Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma

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Background: Epithelial barrier dysfunction and increased permeability may contribute to antigen sensitization and disease progression in asthma. Claudin-18.1 is the only known lung-specific tight junction protein, but its contribution to airway barrier function or asthma is unclear.

Objectives: We sought to test the hypotheses that claudin-18 is a determinant of airway epithelial barrier function that is downregulated by IL-13 and that claudin-18 deficiency results in increased aeroantigen sensitization and airway hyperresponsiveness.

Methods: Claudin-18.1 mRNA levels were measured in airway epithelial brushings from healthy controls and patients with asthma. In patients with asthma, claudin-18 levels were compared with a three-gene-mean marker of T_H2 inflammation. Airway epithelial permeability changes due to claudin-18 deficiency were measured in 16HBE cells and claudin-18 null mice. The effect of IL-13 on claudin expression was determined in primary human airway epithelial cells and in mice. Airway hyperresponsiveness and serum IgE levels were compared in claudin-18 null and wild-type mice following aspergillus sensitization.

Results: Epithelial brushings from patients with asthma (n = 67) had significantly lower claudin-18 mRNA levels than did those from healthy controls (n = 42). Claudin-18 levels were lowest among T_H2-high patients with asthma. Loss of claudin-18 was sufficient to impair epithelial barrier function in 16HBE cells and in mouse airways. IL-13 decreased claudin-18 expression in primary human cells and in mice. Claudin-18 null mice had significantly higher serum IgE levels and increased airway responsiveness following intranasal aspergillus sensitization.

Conclusions: These data support the hypothesis that claudin-18 is an essential contributor to the airway epithelial barrier to aeroantigens. Furthermore, T_H2 inflammation suppresses claudin-18 expression, potentially promoting sensitization and airway hyperresponsiveness. (J Allergy Clin Immunol 2017;139:72-81.)

Key words: *Asthma, epithelium, epithelial barrier function, tight junction, antigen sensitization, airway hyperresponsiveness*

Genetic and environmental factors influence asthma development, progression, and severity. Moreover, recent work has begun to parse the clinical syndrome of asthma into distinct endotypes that may vary in pathogenesis, progression, and response to therapy.[1] Amid this complexity, airway epithelial barrier impairment is a common feature of asthma that has been postulated to contribute to immune and parenchymal cell activation, antigen sensitization, and airway hyperresponsiveness.[2,3] In severe asthma, epithelial cell loss has been reported, but previous work has also demonstrated that more subtle changes in epithelial cell junctions may account for impaired barrier function in mild-moderate asthma.[4] For example, infections, toxins, and environmental proteins, such as Dpr1, modify cell junctions to impair barrier function.[5-7] Moreover, recent work also supports the hypothesis that differences in tight junction protein expression or trafficking account for differences in barrier function in the epithelium of those with asthma. For example, biopsy samples from subjects with asthma exhibit tight junction disruption with reduced expression of occludin and zona occludens-1—key structural components of tight junctions.[8] In parallel, cultured epithelial monolayers derived from airways of subjects with asthma had increased macromolecule permeability compared with monolayers derived from healthy subjects.[8] Because the airway epithelium constitutes a barrier to aeroantigens, these findings raise the possibility that increased epithelial permeability could contribute to allergic inflammation by permitting greater exposure of the subepithelial compartment to inhaled allergens. Alternatively, loss of epithelial compartmentalization and polarity could impact cell signaling through dysregulation of normally segregated receptors and ligands. The mechanisms of airway epithelial tight junction dysfunction in asthma, and the contribution of this abnormality to allergic sensitization and airway hyperresponsiveness, remain incompletely understood.

It is noteworthy that previous studies have established that T_H2-mediated changes in tight junction claudin expression contribute to impaired epithelial barrier function in the gut and

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Abbreviations used
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
OVA: Ovalbumin
P_{app}: Apparent permeability
PAS: Periodic acid–Schiff
UCSF: University of California, San Francisco

other organs.[9-11] It is less clear whether this mechanism contributes to airway epithelial barrier abnormalities in asthma; however, a previous report found that IL-4 and IL-13 impaired epithelial barrier function in 16HBE cells.[12] T_H2-high asthma is characterized by higher expression levels of IL-13–dependent genes, greater airway hyperresponsiveness, and higher IgE levels compared with T_H2-low asthma.[13,14] It remains unclear whether tight junction composition and epithelial barrier function differ among subjects with T_H2-high and T_H2-low asthma.

Claudins are essential to tight junction formation and are a primary determinant of paracellular permeability through intact tight junctions. Differential claudin expression accounts for the differences in epithelial permeability in diverse epithelia, and mutations in claudin genes and changes in claudin expression result in clinical disease.[15,16] The claudin-18 gene encodes 2 variants that differ in the first exon. Claudin-18.1 expression requires the transcription factor NKX2-1 and is exclusive to lung epithelia.[17] At present, claudin-18.1 is the only known lung-specific tight junction gene product. The expression of specific claudin family members may vary in different airway epithelial cell types, but claudins-1, -4, and -7 are expressed at high levels in airway epithelium.[18,19] Although claudin-18 is also normally expressed in airway epithelial cells, its contribution to the permeability barrier in the airways has not been fully defined.

This study was undertaken to determine the contribution of claudin-18 to airway epithelial barrier function and whether claudin-18 expression is regulated by T_H2 inflammation. In addition, the consequences of claudin-18 deficiency to aeroantigen sensitization and airway hyperresponsiveness were examined. The presented data substantiate the hypothesis that claudin-18 is a central barrier-forming component of tight junctions and show that IL-13 downregulates claudin-18. These data also suggest that the loss of claudin-18 is associated with increased sensitization to aeroantigens and airway responsiveness.

METHODS

Airway mRNA expression studies

Aliquots of RNA extracted from airway epithelial brushings and stored in the Airway Tissue Bank at the University of California, San Francisco (UCSF) were analyzed by quantitative PCR as previously described.[20] The UCSF Committee on Human Research approved the policies and procedures of the UCSF Airway Tissue Bank and use of samples for this study. These epithelial brushings had been collected during research bronchoscopy from 67 nonsmoking subjects with asthma and 42 healthy nonsmoking control subjects (Table I). All subjects signed an Airway Tissue Bank informed consent form approved by the UCSF Committee on Human Research. Subjects with asthma had a prior physician's diagnosis of asthma, a PC_{20} methacholine value of less than 8 mg/mL, and were using only inhaled β-agonist medications for therapy. RNA was reverse transcribed with random hexamer primers and then amplified in a multiplex reaction with custom primers (see Table E1 in this article's Online Repository at www.jacionline.org). The amplified cDNA was then used for quantitative PCR with custom primers and Taqman-based probes run in separate batches.[13,21] Normalization was done using the geometric mean of the value of 3 housekeeping genes as previously described.[21,22] Log2 transformed, normalized, relative expression is reported. The three-gene-mean marker of T_H2 inflammation was determined as described previously.[13,14] This measure is based on the geometric mean of mRNA expression levels of CLCA1, SERPINB2, and POSTN. Claudin-18 mRNA expression levels were compared with serum IgE levels and blood eosinophil counts obtained from subjects with asthma at the time of bronchoscopy.

Primary cell culture and immunostaining

Cadaveric airway tissues from lungs rejected for transplantation were obtained in accordance with UCSF Committee on Human Research approval from the Northern California Organ Donor Network. Normal airway surface epithelial cells were harvested from 8 individual donors and expanded and cultured at an air-liquid interface as described previously.[23,24] Treatment with IL-13 (10 ng/mL) was begun at 2 weeks, once air-liquid interface cultures had established confluence, and continued for 7 days. Recombinant human IL-13 (R&D Systems, Minneapolis, Minn) was reconstituted in sterile PBS containing 0.1% BSA. For primary human airway epithelial cells, quantitative PCR was done without preamplification. Normalization was done as above using EEF1A1 and PPIA. Data are reported as relative mRNA expression normalized to the housekeeping genes. Claudin-18 protein levels were compared in cell lysates using immunoblot densitometry normalized to tubulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Blots were incubated with claudin-18 and tubulin primary antibodies in series and images developed such that both bands could be visualized on the same blot. In addition, separate, equally loaded blots were probed for either claudin-18 or GAPDH. Data normalized to tubulin or GAPDH were similar and the GAPDH data are shown. For immunostaining, tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were stained for beta catenin (G10, Santa Cruz Biotechnology, Santa Cruz, Calif) and claudin-18 (ZMD.385, Life Technologies, Waltham, Mass) and counterstained with 4′-6-diamidino-2-phenylindole, dihydrochloride.

16HBE cell culture

16HBE cells (a gift from Dr Dieter Gruenert, UCSF) were cultured in minimum essential medium Eagle's with Earle's basic salt solution medium supplemented with 10% BSA, 1% penicillin/streptomycin, and 1% glutamine. A coating medium comprising LHC basal media, 0.1% type 1 collagen, 0.1% BSA, and 0.1% fibronectin was used to coat the culture plates. Cells were cultured until they reached 80% confluence, and 250,000 cells/cm^2 were then passaged on to 1.13-cm^2 Transwell polycarbonate inserts (3407, Corning Costar, Sigma Aldrich, St Louis, Mo) treated with coating medium. Claudin-18 and tubulin protein expression levels were measured using Western blotting, and densitometry data were analyzed using ImageJ (National Institutes of Health, Bethesda, Md). Data are reported as claudin-18/tubulin. Transepithelial electrical resistance was measured using a voltohm meter (World Precision Instruments, Sarasota, Fla). Permeability to the 500 Dalton fluorescent tracer pyranine was measured by adding 10 μg/mL pyranine to the apical chamber of transwells and measuring fluorescence recovery in the basolateral chamber.[25] Data are reported as apparent permeability (P_{app}).

shRNA studies

Claudin 18 shRNA sequences (TRCN0000116737 to TRCN 000011673741) were cloned into the third-generation lentiviral vector pLKO.1-puro (Sigma Mission shRNA library) and transfer plasmids were cotransfected with packaging plasmids and VSV-G–expressing envelope plasmid into human 293 cells at low passage and 30% confluence. When the cells became confluent (36-48 hours), the culture medium was collected and filtered. Supernatants were aliquoted and kept at −80°C. After confirmation of posttransduction knock down by immunoblot,

TABLE I. Airway brush specimen subject data

Characteristic	Healthy	Asthma
Sample size	42	67
Sex: female	22	35
Age (y)	34 (27-40)	33 (24-42)
Body mass index	25.7 (22.6-29.6)	26.9 (23.9-29.5)
FEV_1 (percent)	102.5 (94.3-113.5)	85.4 (79.5-94.0)*
Forced vital capacity (percent)	108.2 (99.7-115.0)	100.0 (92.4-109.0)*

Data are median and 25% to 75% quartile range.
*$P < .05$ by Wilcoxon rank-sum test for each pair.

transepithelial electrical resistance of stable monolayers and paracellular permeability to pyranine were measured as previously described.[25]

Mouse studies

Animal studies were done in accordance with local Institutional Animal Research Committee approval. Constitutive Cldn18$^{-/-}$ mice on the C57/b6 background were generated by the trans-NIH Knock-Out Mouse Project. The knock-out strategy targeted a 17-kB DNA genome segment encompassing the 5 exons of claudin-18.1. Isoflurane-anesthetized mice were exposed to aspergillus 100 μg antigen (Hollister, Spokane, Wash) in 40 μL saline or saline alone intranasally 3 times per week for 3 weeks as previously described.[26] One day after the final aspergillus administration, mice were anesthetized with ketamine/acepromazine (90/2 mg/kg) and received pancuronium (1 mg/kg). Airway resistance was measured with a FlexiVent system (Scireq, Montreal, Quebec, Canada) before and after increasing doses of acetylcholine (intravenous). Total serum IgE level was measured as previously described.[26] Separate claudin-18 null and wild-type mice were sensitized intraperitoneally with 20 mg of aspergillus mixed with 20 mg of aluminum potassium sulfate (alum) on days 0 and 14. Control animals received an equal volume of alum alone. Mice were euthanized for serum harvesting 3 days after the second sensitization. Additional mice were sensitized on days 0, 7, and 14 by intraperitoneal injection of 50 μg ovalbumin (OVA, Sigma Aldrich) emulsified in 1 mg alum. Control animals received an equal volume of alum alone. Mice were euthanized for serum harvesting 7 days after the third sensitization. OVA-specific IgE levels were measured by ELISA using microplates coated with OVA. Diluted serum samples were added to each well, and the bound IgE was detected with biotinylated antimouse IgE (R35-118; Pharmingen, Stamford, Conn).

To further explore the effect of IL-13 on claudin-18 expression *in vivo*, previously described IL-13–overexpressing transgenic mice and wild-type C57/b littermate controls were compared.[27,28] Lung tissue was collected and mRNA and protein extracted. Claudin-18 mRNA and protein abundance was determined as described above.

Tracheal epithelial permeability to macromolecules was measured as previously described[29] with slight modification. Briefly, tracheas from claudin-18 null and wild-type littermates were cannulated at both ends with PE50 tubing and instilled with 50 μL Krebs buffer containing 1 mg/mL of the 0.5-kD fluorophore pyranine. Temperature was maintained at 37°C for 30 minutes. Tracheas were then gently flushed with 300 μL tracer-free Krebs buffer and homogenized in 250 μL fresh buffer. Before homogenization, tracheal segment width and length, spanning the distance between the sutures securing the PE50 tubing, were recorded for determination of tracheal epithelial surface area. Homogenates were centrifuged for 10 minutes at 12,000*g* and fluorescence was measured in 100-μL aliquots of supernatant. Tracheal epithelial permeability is reported as P_{app} or $(dQ/dT)/CA$, where dQ/dT is the permeation rate of tracer as a mass per time, C is the initial concentration of tracer in the buffer, and A is the tracheal epithelial surface area.

Histology

Lungs were inflated with 10% buffered formalin to a pressure of 25 cm H_2O. Sections (5 μm) of the entire lung were stained with hematoxalin and eosin, periodic acid–Schiff (PAS), or Sirius red. PAS staining was scored in a semi-quantitative fashion as previously reported.[30] Airway fibrosis was quantified using the area/perimeter method as previously reported.[31]

Statistics

Comparisons between 2 groups were done by *t* test for normally distributed data or by the Wilcoxon rank-sum test. Claudin-18 mRNA expression in airway brush samples was compared using ANOVA with posttest for linear trend and *post hoc* Bonferroni correction for multiple comparisons. For airway resistance data, ANOVA and pairwise, *post hoc* Bonferroni correction was done. For primary cell culture studies, Friedman test with Dunn correction for multiple comparisons was used. *P* values of less than .05 were considered statistically significant. Statistics were calculated using Prism v6.04 (GraphPad Software, La Jolla, Calif). Data are shown as mean ± SEM unless otherwise indicated.

RESULTS

Claudin-18 expression is decreased in asthma and is inversely associated with T_H2 inflammation

Claudin-18 mRNA levels were lower in epithelial brush samples from subjects with asthma (n = 67) than from healthy controls (n = 42) (Fig 1, *A*). Claudin-18 levels were lower in subjects with asthma with low three-gene-mean values than in healthy controls and were lowest in subjects with asthma with higher three-gene-mean values ($P < .0001$ by ANOVA with a significant linear trend). T_H2-high subjects with asthma (n = 44) had the lowest claudin-18 mRNA levels ($P < .05$ compared with healthy subjects following *post hoc* Bonferroni test for multiple comparisons). Consistent with previous reports,[13] most subjects with asthma were T_H2-high (44 of 67) as defined by a three-gene-mean of greater than 0.1. Although protein samples were not available from the epithelial brush samples used in this study, the presence of claudin-18 protein was examined in airway epithelium from cadaveric tracheal tissue (Fig 1, *B*). Claudin-18 immunolocalized to the epithelium as defined by the presence of beta catenin. Among subjects with asthma, there was a modest inverse correlation between serum IgE levels and claudin-18 levels in the airway brush samples (rs = −0.31) (Fig 1, *C*). There was also an inverse correlation between claudin-18 levels and blood eosinophil counts among subjects with asthma (rs = −0.42) (Fig 1, *D*).

IL-13 decreases claudin-18 mRNA and protein levels in primary human airway epithelial cells

In primary human airway epithelial cells, IL-13 induced a significant decrease in claudin-18 protein expression compared with vehicle-treated controls (Fig 2, *A*). IL-13 significantly decreased claudin-18 mRNA levels, but did not significantly change mRNA levels of claudin-1, claudin-4, or claudin-7 (n = 8 separate donors) (Fig 2, *B*).

Loss of claudin-18 impairs epithelial barrier function

In 16HBE cells, claudin-18–targeted shRNA decreased claudin-18 protein levels by approximately 50% (Fig 3, *A*), but did not significantly affect protein levels of claudin-1, claudin-4, or claudin-7. Claudin-1 protein expression in cldn18-targeted shRNA-treated cells was 110% ± 20% of control shRNA-treated cells. Claudin-4 protein expression was 91% ± 15% and claudin-7 protein expression was 114% ± 10% of control

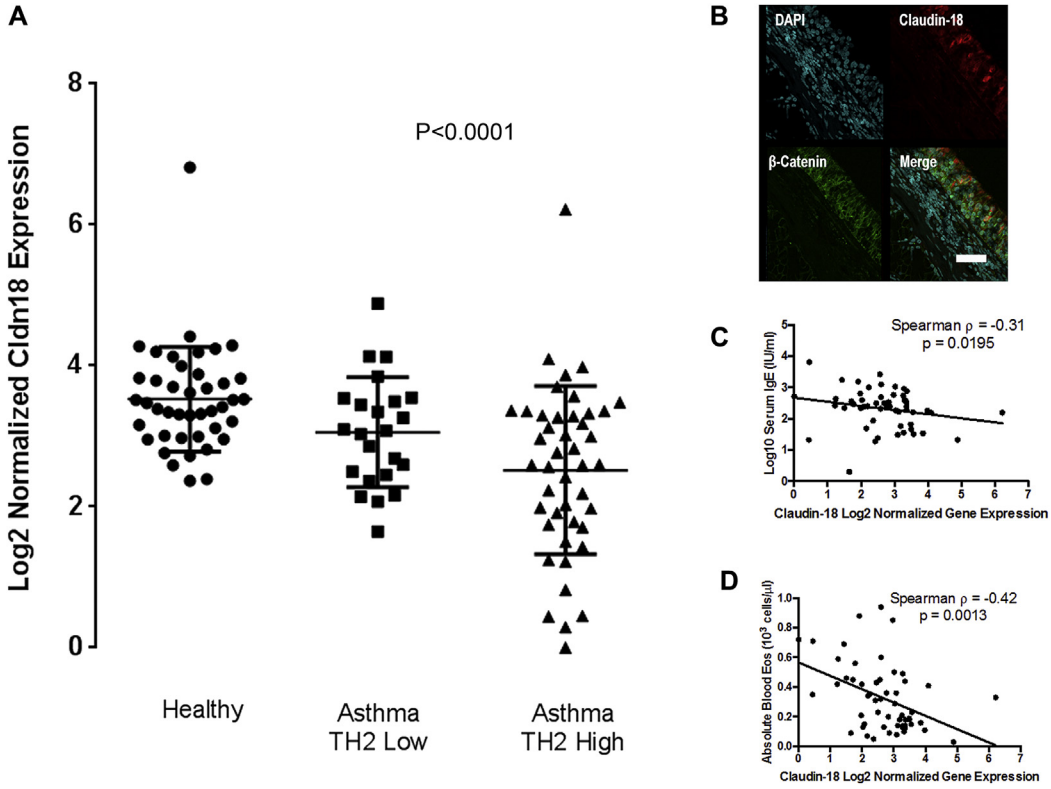

FIG 1. Airway claudin-18 expression in patients with asthma. **A,** Claudin18.1 levels in patients with asthma and healthy controls. Claudin-18.1 levels are lowest in T_H2-high patients with asthma, $P < .0001$ by ANOVA and significant posttest for linear trend. **B,** Claudin-18 immunolocalizes to the epithelium (beta catenin positive cells) in tracheal tissue. Serum IgE **(C)** and blood eosinophils **(D)** are inversely correlated with airway claudin-18 levels in patients with asthma.

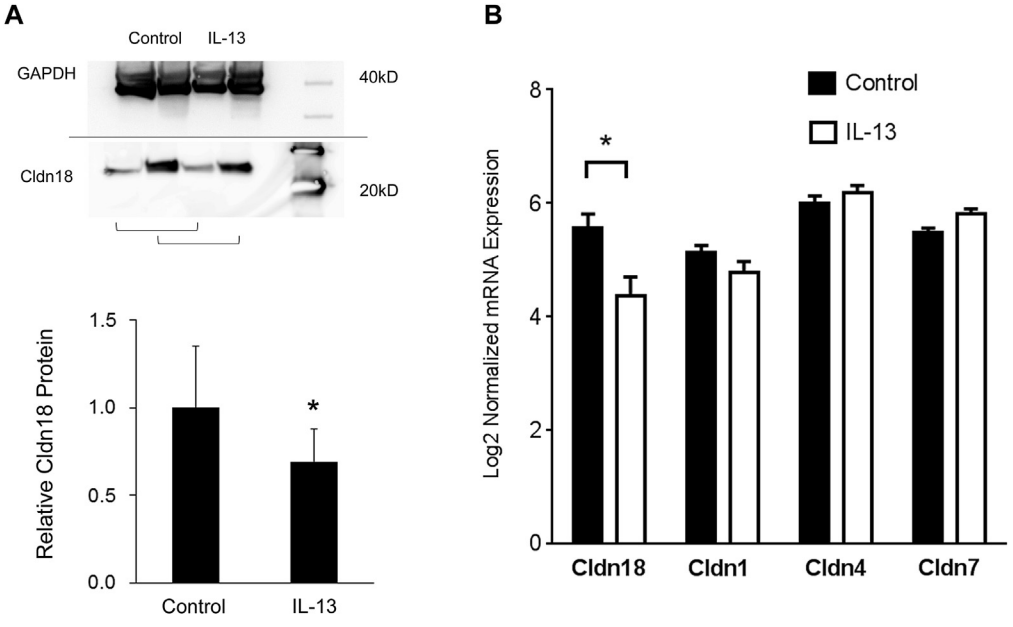

FIG 2. IL-13 decreases claudin-18 expression in primary human airway epithelial cells. **A,** IL-13 decreased claudin-18 protein levels (representative blot shown, brackets indicate cells from the same donor) (n = 8, $*P < .05$ by paired analysis). **B,** IL-13 decreased claudin-18.1 mRNA levels, but did not significantly change claudin-1, claudin-4, or claudin-7 mRNA abundance (n = 8, $*P < .05$).

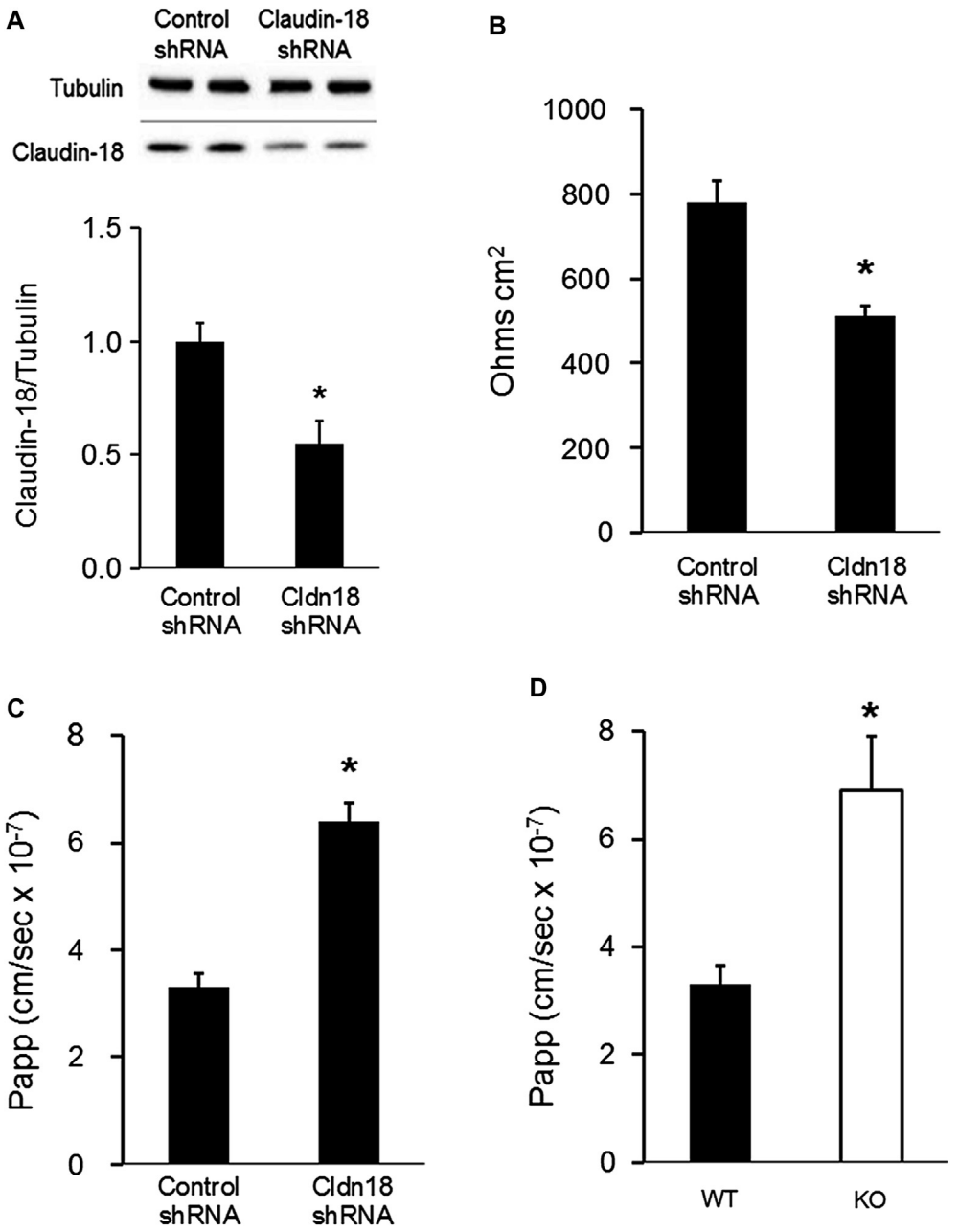

FIG 3. Claudin-18 is required for the airway epithelial permeability barrier. **A,** Claudin-18-shRNA decreased claudin-18 protein levels in 16HBE cells (*$P < .05$). Representative blot above with densitometry (n = 6) below. Claudin-18 knock down decreased transepithelial electrical resistance (TER) **(B)** and increased P_{app} (*$P < .05$) **(C)**. **D,** Tracheal epithelial permeability in claudin-18 null and wild-type mice (n = 6 in each group, *$P < .01$). *KO,* Knockout; *WT,* wild-type.

shRNA-treated cells based on densitometry normalized to tubulin (n = 6 biologic replicates).

Knock down of claudin-18 resulted in a significant decrease in transepithelial electrical resistance and a significant increase in epithelial permeability to the 0.5-kD tracer pyranine in 16HBE cells ($P < .05$) (Fig 3, *B* and *C*).

Tracheal epithelial permeability to pyranine was assessed in wild-type and claudin-18 null mice (n = 6 in each group). This method yielded permeability values comparable to those in previous studies of airway permeability in wild-type mice ($P_{app} = 3.3 \pm 0.3 \times 10^{-7}$ cm/s). Consistent with the *in vitro* claudin-18 knock-down studies, tracheal epithelial permeability was more than 2-fold higher in claudin-18 null mice ($P < .05$) (Fig 4, *D*).

Claudin-18 deficiency increased sensitization to aeroantigens and airway responsiveness in mice

To investigate the association between claudin-18, epithelial barrier function, and asthma, claudin-18 knock-out mice were

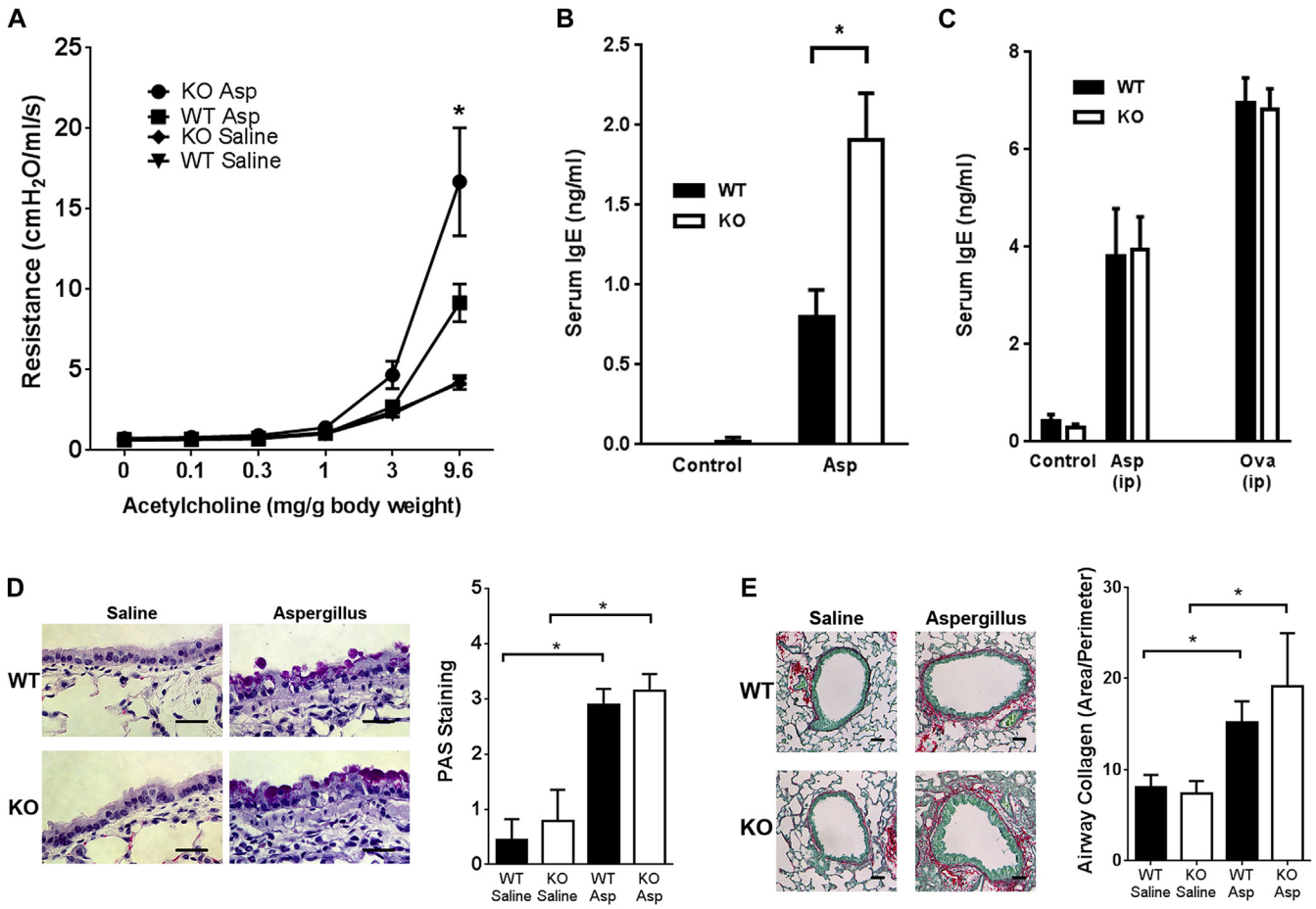

FIG 4. Airway responsiveness and serum IgE levels after aspergillus (Asp) sensitization in mice. **A**, Following intranasal Asp sensitization, airway resistance responses were greater in claudin-18 null mice (*$P < .05$). Serum IgE following intranasal Asp (*$P < .05$) **(B)** and intraperitoneal (ip) Asp or ova **(C)**. PAS staining **(D)** and collagen deposition **(E)** before and after intranasal Asp sensitization (*$P < .05$ vs saline). *KO*, Knockout; *WT*, wild-type.

exposed to intranasal aspergillus antigen. Following the final intranasal exposure, serum IgE levels and airway resistance changes in response to intravenous acetylcholine were measured. Compared with wild-type mice, claudin-18 null mice showed increased airway responsiveness to acetylcholine following intranasal aspergillus sensitization (Fig 4, *A*) ($P < .01$). Control, saline-treated, claudin-18 knock-out mice were not different from saline-treated wild-type mice (n = 6-8 in each group).

Serum IgE levels in intranasally sensitized cldn18 null mice were significantly higher than in aspergillus-treated wild-type mice and saline-treated control mice (Fig 4, *B*). To determine whether the airway route of administration accounted for the difference in sensitization to aspergillus, additional mice were sensitized to aspergillus antigen using the intraperitoneal route. Serum IgE levels were not different in knock-out and wild-type mice given intraperitoneal aspergillus antigen (Fig 4, *C*). To further evaluate for baseline differences in antigen sensitization, additional mice were sensitized to OVA via the intraperitoneal route and OVA-specific IgE levels were measured in serum. OVA-specific IgE levels were not different between knock-out and wild-type mice (Fig 4, *C*).

On lung histology, there was no difference in airway mucin between genotypes at baseline as quantified by PAS staining. Intranasal aspergillus sensitization increased PAS staining to a similar degree in both genotypes (Fig 4, *D*). Similarly, aspergillus sensitization increased airway collagen to a comparable degree in both genotypes. Although airway collagen was slightly higher in knock-out mice after aspergillus sensitization, the difference did not reach statistical significance (n = 6-8 in each group) (Fig 4, *E*).

IL-13 overexpression *in vivo* decreases claudin-18 mRNA and protein expression

To test whether IL-13 decreases lung claudin-18 expression *in vivo*, previously described transgenic IL-13–overexpressing mice were compared with wild-type littermates. Claudin-18 protein expression, normalized to tubulin, was significantly decreased in IL-13 transgenic mice compared with wild-type mice (Fig 5, *A*). Using quantitative PCR, it was found that claudin-18 mRNA levels, normalized to beta actin, were also significantly decreased (Fig 5, *B*). Claudin-4 mRNA expression was significantly increased in IL-13–overexpressing

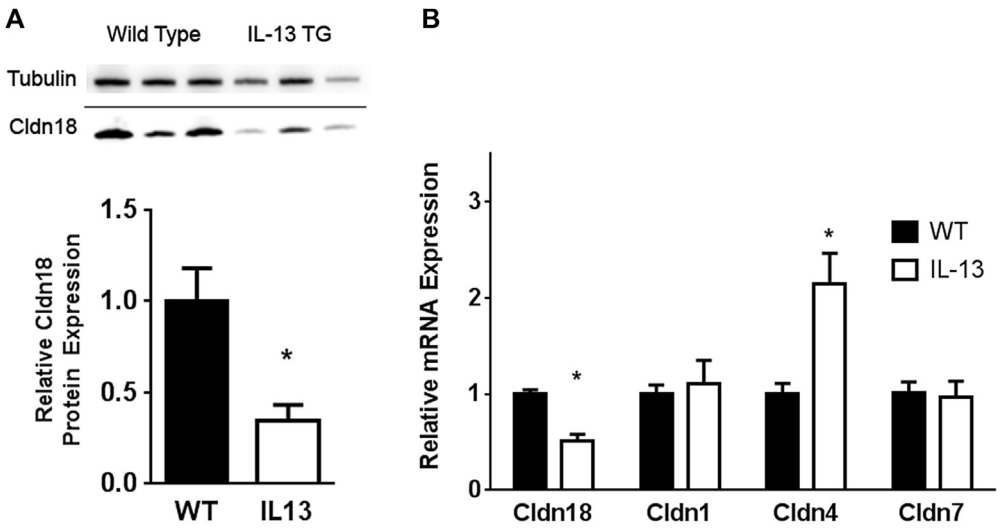

FIG 5. IL-13 decreases claudin-18 expression in mice. Mice hemizygous for a transgene that overexpresses IL-13 (IL-13 TG) have lower claudin-18 protein (*$P < .05$) **(A)** and mRNA levels than do wild-type littermates **(B)**. *WT,* Wild-type.

transgenic mice and claudin-1 and claudin-7 mRNA levels were not different between the genotypes (n = 6 in each group) (Fig 5, *B*).

DISCUSSION

The overall objective of this study was to establish the role of claudin-18 in airway epithelial barrier function and determine whether claudin-18 is regulated by mediators of T_H2 inflammation. Based on the hypothesis that claudin-18 is a requirement for the epithelial permeability barrier, the contribution of claudin-18 to aeroantigen sensitization and airway responsiveness was investigated with the prediction that serum IgE and airway responsiveness would be increased in the setting of claudin-18 deficiency. The primary findings of this study are that claudin-18 levels are decreased in subjects with asthma in association with T_H2 inflammation and that IL-13 acts to decrease claudin-18 expression in primary human cells and in mice. In addition, subjects with asthma with low claudin-18 mRNA levels had higher serum IgE levels and blood eosinophil counts. Claudin-18–deficient mice developed a greater increase in serum IgE following intranasal but not intraperitoneal aspergillus antigen sensitization. Claudin-18–deficient mice also developed greater airway hyperresponsiveness compared with wild-type mice following intranasal aspergillus sensitization. Together these data support the hypothesis that claudin-18 is a requirement for airway epithelial barrier function and suggest that increased epithelial permeability may contribute to greater antigen sensitization and airway responsiveness in asthma. Because T_H2 inflammation decreases claudin-18 expression, the data in this study are consistent with the hypothesis that T_H2 inflammation and epithelial barrier dysfunction can participate in a feed forward loop in asthma that includes the downregulation of claudin-18.

The contributors to disease development and severity in asthma are manifold; however, epithelial barrier dysfunction may represent a common physiological feature of asthma that is downstream to diverse genetic and environmental inputs. Compromised epithelial permeability function represents a partial loss of compartmentalization and potentially epithelial polarization as well. These changes in epithelial function may permit increased sensitization to aeroantigens and alter cell signaling pathways that are normally regulated by segregated receptors and ligands. There are several examples of the causative role epithelial barrier dysfunction can play in disease progression outside of the airway.[10,32,33] T_H2 inflammation appears to be a potent modulator of epithelial permeability, not only in the gut but also in the skin, nasal epithelium, and 16HBE cells.[9,11,12] In the context of asthma, previous studies have reported decreased expression levels of certain junctional proteins, including epithelial cadherin, occluding, and zona occludens-1.[8,34-36] Previous work has also shown that innate immune mediators such as TNF-α can influence claudin expression in airway epithelial cells.[18] The data from the present study and others implicate a crucial role for epithelial barrier dysfunction in asthma.

To date, relatively little is known about the regulation of claudins in asthma or the consequences of any changes in claudin expression to disease. Although numerous proteins make contributions to the epithelial permeability barrier, the claudin family of proteins has particular importance. Claudins form the band-like meshwork within the apical junction complex that accomplishes the sealing function between cells as well as epithelial polarization within the membrane. Claudin-18.1 is of interest because it is uniquely expressed in lung epithelia. The recent development of claudin-18 null mice has begun to advance our understanding of the function of this protein in the lung. For example, claudin-18 null mice exhibit alveolar epithelial barrier defects. In the alveolar epithelium, loss of claudin-18 results in increased paracellular permeability and altered tight junction structure between type 1 pneumocytes.[37,38] In cultured alveolar epithelial cells the permeability-limiting function of claudin-18 is not replaced by increased expression levels of other claudin family members.[37] Interestingly, claudin-18 deficiency results in impaired alveolarization postnatally; the phenotype of claudin-18 null mice shares some similarity with animal models of bronchopulmonary dysplasia.[37] Although claudin-18 is expressed in the airways, the contribution of claudin-18 to airway barrier

function has not been previously reported in detail. Ongoing development of conditional claudin-18 knock-out mice may help to separate the effects of claudin-18 deficiency on the alveolar and airway epithelium, but the data presented in this study indicate that airway epithelial permeability is higher in the absence of claudin-18.

A key finding of this study is that claudin-18 mRNA levels are reduced in the airways of subjects with asthma and that claudin-18 expression is inversely associated with T_H2 inflammation (Fig 1, *A*). These data are consistent with the hypothesis that IL-13 suppresses claudin-18 expression in airway epithelial cells. The observed decrease in claudin-18 mRNA levels could be the result of a direct effect of IL-13 on claudin-18 mRNA expression or stability. Alternatively, this finding could be the result of a change in the relative abundance of particular cell populations within the epithelium that differ in claudin-18 expression levels. Claudin-18 protein levels were not examined in this study because the airway brush specimens used were too small to allow for protein-level analysis. However, there was a consistent correlation between claudin-18 mRNA and protein levels in primary airway epithelial cells and in 16HBE cells.

Atopic status is an important phenotypic feature of asthma that influences treatment decisions, but the mechanisms underpinning the association between atopy and asthma pathogenesis or progression are not entirely understood. Because environmental antigens drive T_H2 inflammation in atopic individuals, increased antigen exposure due to epithelial barrier dysfunction could potentially increase asthma severity in these patients. The findings of this study raise the possibility of an inverse association between claudin-18 levels and sensitization to inhaled environmental antigens. It should be acknowledged that the magnitude of antigen exposure is not the only determinant of serum IgE or eosinophilia, and differences among subjects with asthma could result from other factors, including T_H2 status.

To more directly test the effect of IL-13 on claudin-18 expression, primary human airway epithelial cells were cultured in the presence of IL-13. The effect of IL-13 on claudin-18 expression was also examined *in vivo* in IL-13–overexpressing transgenic mice. Although mouse models of asthma should be interpreted with caution, these mice are known to exhibit airway hyperresponsiveness and increased serum IgE levels following antigen exposure.[27,28] In each of these experiments, IL-13 decreased claudin-18 levels. In contrast to studies in primary human airway epithelial cells, claudin-4 mRNA levels were significantly higher in IL-13 transgenic mice compared with wild-type mice. These data suggest that IL-13 exposure results in additional changes in airway claudin expression in mice. Barrier function was not assessed in this experiment, but previous work has demonstrated that IL-13 impairs airway epithelial barrier function.[12]

To better understand the specific contribution of claudin-18 to airway epithelial barrier function, the human airway epithelial cell line 16HBE was used in loss-of-function studies. 16HBE cells form tight junctions and have been used as a model of the airway epithelial barrier.[39] In these cells, the loss of claudin-18 is sufficient to impair epithelial barrier function, including increased permeability to macromolecules. In additional experiments, airway epithelial permeability was examined in intact tracheas from claudin-18 null and wild-type mice *in situ*. Similarly, claudin-18 null mouse tracheas had significantly higher permeability. The mechanism by which claudin-18.1 uniquely limits epithelial permeability is not yet certain. In stomach epithelium, expression of claudin18.2 results in the formation of a distinct apical tight junction strand[40]; however, previous studies of airway epithelial tight junction ultrastructure have not reported distinct apical strands analogous to those reported in stomach epithelium.[41,42] Claudin-18.1 (lung) and claudin-18.2 (stomach) differ in sequence at the first of 2 extracellular domains. Although claudin-18.1 deficiency alters alveolar epithelial type 1 cell tight junction structure,[37] it is not yet clear whether claudin-18.1 deficiency results in ultrastructural changes to airway epithelial tight junctions. It remains possible that the loss of claudin-18 results in larger changes in tight junction organization or composition that compromise the permeability barrier in the airway. Although the structural relationship between claudin-18 and tight junction function requires additional investigation, the data from the present study demonstrate that claudin-18 is a requirement for normal airway epithelial permeability.

The contribution of claudin-18 deficiency to aeroantigen sensitization and airway hyperresponsiveness was examined in claudin-18 null mice using the intratracheal aspergillus sensitization model. Because claudin-18.1 expression is restricted to the lung epithelium, if the barrier defect resulting from claudin-18 deficiency augments antigen sensitization, it would follow that sensitization to antigens delivered via a route other than the airway would be similar in wild-type and clauidn-18 null mice. Therefore, mice were also sensitized with intraperitoneal aspergillus and serum IgE levels were measured. In contrast to the results of the intraairway sensitization experiments, wild-type and claudin-18 null mice had similar serum IgE levels following intraperitoneal aspergillus or OVA sensitization (Fig 4, *C*). These data indicate that sensitization responses in claudin-18 null mice are not intrinsically different from those in wild-type mice, but sensitization to aeroantigens is greater in the absence of claudin-18. Although airway responsiveness and serum IgE levels differed between wild-type and knock-out mice, mucous cell hyperplasia and airway fibrosis did not significantly differ following airway aspergillus sensitization (Fig 4, *D* and *E*). In sum, these mouse studies show that claudin-18 deficiency results in increased airway permeability and increased serum IgE levels following intraairway, but not intraperitoneal antigen exposure. Murine models of asthma have important limitations. Mouse models do not necessarily recapitulate responses observed in human subjects or tissues. Although the data from this study show a significant increase in airway permeability in human cells with clauidn-18 depletion, the sensitization responses observed in the mouse and human studies may not result from identical mechanisms.

A growing body of evidence has focused attention on airway epithelial barrier dysfunction as a central feature of asthma. The potential contributions of increased epithelial permeability and loss of epithelial polarity to asthma include heightened exposure to air space antigens and altered cell signaling due to the loss of segregation of normally polarized receptors and ligands. These abnormalities may serve to propagate allergic inflammation, increase parenchymal cell activation, and promote hyperresponsiveness. Previous studies have found that specific structural components of cell-cell junctions exhibit decreased abundance or abnormal localization in the airways of subjects with asthma. Data from this study suggest that decreased claudin-18 expression

mediated by IL-13 is an additional junctional abnormality in some patients with asthma, particularly those with T_H2-high asthma. Claudin-18 appears to play a nonredundant role in the epithelial permeability barrier, potentially through effects on tight junction organization, or a more selective effect on paracellular permeability. A better understanding of the regulation of claudin-18 in disease may provide new insights for targeted therapeutics.

Key messages

- **Claudin-18 is a key barrier-forming protein in the airway epithelium that is expressed at lower levels in subjects with asthma compared with healthy controls. T_H2 inflammation markers are inversely associated with claudin-18 levels in those with asthma.**
- **IL-13–mediated loss of claudin-18 may contribute to increased epithelial permeability, increased sensitization to aeroantigens, and airway hyperresponsiveness.**

REFERENCES

1. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol 2011;127:355-60.
2. Holgate ST. Pathophysiology of asthma: what has our current understanding taught us about new therapeutic approaches? J Allergy Clin Immunol 2011;128:495-505.
3. Georas SN, Rezaee F. Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation. J Allergy Clin Immunol 2014;134:509-20.
4. Swindle EJ, Collins JE, Davies DE. Breakdown in epithelial barrier function in patients with asthma: identification of novel therapeutic approaches. J Allergy Clin Immunol 2009;124:23-34; quiz 5-6.
5. Wan H, Winton HL, Soeller C, Gruenert DC, Thompson PJ, Cannell MB, et al. Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. Clin Exp Allergy 2000;30:685-98.
6. Antony AB, Tepper RS, Mohammed KA. Cockroach extract antigen increases bronchial airway epithelial permeability. J Allergy Clin Immunol 2002;110:589-95.
7. Heijink IH, Nawijn MC, Hackett TL. Airway epithelial barrier function regulates the pathogenesis of allergic asthma. Clin Exp Allergy 2014;44:620-30.
8. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al. Defective epithelial barrier function in asthma. J Allergy Clin Immunol 2011;128:549-56, e1-12.
9. Wise SK, Den Beste KA, Hoddeson EK, Parkos CA, Nusrat A. Sinonasal epithelial wound resealing in an in vitro model: inhibition of wound closure with IL-4 exposure. Int Forum Allergy Rhinol 2013;3:439-49.
10. Heller F, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B, et al. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology 2005;129:550-64.
11. Wise SK, Laury AM, Katz EH, Den Beste KA, Parkos CA, Nusrat A. Interleukin-4 and interleukin-13 compromise the sinonasal epithelial barrier and perturb intercellular junction protein expression. Int Forum Allergy Rhinol 2014;4:361-70.
12. Saatian B, Rezaee F, Desando S, Emo J, Chapman T, Knowlden S, et al. Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells. Tissue Barriers 2013;1:e24333.
13. Bhakta NR, Solberg OD, Nguyen CP, Nguyen CN, Arron JR, Fahy JV, et al. A qPCR-based metric of Th2 airway inflammation in asthma. Clin Transl Allergy 2013;3:24.
14. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009;180:388-95.
15. Capaldo CT, Nusrat A. Claudin switching: physiological plasticity of the Tight Junction. Semin Cell Dev Biol 2015;42:22-9.
16. Angelow S, Ahlstrom R, Yu AS. Biology of claudins. Am J Physiol Renal Physiol 2008;295:F867-76.
17. Niimi T, Nagashima K, Ward JM, Minoo P, Zimonjic DB, Popescu NC, et al. Claudin-18, a novel downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor, encodes lung- and stomach-specific isoforms through alternative splicing. Mol Cell Biol 2001;21:7380-90.
18. Coyne CB, Vanhook MK, Gambling TM, Carson JL, Boucher RC, Johnson LG. Regulation of airway tight junctions by proinflammatory cytokines. Mol Biol Cell 2002;13:3218-34.
19. Xu R, Li Q, Zhou J, Zhou XD, Perelman JM, Kolosov VP. The degradation of airway tight junction protein under acidic conditions is probably mediated by transient receptor potential vanilloid 1 receptor. Biosci Rep 2013;33. http://dx.doi.org/10.1042/BSR20130087.
20. Dougherty RH, Sidhu SS, Raman K, Solon M, Solberg OD, Caughey GH, et al. Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. J Allergy Clin Immunol 2010;125:1046-53.e8.
21. Dolganov GM, Woodruff PG, Novikov AA, Zhang Y, Ferrando RE, Szubin R, et al. A novel method of gene transcript profiling in airway biopsy homogenates reveals increased expression of a Na+-K+-Cl- cotransporter (NKCC1) in asthmatic subjects. Genome Res 2001;11:1473-83.
22. Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proc Natl Acad Sci U S A 2007;104:15858-63.
23. Widdicombe JH, Sachs LA, Morrow JL, Finkbeiner WE. Expansion of cultures of human tracheal epithelium with maintenance of differentiated structure and function. Biotechniques 2005;39:249-55.
24. Sachs LA, Finkbeiner WE, Widdicombe JH. Effects of media on differentiation of cultured human tracheal epithelium. In Vitro Cell Dev Biol Anim 2003;39:56-62.
25. LaFemina MJ, Rokkam D, Chandrasena A, Pan J, Bajaj A, Johnson M, et al. Keratinocyte growth factor enhances barrier function without altering claudin expression in primary alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 2010;299:L724-34.
26. Gordon ED, Sidhu SS, Wang ZE, Woodruff PG, Yuan S, Solon MC, et al. A protective role for periostin and TGF-beta in IgE-mediated allergy and airway hyperresponsiveness. Clin Exp Allergy 2012;42:144-55.
27. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med 2002;8:885-9.
28. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Investig 1999;103:779-88.
29. Sidhaye VK, Schweitzer KS, Caterina MJ, Shimoda L, King LS. Shear stress regulates aquaporin-5 and airway epithelial barrier function. Proc Natl Acad Sci U S A 2008;105:3345-50.
30. Sugimoto K, Kudo M, Sundaram A, Ren X, Huang K, Bernstein X, et al. The alphavbeta6 integrin modulates airway hyperresponsiveness in mice by regulating intraepithelial mast cells. J Clin Investig 2012;122:748-58.
31. Reinhardt AK, Bottoms SE, Laurent GJ, McAnulty RJ. Quantification of collagen and proteoglycan deposition in a murine model of airway remodelling. Respir Res 2005;6:30.
32. Yu HS, Kang MJ, Kwon JW, Lee SY, Lee E, Yang SI, et al. Claudin-1 polymorphism modifies the effect of mold exposure on the development of atopic dermatitis and production of IgE. J Allergy Clin Immunol 2015;135:827-30.e5.
33. Weidinger S, Illig T, Baurecht H, Irvine AD, Rodriguez E, Diaz-Lacava A, et al. Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. J Allergy Clin Immunol 2006;118:214-9.
34. Ierodiakonou D, Postma DS, Koppelman GH, Boezen HM, Gerritsen J, Ten Hacken N, et al. E-cadherin gene polymorphisms in asthma patients using inhaled corticosteroids. Eur Respir J 2011;38:1044-52.
35. Trautmann A, Kruger K, Akdis M, Muller-Wening D, Akkaya A, Brocker EB, et al. Apoptosis and loss of adhesion of bronchial epithelial cells in asthma. Int Arch Allergy Immunol 2005;138:142-50.
36. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. Can J Physiol Pharmacol 2008;86:105-12.
37. LaFemina MJ, Sutherland KM, Bentley T, Gonzales LW, Allen L, Chapin CJ, et al. Claudin-18 deficiency results in alveolar barrier dysfunction and impaired alveologenesis in mice. Am J Respir Cell Mol Biol 2014;51:550-8.
38. Li G, Flodby P, Luo J, Kage H, Sipos A, Gao D, et al. Knockout mice reveal key roles for claudin 18 in alveolar barrier properties and fluid homeostasis. Am J Respir Cell and Mol Biol 2014;51:210-22.

39. Wan H, Winton HL, Soeller C, Stewart GA, Thompson PJ, Gruenert DC, et al. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o. Eur Respir J 2000;15:1058-68.
40. Hayashi D, Tamura A, Tanaka H, Yamazaki Y, Watanabe S, Suzuki K, et al. Deficiency of claudin-18 causes paracellular H+ leakage, up-regulation of interleukin-1beta, and atrophic gastritis in mice. Gastroenterology 2012;142:292-304.
41. Godfrey RW, Severs NJ, Jeffery PK. Freeze-fracture morphology and quantification of human bronchial epithelial tight junctions. Am J Respir Cell Mol Biol 1992;6:453-8.
42. Schneeberger EE. Heterogeneity of tight junction morphology in extrapulmonary and intrapulmonary airways of the rat. Anat Rec 1980;198:193-208.

TABLE E1. PCR primers and probes

Gene category	Gene	Probe	Sequence	Probe label
Claudin family	CLDN1	Outer forward	CCACAGCATGGTATGGCAATAG	
		Outer reverse	TGGTGTTGGGTAAGAGGTTGTTT	
		Inner forward	CAGTCAATGCCAGGTACGAATTT	
		Probe	TCAGGCTCTCTTCACTGGCTGGGC	5-FAM/3-BHQ
		Inner reverse	AAGTAGGGCACCTCCCAGAAG	
	CLDN4	Outer forward	GAGATGGGTGCCTCGCTCTAC	
		Outer reverse	AAGAACAAAGCAGAGAGGAACAGAGT	
		Inner forward	GGCTGCTTTGCTGCAACTG	
		Probe	CCACCCCGCACAGACAAGCCTT	5-FAM/3-BHQ
		Inner reverse	CAGAGCGGGCAGCAGAATAC	
	CLDN7	Outer forward	TGATGAGCTGCAAAATGTACGA	
		Outer reverse	CAGCGCGTGCACTTCATG	
		Inner forward	GGCCACTCGAGCCCTAATG	
		Probe	CACAAACATGGCCAGGAAGCCCA	5-FAM/3-BHQ
		Inner reverse	TGCACTTCATGCCCATCGT	
	CLDN18.1	Outer forward	CCTATTTCACCATCCTGGGACTT	
		Outer reverse	GAGGTCAGTGTCATGTTGGCTTT	
		Inner forward	CCTGATGATCGTAGGCATCG	
		Probe	TACCAGGAGGCCAATGGCACCC	5-FAM/3-BHQ
		Inner reverse	TGCATTTCAGGGCAAAGATG	
Three-gene-mean	POSTN	Outer forward	GCAAACCACCTTCACGGATCT	
		Outer reverse	TTATTCACAGGTGCCAGCAAAG	
		Inner forward	CGGATCTTGTGGCCCAATT	
		Probe	CTTGGCATCTGCTCTGAGGCC	5-FAM/3-BHQ
		Inner reverse	AGGTGCCAGCAAAGTGTATTCTC	
	CLCA1	Outer forward	CCAGGCATTGCTAAGGTTGG	
		Outer reverse	ACTGGCCCTGAGAATTGGG	
		Inner forward	CCTTGACCCTGACTGTCACGT	
		Probe	TGCGTCCAATGCTACCCTGCCTC	5-FAM/3-BHQ
		Inner reverse	TTGTTCGTTTTGGAAGTCACTGTAA	
	SERPINB2	Outer forward	CTGAAGTGTTCCACCAAGCCA	
		Outer reverse	CAAACTGTGGGCCTCCATGT	
		Inner forward	GTGAATGAGGAGGGCACTGAA	
		Probe	TAACACCTCCTGTGCCAGCGGCTG	5-FAM/3-BHQ
		Inner reverse	CCATGTCCAGTTCTCCCTGTC	
Housekeeping genes	RPL13A	Outer forward	GGACCGTGCGAGGTATGCT	
		Outer reverse	TTCAGACGCACGACCTTGAG	
		Inner forward	TATGCTGCCCCACAAAACC	
		Probe	CAGAGCGGCCTGGCCTCGCT	5-FAM/3-BHQ
		Inner reverse	TGCCGTCAAACACCTTGAGA	
	PPIA	Outer forward	ATGAGAACTTCATCCTAAAGCATACG	
		Outer reverse	TTGGCAGTGCAGATGAAAAACT	
		Inner forward	ACGGGTCCTGGCATCTTGT	
		Probe	ATGGCAAATGCTGGACCCAACACA	5-FAM/3-BHQ
		Inner reverse	GCAGATGAAAAACTGGGAACCA	
	EEF1A1	Outer forward	TGCTAACATGCCTTGGTTCAAG	
		Outer reverse	TTGGACGAGTTGGTGGTAGGAT	
		Inner forward	CCTTGGTTCAAGGGATGGAA	
		Probe	CACTGGCATTGCCATCCTTACGGG	5-FAM/3-BHQ
		Inner reverse	GCCTCAAGCAGCGTGGTT	