Series editors: Joshua A. Boyce, MD, Fred Finkelman, MD, and William T, Shearer, MD, PhD

Epithelial barrier function: At the front line of asthma immunology and allergic airway inflammation

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Airway epithelial cells form a barrier to the outside world and are at the front line of mucosal immunity. Epithelial apical junctional complexes are multiprotein subunits that promote cell-cell adhesion and barrier integrity. Recent studies in the skin and gastrointestinal tract suggest that disruption of cell-cell junctions is required to initiate epithelial immune responses, but how this applies to mucosal immunity in the lung is not clear. Increasing evidence indicates that defective epithelial barrier function is a feature of airway inflammation in asthmatic patients. One challenge in this area is that barrier function and junctional integrity are difficult to study in the intact lung, but innovative approaches should provide new knowledge in this area in the near future. In this article we review the structure and function of epithelial apical junctional complexes, emphasizing how regulation of the epithelial barrier affects innate and adaptive immunity. We discuss why defective epithelial barrier function might be linked to T_H2 polarization in asthmatic patients and propose a rheostat model of barrier dysfunction that implicates the size of inhaled allergen particles as an important factor influencing adaptive immunity. (J Allergy Clin Immunol 2014;134:509-20.)

Key words: *Airway epithelium, asthma, barrier defect, mucosal immunity, tight junction, adherens junction, innate immunity, allergy*

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Airway epithelial cells are an important part of the innate immune system in the lung. In addition to establishing mucociliary clearance, epithelial cells produce antimicrobial peptides, chemokines, and cytokines that recruit and activate other cell types and promote pathogen clearance.[1] Recent studies have emphasized the importance of epithelium-derived cytokines in promoting T_H2 immune responses, at least in part by conditioning local dendritic cells (DCs).[2,3] Epithelial cells also form a barrier to the outside world comprised of airway surface liquids, mucus, and apical junctional complexes (AJCs) that form between neighboring cells. AJCs consist of the apical ***tight junctions*** (TJs) and underlying ***adherens junctions*** (AJs) that bind together through homotypic and heterotypic interactions (Fig 1). Epithelial TJs and AJs establish cell-cell contact and cell polarity and also regulate the paracellular movement of ions and macromolecules. Recent studies have documented the presence of dysfunctional epithelial AJCs in the airways of asthmatic patients, although the precise mechanisms involved and consequences for airway inflammation are not clear. Interestingly, inhaled allergens, pollution particles, and respiratory tract viruses can disrupt barrier integrity, which might represent a risk factor for allergen sensitization. Certain inflammatory cytokines can also cause barrier dysfunction, potentially creating a positive feedback loop. In addition to allowing better penetration of inhaled allergens and particles, airway barrier dysfunction likely initiates signal transduction cascades, affecting epithelial activation and differentiation. Therefore regulation of airway epithelial barrier function is emerging as an important checkpoint in asthma immunology. Before considering the mechanisms and consequences of barrier dysfunction for allergic airway inflammation, a brief overview of junctional structure is in order.

Abbreviations used

AJ:	Adherens junction
AJC:	Apical junctional complex
ALI:	Air-liquid interface
CAR:	Coxsackie adenovirus receptor
CCSP:	Clara cell secretory product
DC:	Dendritic cell
HDM:	House dust mite
JAM:	Junctional adhesion molecule
MDCK:	Madin-Darby canine kidney
PBEC:	Primary bronchial epithelial cell
RSV:	Respiratory syncytial virus
TAMP:	Tight junction–associated MARVEL protein
TJ:	Tight junction
ZO:	Zonula occludens

AJCs: BASIC STRUCTURE AND FUNCTION

Junctions between neighboring cells were first discovered by using electron microscopy and appear as apposing strands that eliminate the intercellular space.[4] Junctional complexes contain

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Disclosure of potential conflict of interest: S. N. Georas has received research support from the National Institutes of Health (NIH; R01 HL071933, T32 HL066988, and P30 ES001247) and holds a provisional patent for a noninvasive way for monitoring outside-in barrier function. F. Rezaee has received research support from the NIH (K12 HD068373).

Received for publication April 11, 2014; revised May 30, 2014; accepted for publication May 30, 2014.

Available online July 29, 2014.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2014.05.049

Terms in boldface and italics are defined in the glossary on page 510.

the most apical TJs and underlying AJs, which are both linked to perijunctional ***actin*** filaments (Fig 1).[5,6] TJs regulate paracellular transport of ions and certain small molecules, whereas AJs are important for initiation and maintenance of cell-cell adhesion.[7,8] TJs and AJs interact to establish apical versus basolateral membrane domains (ie, cell polarity) and also regulate each other's structure. Both TJs and AJs are involved in numerous signal transduction cascades.[9] A current model proposes that there are 2 pathways for paracellular movement of molecules across TJs. The claudin-containing "pore" controls movement of ions in a charge and size-selective manner, whereas the "leak" pathway allows limited movement of larger macromolecules.[8] The precise molecular basis for size and charge discrimination by different junctional components is currently under active investigation, and several reviews are available on this topic.[8,10,11]

Our current understanding of junctional structure and function comes largely from studies of epithelial monolayers *in vitro*, and a current challenge is to understand how findings in model epithelia extrapolate to the multicellular epithelium in real-world conditions. *In vitro* studies typically grow epithelial monolayers to confluence on semipermeable membranes and compare epithelial barrier function with junctional structure, as determined by using microscopy. Different functional assays can be used to study barrier integrity. ***Transepithelial electrical resistance (TEER)*** is easy to measure and commonly used to assess junctional integrity because intact junctions will be relatively impermeable to ion flux (ie, high TEER). However, low TEER does not always imply higher macromolecular permeability (discussed in Rezaee et al[12]), and consequently, multiple approaches should be used to provide a complete picture of junctional integrity. In later sections of this review, we discuss additional assays that have been used to study outside-in airway barrier function in living organisms.

TJs and AJs are macromolecular complexes that bind together in the intercellular space and also make numerous intracytoplasmic protein-protein interactions. Table I summarizes the major families of junctional complex proteins, including the 3 TJ families. New junctional components and protein interactions are being discovered regularly, and Table I is meant to be illustrative rather than comprehensive.

First, claudins are a large family of tetraspanning transmembrane proteins that are expressed in a tissue- and cell type–selective manner and interact in a homotypic or heterotypic fashion in the extracellular space. Claudins can be either barrier promoting or barrier disrupting (or "leaky"). For example, claudin-1, the founding family member,[13] is necessary and sufficient for junction formation function.[14] Claudin-1–deficient mice die soon after birth and have excessive ***transepidermal water loss (TEWL)***.[15] This study established a key role for keratinocyte TJs in skin barrier function. Interestingly, defective expression of epidermal claudin-1 was observed in the skin of patients with atopic dermatitis,[16] where it can serve as a risk factor for viral infection and allergen sensitization.[17,18] Claudin-2, in contrast, is an example of a leaky claudin associated with increased permeability in the intestine, where it is induced by ***IL-13*** in a signal transducer and activator of transcription 6–dependent manner.[19] Although IL-4 and IL-13 also enhance airway epithelial permeability and barrier dysfunction, they do so without inducing claudin-2 in 16HBE airway epithelial cells.[20] These studies indicate that T_H2 cytokine–induced epithelial barrier dysfunction can occur in the intestine and airway through different mechanisms. Other claudins expressed in the respiratory tract include claudin-1, claudin-3, claudin-4, claudin-7, and claudin-18, the expression and function of which are under active study.[10-12,21,22]

The second group of TJ proteins is the tight junction–associated MARVEL protein (TAMP) family, which has 3 members: occludin, tricellulin, and MarvelD3.[23] In contrast to claudins, TAMP family members are not essential for normal epithelial development and barrier function, although they appear

GLOSSARY

ACTIN: A protein found especially in microfilaments and active in cellular movement and maintenance of cell shape. A belt of actin below the plasma membrane helps maintain the integrity of cellular junctions.

ADHERENS JUNCTION: These junctional structures form below tight junctions and help establish barrier function and epithelial polarity.

***Alternaria alternata*:** An aeroallergen of the Ascomycota phyla. Its spores have characteristic, elongated, beak-like chains. Spores are capable of traveling hundreds of miles and are found in grain-growing regions of temperate climates, with a peak in the late summer and fall. It is one of the most common spores found in dust from North American homes.

EPITHELIAL-MESENCHYMAL TRANSITION (EMT): A biologic process in which polarized epithelial cells assume a more mesenchymal phenotype characterized by migration and invasiveness. An early event in EMT is loss of junctional protein expression, including E-cadherin.

IL-13: A cytokine produced by T_H2 and type 2 innate lymphoid cells capable of inducing the IgE isotype switch. Its receptor is not found on mast cells (as is the case for IL-4), but IL-13 is more widely produced than IL-4. IL-13 contributes to airway mucus hypersecretion and airway hyperreactivity in mouse models.

PROTEASE-CONTAINING ALLERGENS: Cysteine and seine proteases are found in many common allergens, including fungal and insect extracts (eg, dust mite and cockroach). Allergen-associated proteases might promote allergic sensitization by disrupting epithelial junctional structures.

γδ T CELLS: A subset of T cells whose T-cell antigen receptors (TCRs) have γ and δ chains. These cells express a restricted repertoire of TCRs. They are capable of responding to nonpeptide and nonprocessed antigens, such as lipids, and appear to recognize antigens directly (independent of class I or class II MHC).

TIGHT JUNCTION: A multisubunit complex of transmembrane proteins that interact in the intercellular space to promote epithelial apposition. Tight junctions are comprised of different family members (eg, claudins and occludin) and link to the actin cytoskeleton.

TOLL-LIKE RECEPTOR 4 (TLR4): The first TLR identified. TLR4 binds to bacterial endotoxin (an LPS in the cell membrane of gram-negative bacteria) and viral coat proteins. Binding to TLR4 activates signal transduction through the MyD88 adaptor protein.

TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER): Opposition of the epithelium to the passage of a steady electrical current, which measures instantaneous ion flux. High TEER implies low ion flux and a tight epithelial barrier.

TRANSEPIDERMAL WATER LOSS (TEWL): A noninvasive measurement that uses vapor pressure gradient estimation. Humidity and temperature affect its measurement. TEWL is increased in patients with atopic dermatitis, reflecting defective skin barrier properties.

The Editors wish to acknowledge Daniel Searing, MD, for preparing this glossary.

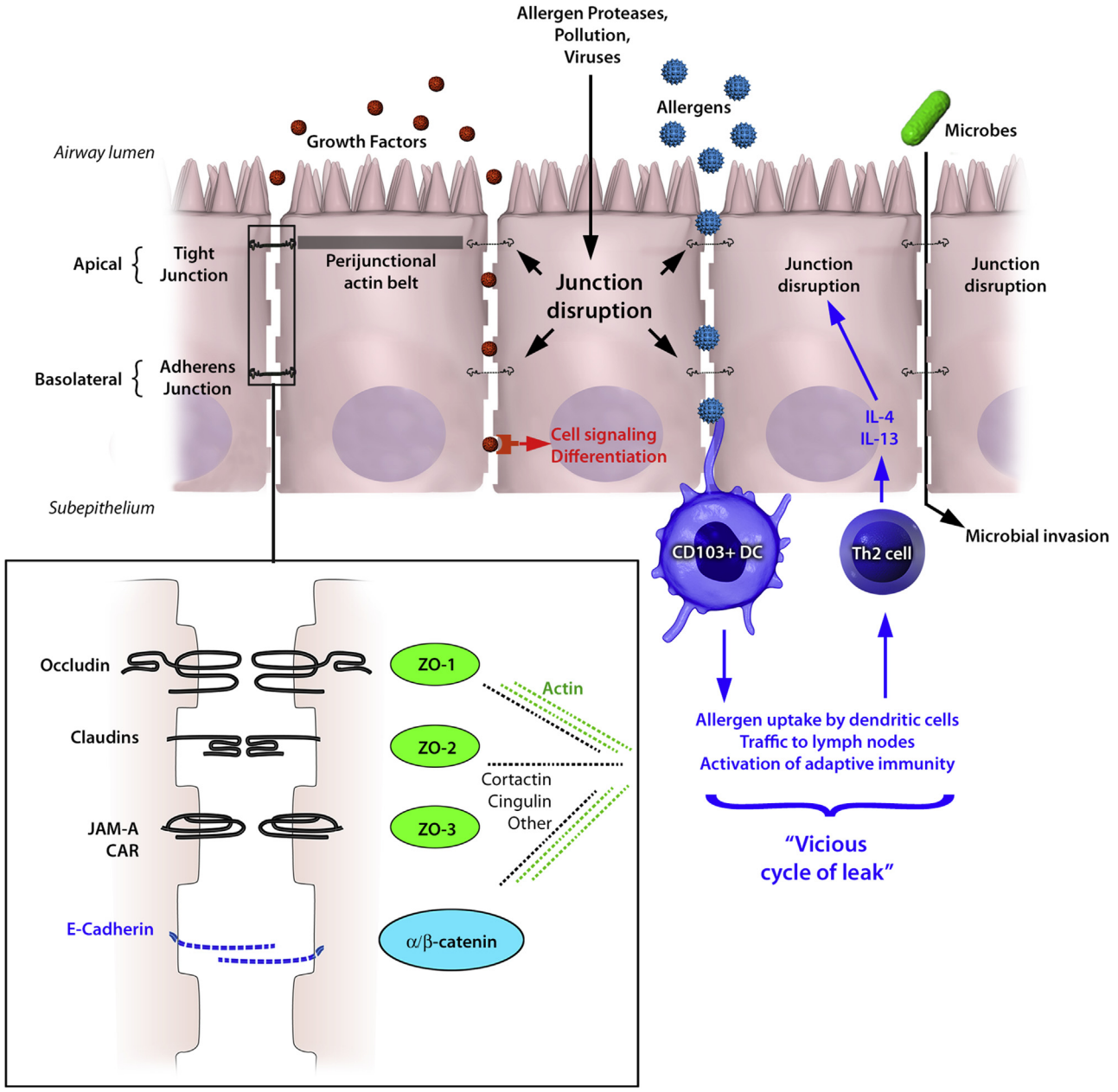

FIG 1. Cartoon diagram of airway epithelial cells indicating junctional structures, including TJs *(black)* and AJs *(blue)*, which are intimately linked with perijunctional actin filaments. The *inset* shows an enlarged schematic of protein-protein interactions in TJs *(black text)* and AJs *(blue text)*, including the ability of ZO proteins to interact with intracytoplasmic domains. The *inset* also indicates that junctional proteins are linked to the actin cytoskeleton *(green dashed line)* through several potential adaptor proteins *(black dashed line)*. Inhaled allergens, air pollutants, and respiratory tract viruses can cause dysfunction of the epithelial junction, resulting in greater outside-in permeability (see the text and Table II). Barrier dysfunction can lead to epithelial cell signaling or differentiation because it will allow apical growth factors constitutively present in epithelial lining fluids *(red dots)* to interact with their basolateral receptors. In the presence of intact epithelial junctions, these ligand/receptor interactions are prevented. Barrier dysfunction will also allow greater sampling of luminal allergens *(blue stars)* by intraepithelial DCs, including $CD103^+$ DCs, which interdigitate in the epithelium. Allergen-induced T_H2 responses can induce a vicious cycle of leak because T_H2 cytokines perpetuate junctional dysfunction (see the text and Table III). Another consequence of leaky epithelial barriers is increased microbial invasion *(green oval)*, which might predispose susceptible asthmatic patients to exacerbations or lung infections.

to play a role in barrier regulation during inflammation. The intracytoplasmic tails of occludin and other TAMP family members are subject to numerous posttranslational modifications, which are thought to affect interactions with scaffold components, signaling molecules, and the actin cytoskeleton.[24] The observation that occludin-null mice are viable and have intact TJs in numerous epithelia indicate that this molecule was not essential for mucosal barrier function.[25,26] However, more

TABLE I. Major TJ, AJ, and plaque proteins

TJs	AJs	Cytosolic plaque proteins
Claudin family	Cadherin family	ZO family
• Claudin-1 through claudin-27	• E-cadherin	• ZO-1, ZO-2, ZO-3
TAMP family	Nectin family	Catenin family
• Occludin		• α-catenin, β-catenin, p120 catenin
• Tricellulin		
• MarvelD3		Others
		• Cingulin, vinculin, afadin, α-actinin, cortactin
Immunoglobulin family		
• JAM-A		
• CAR		

research needs to be done to study the role of these proteins under conditions of epithelial stress or inflammation. Several recent studies indicate that occludin might have other functions separate from maintenance of epithelial integrity. For example, Huber et al[27] found that the migration of neutrophils across an Madin-Darby canine kidney (MDCK) monolayer was regulated by the occludin N-terminus independent of effects on TEER or paracellular permeability to mannitol. MDCK cells are derived from a canine kidney tumor and are a widely used model of TJ structure and function. Recently, Edelblum et al[28] used intravital imaging with novel transgenic mice and found that γδ intraepithelial lymphocytes expressed occludin and appeared to migrate within the epithelium through homotypic interactions with occludin-expressing epithelial cells.[28] This interesting study suggested a potential role for occludin in immune surveillance. Less is known about the expression and function of tricellulin or MARVELD3 in the lung, and more research is needed into the role of epithelial occludin and the TAMP family in airway inflammation and asthma.

The third group of airway TJs is the immunoglobulin-like family, specifically junctional adhesion molecule (JAM) and the coxsackie adenovirus receptor (CAR). These TJ components are receptors for several important viruses.[29,30] Airway epithelial cells express multiple CAR isoforms, which promote the entry of viral particles. A pioneering study demonstrated that adenovirus binding to CAR caused disassembly of junctional complexes and enhanced epithelial permeability.[31] Because adenoviral particles are shed into the basolateral space, junction dysfunction allows inside-out paracellular leak and escape of virus into the airway lumen, thus promoting infectivity of neighboring cells. Consequently, junctional dysfunction might represent a strategy used by viruses to enhance their replication. JAM-A has a tissue-specific role in regulating epithelial integrity because JAM-A deficiency in mice did not alter steady-state or LPS-induced lung permeability[32] but did enhance intestinal permeability.[33] JAM and CAR proteins regulate cutaneous immune responses after epidermal injury by affecting interactions between keratinocytes and skin ***γδ T cells***.[34,35] Taken together with other recent studies,[36,37] it is apparent that some T-cell subsets in the skin sense and respond to subtle perturbations of barrier structure, even in the absence of microbial invasion or overt inflammation. It will be interesting to determine whether similar events occur in the airway epithelium.

AJs are the second component of the AJC and are located below the TJs in the lateral membrane. AJs are especially important for maintenance of cell-cell adhesion and are comprised of the cadherin and nectin families. In epithelial cells E-cadherin binds to intracellular catenins, including p120 and β-catenin, thus linking AJs with the Wnt signaling pathway. By regulating the stability and nuclear import of β-catenin, this evolutionarily conserved pathway regulates gene expression and chromatin structure implicated in epithelial wound repair responses and differentiation.[38] Sustained loss of E-cadherin leads to epithelial differentiation into a mesenchymal phenotype, a process known as ***epithelial-mesenchymal transition (EMT)***. The molecular basis of EMT is complex and occurs during embryogenesis and in epithelial neoplasia.[39] Emerging data suggest that EMT is also a feature of epithelial cells in asthmatic patients, where it likely contributes to airway remodeling.[40,41] Interestingly, house dust mite (HDM) extracts were shown to induce features of EMT in 16HBE epithelial cells *in vitro*, especially in concert with TGF-β1.[42] In a separate study chronic HDM administration in mice resulted in airway remodeling and features of EMT in large airways, including loss of E-cadherin and occludin.[43] Support for the idea that loss of E-cadherin occurs in asthmatic patients comes from the observation that sputum E-cadherin levels correlated with asthma severity.[44] Although reduced junctional protein expression during EMT might result in greater epithelial cell permeability, the net effect on airway leakiness will also be affected by subepithelial fibrosis and other compensatory structural changes that might occur over time. Consequently, more research is needed to understand how EMT and other changes in epithelial differentiation affect airway barrier properties.

Insights into the role of AJs in intestinal epithelial barrier function were obtained by using conditional deletion approaches in mice. For example, Smalley-Freed et al[45] generated mice lacking p120 catenin in the intestinal epithelium, which resulted in disrupted AJs and TJs.[45] Interestingly, partial loss of p120 catenin resulted in spontaneous intestinal inflammation and gastrointestinal bleeding, probably because of translocation of luminal microbes.[45] In lung endothelial cells p120 is degraded by LPS and also negatively regulates ***Toll-like receptor 4 (TLR4)*** signaling.[46] This important study demonstrated that junctional structures can cross-talk with innate immune signal transduction.

TJs and AJs bind to numerous cytoplasmic proteins and link to the actin cytoskeleton, forming the "cytosolic plaque." Key among the plaque proteins is the zonula occludens (ZO) family, which links the intracellular domains of TJs and AJs with actin-binding proteins (eg, cortactin, α-catenin, vinculin, and α-actinin) and other cytoskeletal components. ZO proteins, which include ZO-1, ZO-2, and ZO-3, are expressed in a tissue-specific manner and contain numerous domains capable of protein-protein interactions with signaling molecules (reviewed in Rezaee and Georas[12] and Gonzalez-Mariscal et al[47]).

MODELS OF INDUCIBLE BARRIER DYSFUNCTION

Increased epithelial permeability is a hallmark of mucosal inflammation and can occur through multiple mechanisms. Any insult that results in epithelial cell death or detachment from the basement membrane will lead to increased permeability. More subtle exposures likely also increase the leakiness of the epithelial

TABLE II. Dangerous allergens: models of airway epithelial barrier disruption

Allergen	Cells	TEER	Permeability (tracer)	AJC expression	Notes	References
Dust mite extracts and allergens						
Dermatophagoides pteronyssinus growth medium Affinity purified Der p 1 (300-3000 μg/mL)	Bovine bronchial sheets	ND	Increased (BSA)	ND	1.5 mmol/L DTT	59
Aged fecal pellets (~30 pellets/cm^2) Purified Der p 1	MDCK 16HBElo-	ND	Increased (mannitol)	Decreased occludin and ZO-1	Occludin cleavage Blocked by E-64	60, 69
HDM extract (50 μg/mL)	16HBElo-	20% decrease in resistance measured by ECIS	ND	No effect on E-cadherin or β-catenin	Dependent on EGFR and ATP Barrier recovery by 1 hour	61-63
Der p 1	PNEC	ND	ND	Decreased JAM-A Claudin-1		68
Pollen extracts						
Giant ragweed *(Ambrosia trifida)*, white birch *(Betula pendula)*, Kentucky bluegrass *(Poa pratensis)*; protein concentration: 6.25-100 mg/mL	MDCK Calu-3	ND	ND	Decreased occludin, claudin-1, ZO-1	Blocked by protease inhibitors	64
Olive *(Olea europaea)*, orchard grass *(Dactylis glomerata)*, Italian cypress *(Cupressus sempervirens)*, and Scots pine *(Pinus sylvestris)*; protein concentration: ~0.1-5 mg/mL	Calu-3 at ALI	ND	Increased (RITC dextran)	Decreased E-cadherin, claudin-1, occludin in allergen-specific manner No effect on ZO-1	Especially pine Blocked by AEBSF	65
Timothy grass (*Phleum pratense* [equivalent to 1 mg of pollen grains])	PBEC	No effect	ND	No effect on ZO-1	Pollen-induced chemokine production depending on donor severity	67
Japanese hop (Hop J [100 μg/mL])	Calu-3	ND	Increased (FITC dextran)	Decreased occludin	Occludin degradation blocked by NAC	66

AEBSF, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride; *DTT*, dithiothreitol; *EGFR*, epidermal growth factor receptor; *FITC*, fluorescein isothiocyanate; *NAC*, N-acetyl cysteine; *ND*, not determined; *PNEC*, primary nasal epithelial cells.

barrier by affecting junctional complex structure and function without causing cell death. In the airway enhanced outside-in permeability will result in greater penetration of inhaled allergens and particles into the subepithelial space, facilitating antigen sampling and innate and adaptive immune responses, and might activate epithelial signal transduction (Fig 1, see below). Epithelial junctional complexes can be disrupted directly by inhaled substances that penetrate the mucus layer or indirectly by cytokines and other inflammatory mediators. Examples of environmental exposures implicated in airway epithelial barrier dysfunction include respiratory tract viruses (eg, coxsackievirus, rhinovirus, and respiratory syncytial virus [RSV][48-51]), air pollution components (eg, ozone and ambient particulate matter[52-54]), cigarette smoke,[55,56] and allergens (considered below).

Viruses appear to cause junction dysfunction through different mechanisms. For example, coxsackievirus causes occludin macropinocytosis driven by Rab GTPases,[48] whereas rhinovirus reduces epithelial occludin gene expression in an NADPH-oxidase–dependent manner.[49,50] We recently showed that RSV disrupts AJC structure and function without markedly affecting the expression of individual junctional components.[51] Rather, RSV infection leads to disassembly of junctional complexes from the cell surface in association with actin remodeling and phosphorylation of the actin-binding protein cortactin. The barrier disruptive effects of RSV were inhibited by antagonists of protein kinase D, similar to our previous work with the viral mimetic polyinosinic-polycytidylic acid.[57] It will be interesting in future studies to determine whether other viruses converge on protein kinase D to cause junctional disruption, which would suggest that antagonists of this versatile signaling molecule might have therapeutic potential. We recently reviewed the mechanisms and consequences of barrier disruption induced by respiratory tract viruses and air pollution components.[12] In the following section we cover the effects of allergens and cytokines on epithelial junctional structure and function.

Dangerous allergens: Protease-dependent epithelial barrier dysfunction

A central tenet of the "protease hypothesis" is that ***protease-containing allergens*** have the potential to directly cleave epithelial TJs and disrupt barrier structures.[58] One of the first examples of this phenomenon was reported in 1995 using extracts of HDMs (Table II).[59-69] A follow-up study demonstrated that aged dust mite fecal pellets or purified Der p 1 increased the permeability in MDCK monolayers and also decreased cell-surface occludin expression in 16HBElo- cells.[60] Because occludin fragments were detected in Der p 1–treated cells by using Western blotting and Der p 1 was able to cleave peptide fragments of occludin and claudin *in vitro*, the authors concluded that Der p 1 directly targeted cell-surface TJs. More recently, Heijink et al[61] used a commercially available HDM extract and found no effects on surface E-cadherin or β-catenin at concentrations up to 50 μg/mL, although a transient 20% reduction in TEER was detectable by using cell impedance sensing.[62,63] Subsequently, Post et al[70] found that the protease activity of different HDM extracts

correlated poorly with their ability to induce barrier dysfunction *in vitro* or lung inflammation after inhalation in mice. Interestingly, however, the ability to cause barrier disruption in model epithelia *in vitro* predicted inflammatory potential *in vivo*, showing that these 2 properties are closely aligned.[70] Thus even independent of protease activity *per se*, these authors concluded that barrier disruption is a key feature of proinflammatory allergen extracts.

In addition to dust mite extracts, several studies have demonstrated that extracts of pollens can disrupt junctional structure or function by using model epithelia *in vitro* (Table II). For example, diffusates of giant ragweed *(Ambrosia trifida)*, white birch *(Betula pendula)*, and Kentucky bluegrass *(Poa pratensis)* decreased expression of different TJ components in MDCK and Calu-3 cells, although junction function was not specifically investigated in that report.[64] Calu-3 cells are airway epithelial cells derived from a patient with lung adenocarcinoma. In a detailed study Vinhas et al[65] applied diffusates of olive *(Olea europaea)*, orchard grass *(Dactylis glomerata)*, Italian cypress *(Cupressus sempervirens)*, and Scots pine *(Pinus sylvestris)* to Calu-3 cells grown at air-liquid interface (ALI) and studied the effects on epithelial permeability and junctional structure. Extracts of each allergen induced variable degrees of junctional disruption, which could be blocked with protease inhibitors. Interestingly, protease activity, as assessed by using model substrates *in vitro*, did not correlate with barrier disruption (eg, pine extracts with modest specific activity potently enhanced permeability), and there were allergen-specific effects on both junction function and structure. Combinations of allergens were not additive or synergistic, but instead protective effects emerged (eg, cypress extracts tending to neutralize pine extracts).[65] Consequently, this study demonstrates the difficulties in predicting the bioactivity of allergens based on biochemical properties and underscores the need for more empiric research.

Lee et al[66] recently studied the effect of extracts of Japanese hop on permeability of Calu-3 cells. These extracts increased epithelial permeability and occludin degradation in a reactive oxygen species–dependent manner that was blocked by the antioxidant N-acetyl cysteine. In contrast to these studies, Blume et al[67] found that an extract of Timothy grass extract *(Phleum pratense)* had no effect on TEER or ZO-1 expression of human primary bronchial epithelial cells (PBECs) derived from asthmatic donors, although pollen exposure stimulated chemokine secretion. Another report from the Davies laboratory demonstrated that exposure of 16HBE cells to extracts of the allergenic fungus ***Alternaria alternata*** reduced TEER in a dose-dependent manner (eg, approximately 50% reduction at 24 hours by using 100 μg/mL), although PBECs from healthy donors appeared to be resistant to these effects.[71] Interestingly, PBECs derived from patients with severe asthma were more susceptible to *Alternaria* species–induced reductions in TEER, although this effect was transient and only apparent with high extract concentrations (>100 μg/mL).

Taken together, these studies highlight some of the challenges of working with extracts of real-world allergens in models of inducible barrier dysfunction.[59-68,70,71] First, allergen extracts are extremely heterogeneous and vary in protease activity, LPS content, and other danger signals that can activate target cells. Consequently, it is difficult to compare results from different groups using different allergen preparations. Standardization of allergen extracts has been important for clinical allergy diagnostics and immunotherapy, but we need more sustained efforts to standardize allergen extracts used in basic science research.

Another challenge is comparing data using cell lines with primary cultures of differentiated epithelia. Epithelial cell lines do not recapitulate the pseudostratified airway epithelium, with multiple cell types and overlying mucus and surface liquids.

It is also challenging to estimate the concentration of allergen extracts to use in tissue culture experiments. Inhaled allergens must penetrate the mucus layer and escape neutralization by antiproteases or antioxidants in airway surface liquids to directly contact airway epithelial cell surfaces after deposition in the airway. There might be "hotspots" of allergen deposition within the airway, and some subjects with defective antiprotease or antioxidant defenses might be particularly susceptible to allergen-induced barrier dysfunction.[72] However, it seems likely that the local concentration at the apical cell surface is likely extremely low and less than concentrations used in many *in vitro* studies.

Epithelial barrier dysfunction induced by cytokines

It is currently thought that intestinal permeability in patients with inflammatory bowel diseases is caused by cytokine-induced barrier dysfunction in the absence of apoptosis,[73,74] and it is becoming clear that similar pathways likely operate in the airway. Inflammatory cytokines, including IL-4, IL-13, IFN-γ, and TNF-α, have been shown to disrupt airway epithelial barrier function through diverse mechanisms (Table III).[20,75-79] In PBECs cultured at ALI, IFN-γ and TNF-α synergistically disrupt barrier function in association with reduced ZO-1 and JAM expression.[75] Using chemical inhibitors and Western blot assays, these investigators implicated a role for atypical protein kinase C family members. More recently, Hardyman et al[76] reported that TNF-α alone induced PBEC barrier dysfunction without affecting AJC protein expression *per se*. Rather, these investigators found that TNF-α causes marked AJC disassembly in an Src-dependent manner.

We found that both IL-4 and IL-13 induced barrier disruption in 16HBElo- cells by inhibiting surface expression of ZO-1, occludin, E-cadherin, and β-catenin.[20] In contrast to the T_H2-dependent induction of leaky claudin-2 observed in intestinal epithelia, neither IL-4 nor IL-13 induced claudin-2 expression in 16HBElo- cells. The innate type 2 cytokines thymic stromal lymphopoietin, IL-25, and IL-33 also had no effect on airway barrier integrity in our model.

Interestingly, Soyka et al[77] recently reported that IL-4 and IFN-γ disrupted junctional structure and function in primary nasal epithelial cells from patients with chronic rhinosinusitis, whereas IL-17A had no effect. By using immunofluorescence microscopy, cytokine treatment disrupted the integration of ZO-1 and occludin into membrane junctions, without affecting their gene expression.

Parker et al[78] grew bronchial epithelial cells from asthmatic and nonasthmatic children *in vitro* at ALI and studied the effects of IL-9 and IL-13 (alone or in combination) on epithelial differentiation and TEER. The presence of IL-13 in particular affected the cellular composition of epithelial monolayers, with fewer ciliated and more goblet cells detected at the end of culture, which translated into slight reductions in TEER. This study highlights the importance of considering epithelial plasticity and differentiation state when analyzing barrier structure and function.[80,81]

TABLE III. Cytokines implicated in airway epithelial barrier disruption

Stimulus	Cells	TEER	Permeability (tracer)	AJC expression	Notes	Reference
IFN-γ, IL-1β, TNF-α (10-100 ng/mL)	PBEC (normal and CF)	Decreased	Increased (2 kDa of dextran)	Decreased ZO-1 and JAM	Synergy between IFN-γ + TNF-α CF more sensitive Atypical PKC-i	75
IL-4, IFN-γ, TNF-α	Calu-3	Decreased	ND	Decreased ZO-1, occludin	Involvement of EGFR and MAPK	79
IL-4, IL-13, TSLP, IL-25, IL-33 (0.5-50 ng/mL)	16HBElo-	Decreased	Increased (3 kDa of dextran)	Decreased ZO-1, occludin, E-cadherin, β-catenin	Jak dependent No induction of claudin-2 No effect of innate type 2 cytokines	20
IL-4, IFN-γ (10 ng/mL)	PNEC	Decreased	Increased	Decreased ZO-1, occludin	No effect of IL-17 More pronounced in patients with CRSwNP	77
IL-13 (20 ng/mL)	PBEC	Decreased	ND	ND	Long-term exposure	78
TNF-α (10 ng/mL)	PBEC	Decreased	Increased (4 kDa of dextran)	Decreased occludin, claudin-3, claudin-4, and claudin-8 by IF (not WB)	Src kinase	76

CF, Cystic fibrosis; *CRSwNP*, chronic rhinosinusitis with nasal polyposis; *EGFR*, epidermal growth factor receptor; *IF*, immunofluorescence; *MAPK*, mitogen-associated protein kinases; *ND*, not determined; *PNEC*, primary nasal epithelial cells; *PKC*, protein kinase C; *TSLP*, thymic stromal lymphopoietin; *WB*, Western blot.

In addition to cytokines, basophil/mast cell–derived mediators have been shown to disrupt AJC structure and function. Histamine induces transient disruption of barrier function in PBECs and loss of E-cadherin expression, leading to greater infectivity of adenovirus.[82] JAM-A is targeted by mast cell–derived tryptase in intestinal epithelial cells,[83] but whether this same pathway operates in the airway requires further study.

Taken together, these studies demonstrate that cytokines and mediators associated with allergic airway inflammation induce barrier disruption, often by interfering with junctional complex assembly at the apical membrane rather than by interfering with junctional protein expression *per se*. AJC disassembly in the intestine occurs through endocytosis of surface molecules involving complex interactions with cytoskeletal machinery,[6,84] and future studies will be needed to determine the precise mechanisms involved in airway barrier dysfunction during allergic inflammation. The induction of barrier dysfunction by T_H2 cytokines raises the possibility of a vicious cycle in the airway (Fig 1, right). After mucosal allergen sensitization leading to T_H2 polarization, if local secretion of IL-4 and IL-13 causes airway epithelial leakiness, then greater penetration of inhaled allergens and noxious particles could perpetuate the allergic immune response. It will be interesting to determine whether this "cycle of leak" operates in asthmatic patients and whether apical junctions disrupted by allergens or inflammatory mediators can be restored.

EVIDENCE FOR EPITHELIAL BARRIER DYSFUNCTION IN ASTHMATIC PATIENTS

The presence of a skin barrier defect in patients with atopic dermatitis is well established and is now known to involve defects not only in the stratum corneum (eg, filaggrin[85-89]) but also in keratinocyte TJs.[17,18,90] The structure and function of the epidermal barrier can be studied in skin biopsy specimens or explants from affected subjects and monitored noninvasively by measuring TEWL. TEWL is a measure of inside-out barrier function, and increased TEWL reflects defective function of claudins and other TJ components. There is no surrogate of TEWL to monitor inside-out barrier function of the airway in asthmatic patients. Measuring exhaled breath condensate volume is one potential approach, but this does not appear to be enhanced in asthmatic patients compared with control subjects after normalizing for minute ventilation. Clara cell secretory product (CCSP) is normally secreted apically into the airway lumen by airway epithelial cells, and increased serum or urine CCSP concentrations have been used to infer the presence of enhanced outside-in epithelial permeability.[91] For example, serum or urinary CCSP levels increase after exposure to ozone,[92] cold/dry air challenge,[93] and RSV infection in children.[94] In a population study an increased urinary CCSP level was used as a biomarker of permeability caused by ultrafine particulate air pollution.[95] However, CCSP is produced by other glandular epithelia and potentially affected by corticosteroids and lung inflammation[96-98] and remains an indirect measure of barrier integrity. Until other noninvasive measurements of airway permeability are developed, direct analysis of tissue biopsy specimens, explants, or epithelial cell monolayers will be needed to investigate the presence of barrier defects in asthmatic patients.

Several investigators have recently studied airway biopsy specimens, epithelial cells propagated *in vitro* at ALI, or both, and uncovered evidence for defects in AJC structure and function in the asthmatic airway epithelium. Table IV[63,67,78,99-103] summarizes research to date, noting that some studies had small sample sizes and were exploratory in nature. Three studies documented reduced expression of TJ components in the epithelium of asthmatic patients by using immunohistochemistry.[99,101,103] Reduced TEER in epithelial cells obtained from asthmatic donors propagated *in vitro* was observed in some studies[63,67,103] but not others.[78,101,102] The most comprehensive analysis to date was conducted by Xiao et al.[103] These investigators obtained bronchial biopsy specimens or epithelial brushings from healthy control subjects and patients with varying degrees of asthma severity and studied both apical junctional structure by using immunofluorescence microscopy and barrier function with both TEER and permeability assays. Junctional structure was perturbed in both bronchial biopsy specimens and epithelial cells propagated *in vitro* at ALI, as determined by reduced or patchy expression of cell-surface ZO-1 (and trends for reduced occludin). The expression of mRNA for ZO-1 or occludin was not different between groups, arguing for posttranscriptional alterations in AJC formation. Importantly, barrier function was also reduced, with lower

TABLE IV. Evidence for airway barrier dysfunction in asthmatic patients

Authors	Tissue source and subject characteristics	Key findings
Xiao et al[103]	Biopsy specimens and brushings from patients with mild, moderate, and severe asthma (see text)	• Reduced junctional protein expression in asthmatic patients, especially ZO-1 • Reduced barrier function, especially in patients with moderate and severe asthma
de Boer et al[99]	Bronchial biopsy specimens (14 specimens from nonasthmatic subjects, 22 from patients with mild asthma, and 25 from atopic nonasthmatic subjects)	• Statistically significant reduction in expression of α-catenin, E-cadherin, and ZO-1 in superficial epithelial cells in asthmatic patients vs nonasthmatic subjects • Slight reduction in α-catenin in biopsy specimens from atopic nonasthmatic subjects
Parker et al[78,100]	Bronchial brushings (9 specimens from nonasthmatic subjects, 7 from asthmatic patients [children])	• No significant difference in TEER between asthmatic patients vs nonasthmatic subjects at ALI
Post et al[63]	Bronchial brushings (6 specimens from nonasthmatic subjects, 5 from patients with mild asthma)	• Reduction in baseline TEER in asthmatic patients vs nonasthmatic subjects, as determined by using ECIS
Blume et al[67]	Bronchial brushings (21 specimens from nonasthmatic subjects, 15 from patients with severe asthma)	• Reduced TEER at baseline in patients with severe asthma vs nonasthmatic subjects at ALI • No evidence of Timothy grass extract–induced barrier dysfunction
Hackett et al[101,102]	Cadaveric lungs (6 specimens from nonasthmatic subjects, 6 from asthmatic patients) Bronchial brushings (6 specimens from nonasthmatic subjects and 5 from patients with mild asthma)	• Reduced E-cadherin and β-catenin in cadaveric lung sections but no baseline difference in TEER in cells at ALI • No baseline difference but lower TEER after growth factor removal in brushings from asthmatic patients vs nonasthmatic subjects at ALI

ALI, Epithelial cells from brushings or lung digests propagated *in vitro* in defined culture medium for several weeks; *ECIS*, electrical cell impendence sensing.

TEER and higher permeability detected in cells propagated *in vitro* from asthmatic patients, especially those with moderate and severe disease. Treatment with epidermal growth factor restored barrier function toward normal, indicating that defective airway barrier function in asthmatic patients is potentially reversible.[103]

One remarkable aspect of this study is that defects in barrier structure and function were observed in airway epithelial monolayers propagated *in vitro* for several weeks at ALI (as also noted by Post et al[63]). Similar results were observed in nasal epithelium obtained from patients with chronic rhinosinusitis and nasal polyposis, which demonstrated reduced TEER and higher permeability than tissues or cells from control subjects.[77] This indicates that reduced barrier function is a stable property of these cells or at least can be perpetuated *in vitro* under defined culture conditions. The molecular basis for this "hard wiring" of leaky epithelial cells remains to be determined, but one possibility is that it is encoded in epithelial stem cells.[104] Taken together with the now well-established role for barrier defects in patients with atopic dermatitis, these exciting studies indicate that dysregulation of epithelial junctional complex structure and function might be a unifying feature of allergic diseases.

CONSEQUENCES OF BARRIER DYSFUNCTION FOR ALLERGIC AIRWAY INFLAMMATION AND ASTHMA IMMUNOLOGY

Although there is growing evidence for disruption of epithelial barrier structures in patients with airway diseases, the pathophysiologic significance of these observations is currently unknown. Three general downstream effects of relevance to asthma immunology and allergic airway inflammation can be envisioned, which are not mutually exclusive (Fig 1).

First, AJC dysfunction could promote outside-in permeability of inhaled particles and allergens into the subepithelial space. By subtly altering epithelial structure and facilitating sampling of luminal contents by intraepithelial DCs, AJC dysfunction likely promotes innate and adaptive immune responses. Growing evidence suggests the junction disruption might even be required to initiate mucosal immunity in naive hosts. Second, a leaky barrier could be a risk factor for infections by facilitating the penetration of microbes or viruses beyond the epithelial surface. Third, by affecting the polarized distribution of cell-surface receptors and exposing the basolateral membrane to apical mediators (and *vice versa*), epithelial AJC disruption can cause intracellular signaling, secretory activity, and affect differentiation. We consider these possibilities below.

An additional potential consequence of barrier dysfunction might actually be beneficial. If junctional disruption allows better penetration of inhaled medications into the airway, then local concentrations reaching target cells (eg, β-agonists and subepithelial smooth muscle cells) might actually be enhanced.

Enhanced antigen sampling

It seems logical to speculate that defective barriers will allow greater penetration of inhaled allergens or particles into the subepithelial space, where they will encounter intraepithelial DCs and other immune targets. There is a rich network of intraepithelial DCs, including the $CD103^+$ subset that express αEβ7 and bind directly to E-cadherin on epithelial cells.[105-107] Dendrites from these DCs interdigitate between epithelial cells and express integrins and other TJ molecules (eg, claudin-1) that maintain barrier integrity.[105] It is currently not known whether DC dendrites extend beyond TJs in the steady state. This is an active area of investigation but technically challenging because imaging techniques used to visualize epithelial cell–DC interactions in the airway can perturb tissue architecture, activate cells, or both. In the skin confocal microscopy demonstrated that antigen uptake by intradermal Langerhans cells involved subtle protrusion of dendrites with reorganization of keratinocyte TJs.[108] A recent study used minimally invasive intravital 2-photon microscopy with exteriorized bowel loops and concluded that at steady state, intestinal goblet cells (and not

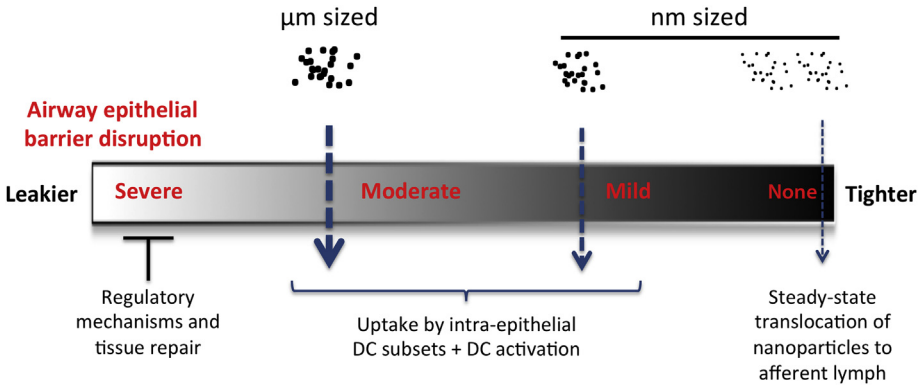

FIG 2. Rheostat model of airway epithelial barrier function. At steady state *(far right)*, airway epithelial cells normally exclude particles greater than approximately 30 to 50 nmol/L in diameter. In the presence of dysfunctional barriers, progressively larger particles will traverse apical junctions *(right to left)*. Barrier dysfunction likely facilitates sampling of luminal contents by DC dendrites (not shown), thus promoting the outside-in translocation of inhaled allergen particles that deposit on the cell surface. In addition to surface properties, the size of inhaled allergen particles might influence their uptake by intraepithelial DC subsets, which in turn could influence the quality and intensity of subsequent adaptive immune responses. The possibility that sustained and persistent barrier disruption leads to reparative responses or increased regulatory tone is indicated on the *far left*.

intraepithelial CD103$^+$ DCs) constitutively sampled luminal contents.[109] These studies raise the possibility that disruption of epithelial junctional complexes might be required for effective sampling of inhaled antigens by intraepithelial DCs. A corollary of this hypothesis is that substances that disrupt barrier function might prove to be effective mucosal adjuvants.

Direct evidence supporting a role for airway leakiness in mucosal allergen sensitization is currently lacking. Future models of outside-in allergen translocation will need to consider that the ability of inhaled particles to penetrate the epithelium depends on their shape, size, and surface chemistry. These properties are being exploited therapeutically to enhance the efficacy of mucosal vaccines,[110] but how the physical properties of inhaled allergens affects their immunogenicity is largely an unexplored area. What is clear is that in addition to affecting deposition within the respiratory tract,[111] particle size affects translocation across lung epithelial barriers. In a rat model inhaled particles of less than 34 nm were rapidly detected in lung draining lymph nodes, likely reflecting direct outside-in translocation into afferent lymph channels.[112] To determine the site of translocation, Blank et al[113] used confocal microcopy and flow cytometry and found that the vast majority of inhaled particles were taken up by alveolar macrophages regardless of size, but a few 20- to 50-nm nanoparticles were detected in intratracheal DCs 24 hours after inhalation. Similarly, Zoltan Veres et al[114] used 2-photon imaging of thick-cut lung slices and found that 1-µm particles were entirely taken up by alveolar macrophages and nonintraepithelial DCs. Taken together, it seems that at steady state, uptake of airway luminal antigens by intraepithelial DCs is uncommon and unlikely to occur with particles greater than approximately 50 nm in diameter. However, future research will be needed to determine how surface chemistry (eg, allergen proteases) or host susceptibility (eg, virus-induced TJ defects) influence allergen sampling and immunogenicity in the respiratory epithelium. Exposure models using low-level aerosol exposure should be particularly insightful because they will mimic physiologically relevant conditions.

Microbial infection

Defective junctional complexes might also facilitate outside-in translocation of luminal microbes or viruses across the airway epithelium. As opposed to inert allergens, bacteria actively penetrate epithelia and secrete toxins that by themselves are barrier disruptive.[115,116] Consequently, even micron-sized bacteria can translocate across epithelial cells, but AJCs still provide a first line of defense. The role of epithelial barriers in maintaining immune homeostasis is a topic of great interest because defects in this regard are linked to microbial dissemination and inflammation, especially in the intestine.[117] However, the term "barrier defects" in this context usually refers to diminished function of epithelial cells or intraepithelial lymphocytes, and few studies have investigated the immunologic consequences of junctional dysfunction *per se*. One exception is the conditional deletion of p120 catenin discussed above, which led to spontaneous intestinal inflammation.[45] Because the microbial burden is less in the lung, deletion of p120 (and other TJ and AJ components) in the airway epithelium should be better tolerated but might affect the lung microbiome or represent a risk factor for mucosal allergen sensitization. Another exception is the recent observation that MyD88-adapter-like (Mal) signaling is required for the expression of occludin, ZO-1, and claudin-3 in intestinal epithelial cells. This helps explain the observation that Mal deficiency predisposes to *Salmonella typhimurium* infection and links TLR signaling directly with AJC integrity.[118] In addition to microbial invasion, AJC dysfunction might be a risk factor for viral respiratory tract infection because basolateral receptors will be more accessible.[29-31] Because viruses can also cause junctional dysfunction,[48-51] this indicates the potential for a positive feedback loop resulting in susceptibility to subsequent viral infections or bacterial superinfection. In fact, Sajjan et al[49] formally demonstrated that rhinovirus infection markedly increased the translocation of bacteria across epithelial monolayers. In future studies, it will be important to determine whether asthmatic patients with leaky airways are particularly prone to respiratory tract infections and pathogen-induced exacerbations.

Epithelial signaling

The idea that TJ disruption can have intrinsic signaling properties is best supported in the case of epithelial growth factors, which are constitutively expressed in apical airway-surface liquids but separated from their basolateral receptors by intact junctions (Fig 1). When epithelial integrity is compromised, ligand/receptor binding can rapidly initiate a wound repair response.[119] One intriguing idea is that junctional disruption could be a T_H2-promoting signal in the airway. A study by Heijink et al[120] supports this possibility because these authors showed that small interfering RNA knockdown of E-cadherin led to the production of the T_H2-promoting cytokines thymus and activation-regulated chemokine and thymic stromal lymphopoietin by airway epithelial cells in an epidermal growth factor receptor–dependent manner. This possibility would help explain the association of epithelial barrier dysfunction with T_H2-driven allergic diseases and is consistent with the hypothesis that T_H2 immunity might have evolved to restore mucosal integrity after parasitic infections.[121] Because T_H2 responses can promote wound healing and fibrosis, persistent junction dysfunction in the airway might also be a risk factor for airway remodeling.

CONCLUDING REMARKS

We propose that epithelial barrier dysfunction is not "all or none" but rather a graded phenomenon with consequences for allergen uptake and processing that might affect subsequent adaptive immune responses (Fig 2). Inducible barrier dysfunction caused by environmental exposures can vary in severity and will affect the penetration and fate of inhaled particles, depending on their size and other physical characteristics. Inhaled allergens themselves might be capable of promoting transient barrier disruption, but sustained dysfunction is likely more common after inhalation of toxic air pollutants and viral respiratory tract infections. Inducible barrier dysfunction is a strategy used by viruses to promote their replication but likely represents a risk factor for allergen sensitization. Future studies of the mechanisms and consequences of airway epithelial barrier dysfunction in asthmatic patients should enhance our understanding of asthma heterogeneity.

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