[3] In

addition to elevated serum IgE, features of atopic asthma include chronic eosinophilic airway inflammation and airway remodelling with increased smooth muscle mass, subepithelial fibrosis, epithelial desquamation and goblet cell hyperplasia. Type-2T-helper (Th2) lymphocytes are key players in the eosinophilic airway inflammatory response of allergen-sensitized individuals, giving rise to the pathological changes and clinical symptoms of asthma.[4] Other asthma endotypes include nonallergic eosinophilic asthma, which may be driven by type-2 innate lymphocytes, mixed-granulocytic asthma, type-1 and type-17-mediated neutrophilic asthma, and paucigranulocytic asthma, without apparent neutrophilia and eosinophilia.[5]

Susceptibility to asthma has a strong genetic component. Many asthma susceptibility genes are expressed in the airway epithelium (eg *IL1RL1*, *IL33*, *TSLP*, *CDHR3*, *PCDH1*, *MUC5AC*, *KIF3A*, *EFHC1* and *GSDMB*, as outlined below), highlighting the importance of the airway epithelium in the development of asthma. Allergens, viruses and other inhaled environmental insults are in first contact with the airway epithelial barrier, which forms a continuous lining of the respiratory system from the nose to the trachea, bronchi, bronchioles and finally the alveoli. The upper airway epithelium has a different developmental origin than the epithelia of the lower airway and alveolar epithelium. The nature of the epithelium changes in the specific regions, being a pseudostratified columnar epithelium in the nose, trachea and bronchi, transitioning into cuboidal cells in the bronchioles and forming a single-cell thick alveolar epithelium. The alveolar epithelium is highly vascularized and responsible for gas exchange. The alveoli receive air from the conducting airways, starting in the trachea, bifurcating into the bronchi and bronchioles and ending in the terminal bronchioles, which divide into the alveolar ducts from which the alveoli arise. The transitional region between terminal bronchioles and alveoli is referred to as the bronchioalveolar duct junction. Alveolar cells can be subdivided into alveolar type 1 (AT1) epithelial cells, flat-shaped epithelial cells that accommodate the transfer of oxygen into the blood stream and cuboidal-shaped AT2 cells that serve as progenitor cells for AT1 cells, contribute to alveolar tissue regeneration upon injury and produce surfactants to reduce the surface tension. The pseudostratified epithelial layer of the conducting airways is separated from the underlying mesenchyme by the basement membrane and consists of different epithelial cell types: basal, club, goblet and ciliated cells being the major ones. Basal cells serve as progenitors, being able to differentiate into secretory club cells, which can further differentiate into mucus producing goblet cells or mucus clearing ciliated cells.[6] Club cells are able to self-renew and generate ciliated cells after injury, repopulating damaged airway tissue. Secretory cells also have the capacity to dedifferentiate into basal cells when these cells are ablated by diphteria toxin, underscoring the remarkable plasticity of the airway epithelium.[7] While some studies have shown that ciliated cells are terminally differentiated,[8] others have shown that ciliated cells can undergo dynamic changes in cell shape and gene expression to re-differentiate into columnar cells upon naphthalene induced injury.[9] In the presence of IL-13, ciliated cells also undergo transdifferentiation into goblet cells.[10] In addition to the physical barrier function and mucociliary clearance of foreign particles, the airway epithelium acts as chemical barrier against environmental insults by secreting, for example antimicrobial peptides, anti-proteases and antioxidants, and is part of the innate immune system. Airway epithelial cells express pattern recognition receptors (PRRs) like toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), protease activated receptor (PAR)-2 and purinergic receptors.[11]

Bullet points outlining future research perspective

- Future research unravelling the molecular mechanisms and regulatory networks underlying abnormal epithelial repair responses after exposure to environmental insults hold promise for the identification of novel intervention strategies in asthma.
- Single-cell RNA-sequencing studies may lead to elucidating the cellular changes and causal gene regulatory networks underlying the different asthma endotypes.
- Analysis of matched single-cell RNA-Sequencing data sets from airway wall biopsies, bronchial brushes and nasal brushes will allow identification of novel biomarkers for disease activity or treatment response using less invasive methodologies.
- Better understanding of (epi)genetic regulatory mechanisms of airway epithelial abnormalities in asthma likely contributes to identification of novel targets for asthma intervention.

Box outlining the major milestone discoveries

- Loss of epithelial junctions not only results in increased susceptibility towards pathogens and allergens, but also propagates pro-inflammatory responses and may contribute to airway remodelling.
- E-cadherin loss and activation of β-catenin per se induce epithelial features reminiscent of the airway epithelium in asthma in in vitro and in vivo models.
- Loss of airway epithelial barrier function in asthma is a consequence of interaction between environmental and genetic factors and epigenetic regulatory mechanisms.
- Expression quantitative trait loci (eQTL) studies in human bronchial epithelial cells and bronchial alveolar lavage identified risk alleles that regulate expression of genes involved in epithelial function, including *IL1RL1*, *IL33*, *TSLP*, *CDHR3*, *MUC5AC*, *KIF3A*, *EFHC1* and *GSDMB*, support the role of the airway epithelium as driver of asthma pathogenesis.

These recognize pathogen-associated molecular patterns (PAMPs) from inhaled microbes, parasites and allergens as well as alarmins/damage-associated molecular patterns (DAMPs) released from dying or damaged cells. Upon recognition of PAMPs or DAMPs, PRRs activate downstream signalling that promotes the release of pro-inflammatory cytokines/chemokines, including IL-6, IL-8, CCL20, CCL17, TSLP, IL-25, IL-33 and GM-CSF. These can attract and/or activate cells from the innate and adaptive immune system. Upon sensing of allergens by various PRRs, including purinergic receptors, multiprotein complexes termed inflammasome can be activated, leading to caspase-1 activity and subsequent cleavage of IL-1β and IL-18 into active forms.[12] In particular, HDM has been shown to activate the nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) inflammasome through PI3K/Akt pathway leading to inflammation in asthma.[13,14] During these allergen-driven inflammatory responses, dendritic cells (DCs) induce the differentiation Th2 cells, which secrete cytokines such as IL-4, IL-5, IL-9 and IL-13 to induce IgE production by B-lymphocytes, eosinophilic infiltration into the airways and goblet cell hyperplasia with excessive mucus production. Epithelial alarmins can drive similar responses (independent of allergens) through activation of type-2 innate lymphoid cells (ILC2).[15]

Upon damage, for example by exposure to allergens, the epithelial barrier is disrupted, promoting epithelial release of growth factors such as epidermal growth factor (EGF) and TGF-β, which activate fibroblasts and myofibroblasts.[16] This promotes excessive deposition of extracellular matrix (ECM) components, for example collagens, in the lamina reticularis just below the basement membrane, termed as subepithelial fibrosis, resulting in airway wall thickening and increased smooth muscle mass.[17] In addition, release of vascular endothelial growth factor (VEGF) by airway epithelial cells increases the size of airway wall vessels and promotes angiogenesis.[18] These structural changes are characteristic of airway remodelling in asthma (Figure 1). Thus, the airway epithelium may be crucial in the pathophysiology of asthma. In this review, we will focus on airway epithelial barrier dysfunction as driver of asthma.

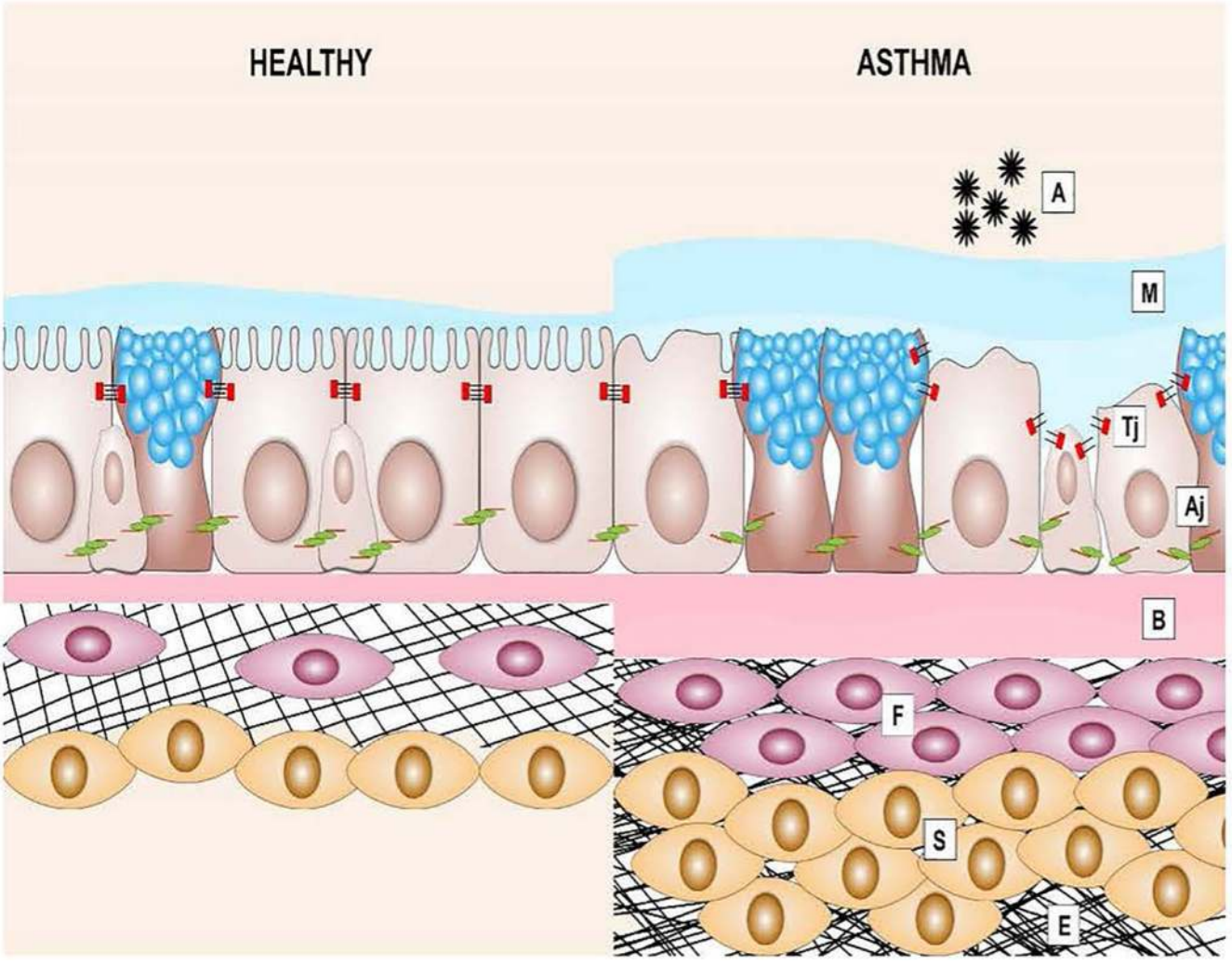

FIGURE 1 Structural changes in the airways of allergic asthma patients: Epithelial barrier dysfunction and airway remodelling. Asthmatic airway epithelium exposed to allergens (A) results in the disruption of adherens junctions (Aj) and tight junctions (Tj), which is accompanied by loss of ciliated cells, mucus hypersecretion (M), thickening of the basal membrane (B), subepithelial fibrosis (F), increased smooth muscle mass (S) and excessive deposition of ECM (E)

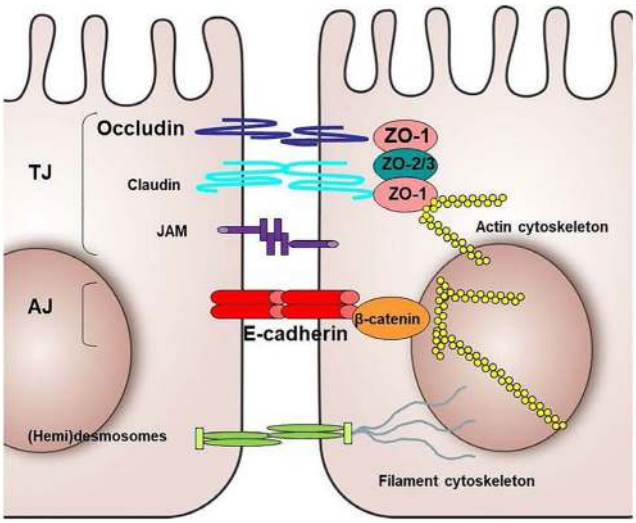

FIGURE 2 Schematic representation of the basic structural components of epithelial junctions. AJ, Adherens Junction; JAM, junctional adhesion molecule; TJ, Tight junction

2 | EPITHELIAL BARRIER DYSFUNCTION IN ASTHMA

The airway epithelial layer in asthma is disrupted, as indicated by detachment of ciliated cells, presence of epithelial cell aggregates (creola bodies) in sputum, increased permeability to allergens and reduced expression of cell-cell adhesion molecule E-cadherin.[19,20] Epithelial damage is a pathological feature observed in all phenotypes of asthma.[21] Structural changes have been observed in the airway epithelium of children with respiratory problems before the onset of airway inflammation and clinical diagnosis of asthma, suggesting that epithelial changes occur early in asthma pathogenesis.[2] This challenged the dogma that chronic airway inflammation induces airway remodelling. One of the key features of epithelial remodelling in asthma is the loss of cell-cell contact proteins, which mechanically connect adjacent epithelial cells, thereby keeping the barrier intact. These intercellular junctions are mainly comprised of tight junctions (TJs), which are located most apically, adherens junctions (AJs) and (hemi)desmosomes, which are located basolaterally (Figure 2). Desmosomes form adhesive bonds with the filament cytoskeleton between adjacent cells or between cells and the lamina propria by nonclassical cadherins.[22] The major constituent of AJs is transmembrane protein E-cadherin. Its extracellular domain binds homotypically to neighbouring cells, while the intracellular domain is linked to the actin cytoskeleton by a microtubule network of p120-catenin, β-catenin and α-catenin proteins, providing mechanical support and intracellular signalling. E-cadherin is thought to be crucial for formation of all other junctions, and its disruption results in delocalization of TJ proteins.[23,24] TJs are composed of the transmembrane proteins zona occludens-1 (ZO-1), occludin, claudins and junction adhesion molecules (JAMs) and are the main regulators of epithelial permeability.[25]

Disrupted expression of E-cadherin, β-catenin, ZO-1 and occludin has been observed in airway epithelium of asthma patients,[20,26,27] leading to impaired barrier function.[19,28] In murine studies, it has been demonstrated that the junctional proteins Zo-1, Tjp2, Occludin and Claudins-5,-8,-18 and -23 are decreased in all the three chronic HDM models of eosinophilic, neutrophilic and mixed granulocyte experimental asthma.[29] Animal models have also demonstrated that lung epithelial-specific deficiency of E-cadherin results in epithelial denudation with specific loss of ciliated cells[30] and that loss of E-cadherin in club cells induces their proliferation while inhibiting differentiation, impairing epithelial repair upon injury.[31] Expression of E-cadherin may not only be critical for the formation of a functionally intact epithelial layer, as downregulation of E-cadherin is also crucial for epithelial plasticity, where cells lose their epithelial phenotype and gain mesenchymal characteristics, termed epithelial-to-mesenchymal transition (EMT).[32] Loss of E-cadherin releases β-catenin into the cytoplasm, where it is normally proteolytically degraded by a destruction complex including glycogen synthase kinase (GSK)-3β. Inactivation of GSK-3β, for example by active WNT signalling or TGF-β, prevents the degradation of β-catenin, resulting in nuclear translocation and transcriptional activation. Active β-catenin, especially when bound to co-activator CREB-binding protein (CBP), promotes the expression of E-cadherin repressors such as Snail and Slug as well as various mesenchymal genes, including fibronectin, EGF receptor (EGFR) and VEGF, which may contribute to airway wall remodelling.[22] The initial induction of a mesenchymal phenotype enables epithelial repair, promoting cell migration and proliferation. After this, cells differentiate into a pseudostratified epithelial layer. In asthma, this repair process may be disturbed, which is supported by the observed increase in basal cell markers (eg cytokeratin 5 and p63)[22] and repair markers (eg TGF-β and EGFR) in the airway epithelium, representing a more proliferative, less differentiated phenotype.[22] HDM facilitates TGF-β-induced EMT in airway epithelial cells *in vitro*[33] and induces EMT-like features in the airway epithelium of mice.[34] In asthma, epithelial cells are more susceptible to undergo TGF-β-induced EMT.[35] The Notch signalling pathway also plays a crucial role in controlling the fate of airway epithelial cells upon injury. Although the mechanisms by which Notch signalling modulates epithelial homeostasis and responses to environmental insults are incompletely understood, various Notch (target) genes are differently expressed in healthy and asthmatic airway epithelium.[36,37]

The inability to reconstitute epithelial barrier function may have important pathophysiological consequences, not only resulting in increased permeability to allergens, but also propagating pro-inflammatory and abnormal repair responses in the airways, leading to airway hyper-responsiveness and airway remodelling[16] (Figure 3). Accordingly, airway epithelial damage has been shown to correlate with the severity of AHR.[38] Furthermore, the knock-down of E-cadherin in vitro resulted in EGFR activation and pro-inflammatory responses.[32] Upon loss of E-cadherin in vivo, the loss of ciliated cells was accompanied by spontaneous goblet cell metaplasia and infiltration of eosinophils and dendritic cells.[22] These features may at least in part be mediated by activation of β-catenin, as inhibition of β-catenin downstream activity attenuated airway inflammation, smooth muscle thickness, supepithelial fibrosis, hyper-responsiveness and

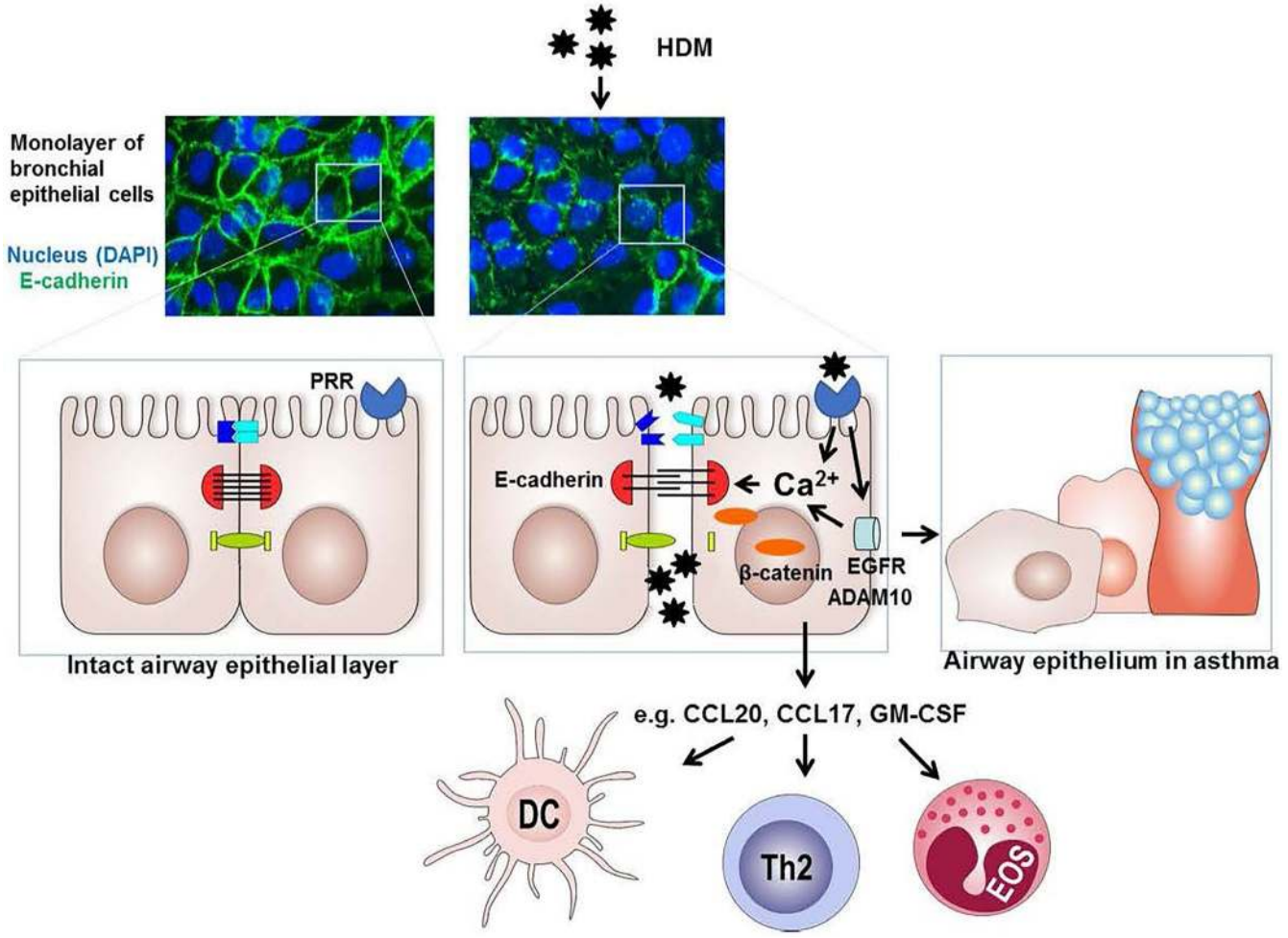

FIGURE 3 Proposed model of house dust mite (HDM)-induced airway epithelium barrier dysfunction. Allergens including HDM can directly cleave epithelial junctions proteolytically or act on various pattern recognition receptors (PRRs), including PAR-2, C-type lectins (CLR) and purinergic receptors. Their activation can induce degradation and/or delocalization of junctional proteins, including E-cadherin, in which intracellular Ca^{2+} signalling and subsequent activation of calpain may be involved and epidermal growth factor receptor (EGFR) activation.[161] EGFR can activate ADAM10, a sheddase of E-cadherin as well as CCL20.[40] In addition, EGFR signalling can induce secretion of pro-inflammatory mediators, such as CCL20, CCL17 and GM-CSF that attract and/or activate dendritic cells (DCs), Th2 cells and eosinophils (EOS).[22] When epithelial repair and re-differentiation is impaired, persistent loss of E-cadherin can result in activation of β-catenin-mediated programs that cause further loss of epithelial characteristics, induction of a more basal/mesenchymal phenotype as well as goblet cell hyperplasia, with loss of ciliated cells, as is also characteristic of the epithelial phenotype in asthma

goblet cell metaplasia in mouse models of asthma.[39] Moreover, we recently demonstrated that inhibition of β-catenin/CBP signalling not only improves epithelial barrier function, but also attenuates HDM-induced airway epithelial pro-inflammatory responses in vitro.[40]

3 | ENVIRONMENTAL RISK FACTORS AND EPITHELIAL BARRIER DYSFUNCTION IN ASTHMA

As described above, the development of asthma results from the interaction between genetic and environmental factors. Various in vitro studies have shown that allergens can disrupt the airway epithelial barrier.[41] Exposure of cultured airway epithelial cells to proteolytically active allergens from house dust mites (eg Der p1), ragweed, white birch, grass and pollen can lead to the cleavage of the junctional proteins.[22] Furthermore, house dust mite (HDM), cockroach, fungi and mould extracts have been shown to disrupt epithelial junctions via activation of PAR-2 and downstream signalling.[42] Accordingly, exposure of human airway epithelial cells to HDM induces rapid, transient reduction in epithelial barrier function,[33] concomitant with delocalization of junctional proteins (Figure 3). Submerged cultures of airway epithelial cells from mild/moderate asthma patients were more susceptible to HDM-induced barrier dysfunction than healthy subject-derived cultures. Surprisingly, this was independent of serine and cysteine proteases.[43,44] Yet to be identified PRRs coupled to Ca^{2+}/calpain-dependent disruption of epithelial junctions may be involved.[43] In addition to direct effects of allergens, allergic sensitization may

lead to epithelial barrier dysfunction as consequence of type-2 mediated airway inflammation associated with atopic asthma. Both Th2 cells and ILC2 may contribute to the compromised epithelial barrier function through IL-13 secretion, which induces many features of the airway epithelium in asthma, including mucus production, and has been reported to disrupt airway epithelial barrier function in vitro.[45,46] In fact, Th2-derived IL-13 and IL-4 and type-2 driving cytokine TSLP[47] have been shown to decrease barrier integrity in air-liquid interface (ALI) cultured primary airway epithelial cells from healthy subjects, with delocalization of TJ proteins.[48] This was not observed in cultures derived from asthma subjects, in which barrier function was already compromised at baseline.[48] This may reflect cell-intrinsic loss of airway epithelial cells in asthma to re-differentiate and form an effective barrier upon ALI culture in vitro, as proposed previously.[2]

In addition to allergens, early-life sensitization to lower respiratory viral infections is an important environmental risk factor for developing asthma in childhood, with the highest risk for progression to persistent asthma when these environmental exposures coincide.[49] Two major respiratory viruses, rhinovirus (RV) and respiratory syncytial virus (RSV), bind to specific receptors on the airway epithelium, for example cadherin-related family member 3 (CDHR3) and ICAM-1 for RV[50] and CX3CR1 for RSV.[51] Upon internalization, uncoating and replication, the virus is recognized by TLR3 and RIG-I like helicase, inducing production of anti-viral type-I interferons (IFNs), which eradicate pathogens and promote pro-inflammatory cytokine release. Impaired epithelial barrier function is accompanied by compromised IFN responses in asthma, resulting in increased viral replication upon rhinovirus infection compared to nonasthma-derived epithelial cultures.[52] Exposure of airway epithelial cells to double-stranded RNA or infection with RV or RSV in vitro induces upregulation of TSLP[53,54] and may thus support type-2 mediated inflammation. This may further impair epithelial barrier function in a vicious circle, viral exposure causing disruption of epithelial cell-cell contacts.[55,56] RV has been shown to disrupt TJ integrity in human bronchial epithelial cell lines and ALI-differentiated primary cultures via loss of ZO-1 from TJs and airway epithelial cells cultures from healthy and asthmatic children, with more pronounced and sustained effects in asthmatic-derived cultures.[57]

Other environmental factors that may impact on epithelial integrity are those associated with nonatopic forms of asthma, for example noneosinophilic, neutrophilic asthma. Besides viral infections, these include smoking[58] and bacterial colonization.[21] Smoke exposure is well known to cause airway epithelial barrier dysfunction by disruption epithelial junctions.[59] Indirectly, smoking-induced Th17-mediated inflammation can reduce epithelial barrier function through Th17 cytokine IL-17.[58] As colonization of the respiratory tract with bacteria, *for example Streptococcus pneumoniae*, *Haemophilus influenzae* or *Moraxella catarrhalis*, may increase the risk of asthma, it is of relevance that also bacteria can cause epithelial barrier dysfunction, as demonstrated for infection with *S pneumonia* in a bronchial epithelial cell line.[60]

Finally, environmental pollutants such as particulate matter and ozone as well as household cleaning products may contribute to the development and/or worsening of asthma[61] and can disrupt the epithelial barrier. Particulate matter has been shown to attenuate ciliary beat frequency in bronchial epithelial cells and degrade TJ proteins in lung epithelial cells.[62] Diesel exhaust particles decreased the expression of TJ proteins and epithelial resistance in primary nasal epithelial cells.[63] Ozone was reported to cause rapid disruption of the epithelial barrier with increased permeability and diminished expression of TJ and AJ proteins in the absence of IL-33.[64] Of interest, also laundry detergents were recently shown to compromise human bronchial epithelial integrity by disruption of tight junctions and may thus contribute to the development of asthma.[65]

4 | GENETIC FACTORS AND THE EPITHELIAL BARRIER IN ASTHMA

As mentioned above, in addition to environmental factors, a heredity component contributes to disease risk, with 35%-95% of susceptibility thought to involve genetic factors. Positional cloning and more recently genome-wide association studies (GWAS) have been highly successful in identifying risk alleles and loci for asthma and related phenotypes.[66]

Expression quantitative trait loci (eQTL) studies in human bronchial epithelial cells and bronchial alveolar lavage identified that risk alleles regulate highly relevant genes involved in epithelial function, for example *IL1RL1*, *IL33*, *TSLP*, *HLA-DQB1*, *CDHR3*, *ZTB10*, *Corf30*, *DEX1* and *GSDMB* levels.[67] Similarly, Luo and colleagues combined asthma GWAS results and small and large airway epithelial eQTL data to demonstrate enrichment of airway epithelial eQTLs.[68] This supports the barrier hypothesis, where genetic alterations influence the ability of the skin and epithelial tissues to form a protective barrier from, for example pathogens and allergens.[1] The finding that the majority of genetic variants associated with risk of developing asthma is shared risk factors for the development of atopic dermatitis and allergic rhinitis[69] further underlines this. Selected genes identified through asthma genetic studies and implicated in epithelial cell function are outlined in Table 1. Genetic changes in the epithelium may thus be important in mediating several aspects of relevance to asthma, including the inflammatory environment, for example *IL33*, *TSLP*, *IL1RL1*, responses to pathogens, for example *CDHR3*,

[1]The shared genetic origin of asthma, rhinitis and eczema was recently analysed in detail.[133] This approach revealed a striking overlap in risk SNPs between these three allergic disorders, with limited disease-specific polymorphisms. The study identified a total of 132 plausible target genes, which were enriched for expression in blood and lung tissue.[133] These results clearly indicate that susceptibility to allergic diseases is mediated by at least in part shared biological mechanisms. Loss of epithelial barrier function has indeed been postulated as a central mechanism in allergic rhinitis[134] and eczema[135] as well, with loss of function variants in epidermal protein filaggrin being identified as major predisposing factor of atopic dermatitis.[136] In addition, GWAS studies have identified epithelial junction protein Desmoglein 1 as susceptibility gene for eosinophilic esophagitis.[137]

TABLE 1 Selected genes identified through genetic studies of asthma implicated in airway epithelial cell homeostasis which may impact barrier properties and inflammation

Chrs	Gene Reported variants	Main Asthma Phenotype(s)	Suggested role in HBEC homeostasis/epithelial gene expression	References
2q12.1	[a]*IL1RL1* *rs3771166*	Asthma, Asthma + Exacerbation, moderate-severe asthma	IL33 receptor, regulates inflammation. Important in innate immune responses including responses to viruses and Type 2 inflammation. Expressed in HBEC	74,76,138,139
5q22.1	[a]*TSLP* *rs1043828*	Asthma, Asthma + Hay fever, moderate-severe asthma	Can drive induction of allergic responses by effects on several cell types including dendritic cells. Regulates an IL-13–dependent increase in bronchial epithelial cell proliferation	138,140-143
5q31.1	*KIF3A* *rs17690965*	Atopic Dermatitis followed by Asthma	Molecular motor that transports molecules along microtubules, role in ciliary function. Role in epithelial apoptosis and inflammation	70,71,144
5q31.3	*PCDH1* *rs3797054* *rs3822357*	Airway hyper-responsiveness	Epithelial adhesion, differentiation, barrier formation	78,79,145
6p12.2	*EFHC1* *rs9357733*	Atopic Dermatitis followed by Asthma	Contains an EF-hand motif which is able to bind Ca^{2+} ions. Involved in ciliary function	70,72,73
7q22.3	[a]*CDHR3* *rs6967330*	Asthma + Exacerbation	Epithelial polarity and cell-cell interactions. Receptor for Rhinovirus C, the most common respiratory virus associated with exacerbations in asthma. Cys529Tyr regulates viral entry	76,77,146
9p24.1	[a]*IL33* *rs1342326*	Asthma, Asthma + Exacerbation, moderate-severe asthma	Epithelium-derived cytokine alarmin, regulates inflammation via interactions with ST2/IL1RL1 on several inflammatory cells. Type 2 inflammation, viral exacerbation. Also activates HBEC via ST2/IL1RL1	74,76,138,147,148
11p15.5	[a]*MUC5AC* *rs11603634*	Moderate-Severe asthma	Oligomeric mucus/gel-forming, a pathogenic mucin linked to allergic airway hyper-reactivity. Elevated in bronchial epithelial cell brushing from severe asthma patients	74,75
15q22.33	*SMAD3* *rs744910*	Asthma, Asthma + Hay fever	Signalling intermediate in the TGF-β_1 induced epithelial–mesenchymal transition	69,74,75,139,149,150
17q21.1	[a]*GSDMB* *rs7216389*	Asthma, childhood asthma + exacerbations, Asthma + Hay fever, childhood asthma, moderate–severe asthma	Member of gasdermin-domain containing protein family, elevated in the airway epithelium in asthma and in mice increased expression led to spontaneous, remodelling and airway hyper-responsiveness. Epithelial cell pyroptosis	76,82,83,140,151
17q21.1	*ORMDL3* *rs7216389*	Asthma, childhood asthma + exacerbations, Asthma + Hay fever, childhood asthma, moderate–severe asthma	Orosomucoid-like protein isoform 3, regulates endoplasmic reticulum (ER) stress. Implicated in epithelial barrier formation, pro-remodelling phenotype in vivo and in vitro. Sphingolipid regulation	69,76,138,151-156
19q23	*PLAUR* *rs4493171 rs2356338* *rs2239372*	Asthma, decline in lung function	Regulates activation of urokinase plasminogen activator (uPA), triggering the plasminogen/plasmin activation cycle. Epithelial repair, proliferation, pro-remodeling phenotype	157-160

Note: For a comprehensive review of asthma related phenotypes, these loci have been associated with see recent reviews.[54,142]

Abbreviations: *CDHR3* cadherin-related family member 3; EFHC1, EF-hand domain containing protein 1; *IL1RL1*, Interleukin 1 Receptor Like 1; IL33, Interleukin 33; *KIF3A*, Kinesin Family Member 3A; *MUC5AC*, Mucin 5AC, Oligomeric Mucus/Gel-Forming; *ORMDL3*, ORMDL sphingolipid biosynthesis regulator 3; *PCDH1*, Protocadherin 1; *PLAUR*, plasminogen activator, urokinase receptor; *SMAD3*, *GSDMB*, gasdermin B; *TSLP*, Thymic stromal lymphopoietin.

[a]Identified in eQTL studies using asthma risk alleles in airway epithelium.

mucociliary clearance, for example *MUC5AC*, *KIF3A*, *EFHC1* and cell homeostasis and epithelial integrity, including proliferation, migration, cell-cell adhesion, apoptosis and repair, for example *PCDH1*, *SMAD3*, *GSDMB*, *ORMDL3 and PLAUR*.

While a discussion of all these genes is beyond this review, it is important to highlight selected genes particularly implicated in barrier function. In the GWAS of atopic dermatitis followed by asthma, two genes thought to be involved in ciliary function were implicated,

that is *KIF3A and EFHC1*.[70] These genes encode for Kinesin Family Member 3A and EF-hand domain containing protein 1, respectively. *KIF3A* is thought to function as a molecular motor transporting molecules along microtubules and has also been implicated in ciliary function in epithelial cells. Interestingly, mice deficient in *KIF3A* in the epithelium is more susceptible to allergen-induced inflammation and epithelial cell apoptosis in an allergic airway model.[71] Mutations within *EFHC1* have been associated with juvenile myoclonic epilepsy via a role in motile cilia and in regulating calcium channels.[72,73] Importantly, *EFHC1* may be of relevance in cilia function in the airways, being expressed in the tracheal epithelium in mice. Therefore, *KIF3A* and *EFHC1* may in part contribute to poor allergen and mucus clearance from the airways. Recently, in a GWAS of moderate-severe asthma, a signal on chromosome 11 was identified that regulates expression of *MUC5AC*,[74] the main mucin found in the airways and linked to severe asthma,[75] emphasizing abnormal mucociliary clearance. In a GWAS of asthma with exacerbation, polymorphisms spanning *CDHR3* were identified, including coding change Cys529Tyr.[76] *CDHR3* is involved in epithelial polarity and cell-cell interactions. As described above, recent data suggest that CDHR3 is the receptor for RV-C and the Cys529Tyr mediates this interaction providing a putative mechanism. Interestingly, *CDHR3* knock-down also influences transepithelial resistance.[77] The *PCDH1* gene also encodes an adhesion molecule localizes to cell-cell junctions especially in differentiated airway epithelial.[78,79] PCDH1 has a dual function, supporting epithelial barrier function[79] and regulating TGF-β/SMAD3 signalling.[80] Hence, PCDH1 may serve as cellular switch between TGF-β driven EMT and epithelial repair vs epithelial differentiation and barrier formation. The gene *ORMDL3* regulates cytosolic Ca^{2+} entry by the sarco-endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA) pump, which we previously showed to be involved in HDM-induced epithelial barrier dysfunction.[43] Moreover, the *ORMDL3* gene product was recently shown to support RV replication in epithelia cells.[81] Finally, *GSDMB* encodes gasdermin B, which is a member of the gasdermin-domain containing protein family linked to epithelial apoptosis. Recently, it has been shown that *GSDMB* is elevated in the airway epithelium in asthma. In mice, increased expression led to spontaneous airway hyper-responsiveness,[82] and the GSDMB protein induces pyroptotic cell death in airway epithelium.[83] Although several asthma genes have been shown to act on airway epithelial function, a clear endotype of asthma driven by the loss of epithelial barrier specifically due to these asthma-associated polymorphisms has not been identified. However, it is important to note that the asthma phenotypes associated with these selected genetic signals include bronchial hyper-responsiveness (*PCDH1*, *PLAUR*, *ORMDL3/GSDMB*) and asthma exacerbation (*IL33*, *IL1RL1*, *CDHR3*, *ORMDL3/GSDMB*), potentially directly by effects on bronchial epithelial function. Similarly, genetic signals associate with blood eosinophil counts (*IL33*, *IL1RL1*, *TSLP*), time to asthma onset (*IL33*, *IL1RL1*, *ORMDL3/GSDMB*), atopic march (*KIF3A*, *EFHC1*) and self-reported allergy (*IL33*, *ORMDL3/GSDMB*, *IL1RL1*), potentially via an indirect mechanism by the production of cytokines from bronchial epithelial cells leading to type-2 inflammation.[84,85] The gene signature of the type-2 high endotype of asthma, characterized by increased blood and BAL eosinophils and basal membrane thickness, lower PC20 threshold and a better lung function improvement after inhaled corticosteroids, identifies this asthma subphenotype as a steroid responsive signature of epithelial cells in asthma,[86] indicating the relevance of the airway epithelial phenotype in the disease. Two of these genes (*CLCA1* and *SERPINB2*) are predominantly expressed in goblet cells, indicating that a true asthma endotype reflecting loss of epithelial barrier function is yet to be identified.

5 | EPIGENETIC FACTORS AND THE EPITHELIAL BARRIER IN ASTHMA

As outlined, asthma-associated polymorphisms can directly alter a gene's coding sequences, thereby altering protein function and, consequently, the biology of the airway epithelium. More frequently, however, asthma-associated SNPs have a regulatory effect on gene expression, acting as eQTLs. A recent study shows that almost 59% of the asthma-associated SNPs identified by the Trans-National Asthma Genetic Consortium (TAGC) study is an eQTL in nasal epithelium and that in almost 90% of these cases, this effect is mediated by CpG methylation.[87] Clearly, epigenetic regulation of gene expression is highly relevant to the translation of disease susceptibility into altered biology of the airway epithelium. Epigenetic marks are highly responsive to environmental exposures relevant to asthma inception or exacerbations, further underscoring the relevance of epigenetics for understanding asthma pathophysiology.[88-91] Three main types of epigenetic marks can be distinguished: CpG methylation, histone modifications and small, noncoding RNAs.

Differences in DNA methylation patterns between asthma patients and healthy controls have been studied in (epi)genome-wide analyses (EWAS). As CpG methylation patterns are also highly cell-type dependent, cell-type composition of the biological sample is an important cofounder of EWAS analyses.[92] Therefore, we here focus on the studies in upper (nasal) airway brushes, that mainly consist of epithelial cells,[93] and which were shown to have the best correlation to the DNA methylation patterns in bronchial epithelial cells.[94] In four studies reported to date,[95-98] methylation of the *GJA4* gene, encoding Connexin37, a protein capable of forming heterotypic gap junctions, was consistently found to be reduced, although an association with altered gene expression levels was not detected.[97] Other genes relevant to epithelial barrier function (*CDH26*, *CDHR3*) were also found differentially methylated.[95,97] In addition to *CDHR3*, another genes selectively expressed in ciliated epithelial cells, *ZMYND10*[91] was found to be differentially methylated, which is consistent with an altered airway epithelial composition in asthma. Only one study to date analysed CpG methylation in bronchial biopsies from asthma patients and healthy controls, but this analysis was focussed on methylation patterns associated with remission of asthma.[99]

Several studies have looked specifically into DNA methylation changes induced by relevant environmental factors, which affect epigenetic regulation of asthma genes.[100-102] RV infection-induced DNA

methylation patterns differed between nasal epithelial cells from asthmatic children and healthy controls, with enrichment for loci carrying genes involved in cell-cell and cell-matrix interactions.[100] Similarly, RV infection-induced DNA methylation patterns differed between nasal epithelial cells from asthmatic adults and healthy controls.[102] In children who had early-life rhinovirus-induced wheezing, specific DNA methylation patterns associated with asthma later in life were identified, including increased methylation at the *SMAD3* locus.[101] Finally, one elegant study analysed the effects of diesel exhaust particle exposure and (segmental) allergen challenge on DNA methylation patterns in airway epithelial cells obtained by bronchial brushing both 48 hours after exposure and after 4 weeks.[103] While both allergen challenge and diesel exhaust particle exposure induced DNA methylation changes in airway epithelial cells, the most pronounced effects were observed in individuals who received an allergen challenge 4 weeks prior to exhaust particles exposure, with genes annotated to cell adhesion being most enriched in the differentially methylated regions.[103] These data clearly indicate the relevance of environmental exposures for epigenetic regulation of gene expression in the airway epithelium and therefore for asthma. As the epigenetic signature of the airway epithelium integrates genetic susceptibility with the life history of relevant environmental exposures, it can be expected to be a strong biomarker for asthma development or even treatment response.[98]

In addition to DNA methylation, epithelial gene expression can be modulated by miRNAs, which are small noncoding RNAs of about 21-25 nucleotides that can bind to target mRNAs, leading to mRNA degradation or translational repression. Altered miRNA profiles have been observed in airway epithelium of asthma patients compared to healthy controls.[104,105] Several of the differentially expressed miRNAs modulate the expression of genes implicated in epithelial barrier function, repair, proliferation or apoptosis. For example, miR-744, miR-19a, miR-221, miR-27a, miR-128 and miR-34/449 are differentially expressed in bronchial epithelial cells from asthma patients compared to controls and have been described to modulate cell proliferation, apoptosis and ciliogenesis by targeting TGF-β1, TGF-βR2, SIRT1, SMAD2 (target of both miR-27a and miR-128) and Cp110, respectively (Figure 4).[106-111] Of interest, the discussed miRNAs were not all identified in patients with the same disease severity. The differential expression of miR-744, miR-221 and miR-19 was shown in HBEC from severe asthma patients with an atopic and eosinophilic phenotype,[106-108] whereas miR-34/449 was identified in mild atopic asthma.[105] While miR-19 was higher in severe atopic eosinophilic asthma, its expression in mild asthma was similar as in healthy controls.[108] Moreover, a miR-19 mimic induced more proliferation in HBEC from severe asthma patients than in control-derived HBEC. The expression of miR-744 was reduced in HBEC from severe asthma, but tended to increase in mild asthma

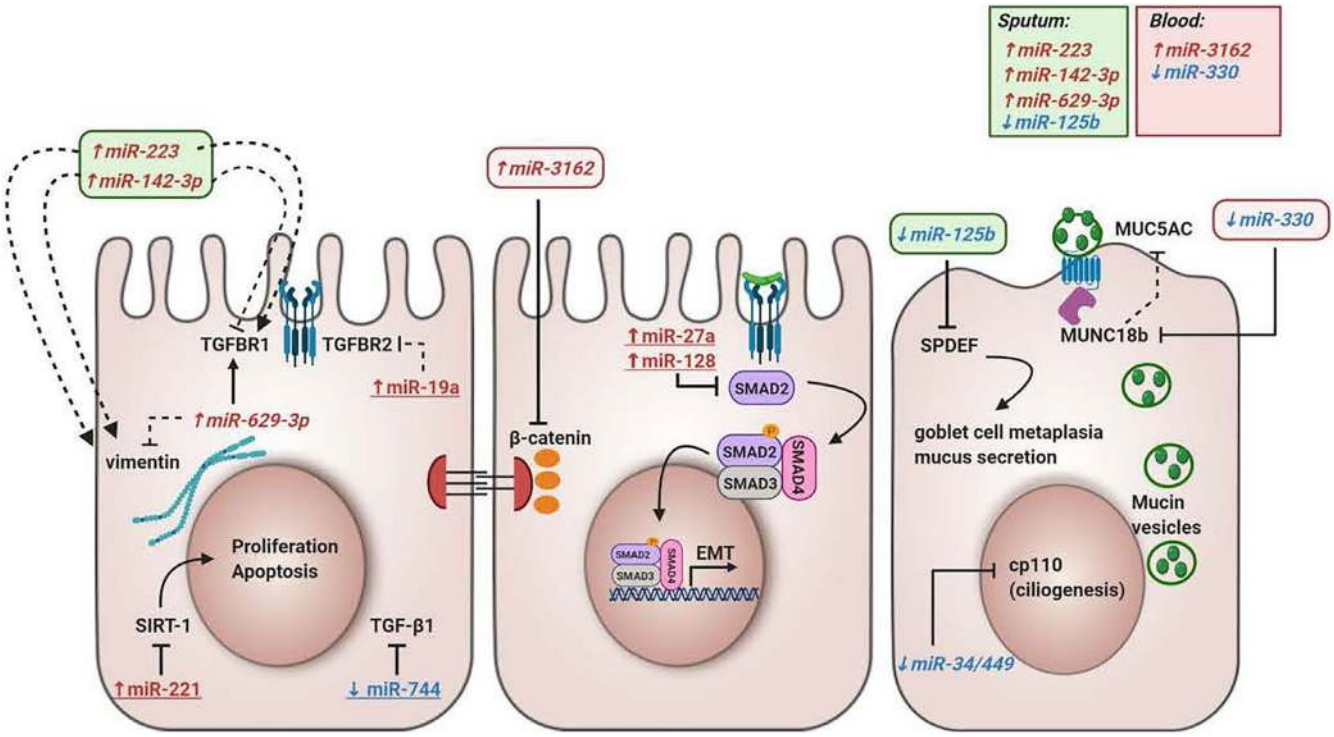

FIGURE 4 The influence of microRNAs in epithelial barrier function. This overview illustrates miRNAs that are differentially expressed in asthma and could contribute to epithelial barrier dysfunction in asthma. miRNAs coloured in red with upward arrow are upregulated in asthma, and miRNAs coloured in blue with downward arrow are downregulated in asthma. miRNAs with an underscore were measured in bronchial epithelial cells, and miRNAs in italic were measured in sputum or blood from asthma patients and controls. Black lines ending with a perpendicular line indicate inhibitory effects, and black lines ending with an arrow indicate a stimulatory effect. Full lines indicate direct effects, and half-full lines indicate indirect effects. EMT, epithelial-mesenchymal transition; LPS, lipopolysaccharide; SIRT-1, Sirtuin 1; SPDEF, SAM Pointed Domain Containing ETS Transcription Factor; TGF-β1, Transforming Growth Factor Beta 1; TGFBR1, Transforming Growth Factor Beta Receptor 1

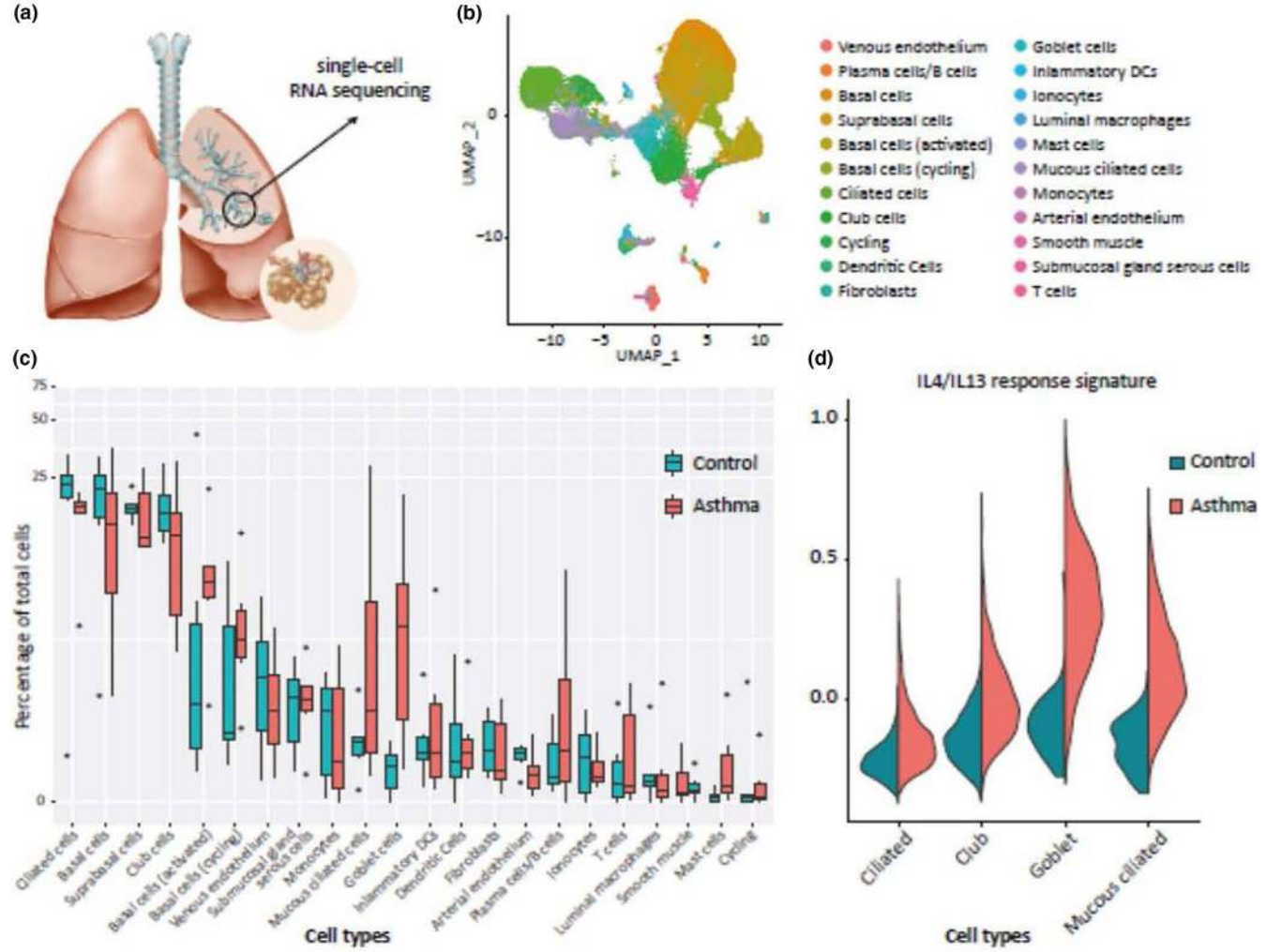

FIGURE 5 Analysis of airway epithelial cells in asthma using single-cell RNA sequencing. (A) Airway wall biopsies are obtained from 5th-7th generation airway through bronchoschopy, followed by tissue digestion and scRNA-Seq analysis. (B) Unsupervised clustering identifies a large number of epithelial and nonepithelial cell types from airway wall. (C) Comparison of relative frequencies of cell types identified increased number of goblet cells and mucous ciliated cells, a novel, disease-associated ciliated epithelial cell phenotype and increased numbers of mast cells and B cells in asthma compared to healthy. (D) Analysis of epithelial cell subset-specific transcriptomes reveals presence of IL4/IL13-induced gene transcription in goblet cells and mucous ciliated cells, specifically in asthma

compared to healthy controls.[106] These observations suggest that the impact of the miRNAs on the epithelium can differ with disease severity; however, this requires further investigation. It is also unknown whether miRNAs that affect epithelial barrier in asthma modify the treatment response, but for miR-34/449, miR-19 and miR-223, there were no correlations between miRNA expression and treatment with inhaled corticosteroids.[105,108,112] Furthermore, miR-155 and miR-223 have been implicated in EMT by altering mesenchymal markers,[112,113] although their exact role in asthma airway epithelial cells is unknown. Also, differential expression of miR-3162, miR-125b, miR-223 and miR-330 in blood or sputum from asthma patients, possibly transported in extracellular vesicles (EVs), can affect the epithelial barrier function by influencing the expression of, for example β-catenin, vimentin and mucins[112,114-117] (Figure 4). Moreover, airway epithelium itself can communicate by secreting EVs. Epithelial-derived EVs play a role in airway homeostasis and airway epithelial remodelling by inducing amongst others mucin hypersecretion.[117] The miRNA signature in epithelial-derived EVs is altered upon stimulation with IL-13 compared to EVs obtained from untreated bronchial epithelial cells.[118] However, it is unclear whether similar changes can be observed in the miRNA profile of epithelial-derived EVs from asthma patients and whether those changes in miRNA expression affect the epithelial barrier. In asthma murine models, lower miR-448-5p and higher miR-106a levels were expressed in lung tissue compared to control mice.[111,119] In vitro up- or downregulation of these miRNAs resulted in altered protein levels of E-cadherin, fibronectin, collagen IV and vimentin in bronchial epithelial cells after TGF-β1 stimulation.[111,119]

Together, the interaction between genetic factors and epigenetic regulatory mechanisms may contribute to abnormalities in the airway epithelium and the development of asthma. Understanding these mechanisms may lead to identification of novel targets in airway epithelium for asthma intervention.

6 | NEW INSIGHTS FROM SINGLE-CELL SEQUENCING DATA

Further insight into the mechanisms of asthma and the role of the airway epithelium may come from technological advances. These include recent progress in single-cell RNA sequencing (scRNA-Seq), greatly enhancing the granularity at which the cellular composition of tissues can be characterized.[120] In addition, scRNA-Seq allows the description of molecular cell phenotypes (or cellular "states"), predict cell-cell interactions and cell state transitions at unprecedented detail. Using these technologies to study lung tissue, the ionocyte has recently been discovered as novel airway epithelial cell.[6,121] The pulmonary ionocyte is a relatively rare cell type, characterized by expression of ion transporters including V-ATPase and the cystic fibrosis *CFTR* gene, indicating a role in regulation of ion and fluid transport across the airway epithelium as well as pH of the mucosal surface. While the application of these technologies to identify all cell types of the healthy human body, including lung, as pursued by the Human Cell Atlas consortium[122,123] are exciting, these novel techniques also hold great potential to increase our understanding of disease pathogenesis. A first description of the cellular landscape of healthy airway wall and the changes thereof in patients with childhood-onset allergic asthma identified a unique disease-associated airway epithelial cell state, as well as a remarkable shift in cell-cell communication.[93] Various known changes in the asthmatic airway wall were recapitulated by scRNA-Seq analysis, such as increased numbers of airway smooth muscle cells, goblet cells and mast cells, underscoring the validity of the approach (Figure 5). The study identified a subset of ciliated epithelial cells in asthma that was characterized by expression of *MUC5AC* and other goblet-cell genes, a molecular phenotype of ciliated cells that was not observed in healthy airway walls.[93] This so-called mucous ciliated cell type was mapped to the ciliated differentiation trajectory. Interestingly, these mucous ciliated cells as well as the goblet cells in asthma lacked expression of Notch target genes, but instead expressed a signature of IL4/IL13-induced genes, which was in contrast to the (few) goblet cells present in airway wall from healthy donors. Therefore, mucous ciliated cells were proposed to represent a transitional cell state in the ciliated lineage—induced by IL-4/IL-13 signalling—leading to a mucous cell phenotype that contributes to mucous cell metaplasia in asthma.[93] As these pathogenic Th2 effector cells were exclusively observed in asthmatic airway walls, and the mucous ciliated cells showed evidence of IL-13-induced gene transcription, it seems likely that Th2 cytokines are responsible for these cell state changes in the asthmatic airway epithelium. Indeed, Th2 effector cells were found to dominate the predicted airway wall cell-cell interactome in asthma.[93] We previously reported that Th2 cytokine production was suppressed by primary bronchial epithelial cells, a regulatory mechanism that seems to be attenuated in asthma.[124] The airway wall cellular interactome analysis also identified cell-cell communication between epithelial cells and other structural or tissue-resident cells, characterized by growth factor signalling. This interaction was present in healthy airway wall, but lost in asthma.[93] Therefore, it will be of great interest to study which cell-cell interactions observed in healthy airway wall maintain the barrier function of airway epithelium, and how these can be restored in the asthmatic condition. Future studies in larger cohorts of patients and controls, as well as in a larger variety of asthma subphenotypes also hold the promise of charting the cellular changes and causal gene regulatory networks underlying a wider variety of asthma endotypes. Moreover, analysis of matched scRNA-Seq data sets from airway wall biopsies, bronchial brushes and nasal brushes will allow design of novel biomarkers for disease activity or treatment response using less invasive methodologies.

7 | THERAPEUTIC STRATEGIES TO IMPROVE BARRIER FUNCTION

Targeting the airway epithelial barrier may constitute a promising novel therapeutic strategy for asthma and related allergic diseases. Intrinsic abnormalities in the airway epithelium of asthmatics culminate in inappropriate immune and inflammatory responses as well as defective repair. Genetically supported targets could double the success rate in clinical development.

A number of pathways involved in maintaining or restoring epithelial barrier function are targetable; these include those (a) enhancing mucosal innate immunity, (b) decreasing epithelial permeability through effective assembly of TJ and AJ proteins and (c) restoring epithelial cell integrity by improving regeneration and regulating mucus production. Modulation of several developmental transcription factors has been shown to improve epithelial differentiation and as a consequence, barrier function. We recently demonstrated that inhibition of β-catenin/CBP signalling inhibits EMT and promotes recovery of epithelial barrier function through restoration of E-cadherin expression.[40,80,125,126] Notch signalling appears to be intimately involved in regulating mucus cell fate and mucus release.[127] Recent studies from our laboratory and others have shown that modulating Notch signalling has a dramatic effect on mucus secretion.[37] In addition, Smad3 inhibitors may reverse airway epithelial abnormalities as observed in asthma, as reviewed previously.[2] Because of the described effects of type-2 cytokines on epithelial barrier function, we anticipate that new biologics may have beneficial effects on airway epithelial barrier function specifically in type-2 driven asthma; however, to the best of our knowledge, there are no studies yet that assessed this.

The majority of patients respond well to a combination of inhaled corticosteroids (ICS) and bronchodilators. Whether or not ICS have direct beneficial effects on epithelial health or barrier function is unclear. Although corticosteroids failed to prevent the TGF-β-induced downregulation of E-cadherin in a bronchial epithelial cell line,[128] findings in primary bronchial epithelial cells indicate that ICS protect against oxidative stress-induced epithelial barrier dysfunction.[129] However, asthma epithelium was found less responsive to ICS.[129] Oxidative stress as well as IL-17 may lead to ICS unresponsiveness by PI3K-dependent post-translational histone deacetylase (HDAC)2 modifications and proteasomal HDAC2 degradation.[130,131] Strategies to restore ICS sensitivity could be beneficial in improving epithelial

barrier function in asthma in combination with ICS, including the use of antioxidants or α-IL-17 antibodies.[132] Endotype-specific therapies that have been recently developed to mitigate symptoms in patients refractory to conventional ICS-based therapy may largely have their impact through effects on immune/inflammatory components though.

8 | CONCLUDING REMARKS

The airway epithelial phenotype induced by the interaction of genotype and environment plays a central role in the pathogenesis of asthma. Accumulating evidence indicates that multiple genetic variants associated with the risk of developing asthma in response to environmental factors regulate proteins of relevance to airway epithelial function, including roles in barrier function, inflammation, mucociliary clearance and homeostasis. In addition, alterations in epigenetic regulation contribute to abnormalities in the biology of the airway epithelium in asthma. Further insight into these regulatory mechanisms, for example by the use of scRNA-seq, holds promise for identifying patients likely to benefit from epithelial-focused therapies and the identification of targets for novel therapies strategies aimed at correcting dysfunctional epithelial barrier.

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CONFLICT OF INTEREST

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