Regulation of bronchial epithelial barrier integrity by type 2 cytokines and histone deacetylases in asthmatic patients

Paulina Wawrzyniak, MSc,[a] Marcin Wawrzyniak, PhD,[a] Kerstin Wanke, PhD,[a] Milena Sokolowska, MD, PhD,[a] Kreso Bendelja, PhD,[d] Beate Rückert, Sci Tec,[a] Anna Globinska, MSc,[a] Bogdan Jakiela, MD, PhD,[b] Jeannette I. Kast, MSc,[a] Marco Idzko, MD,[c] Mübeccel Akdis, MD, PhD,[a] Marek Sanak, MD, PhD,[b] and Cezmi A. Akdis, MD[a] *Davos, Switzerland, Krakow, Poland, Freiburg, Germany, and Zagreb, Croatia*

Background: Tight junctions (TJs) form a barrier on the apical side of neighboring epithelial cells in the bronchial mucosa. Changes in their integrity might play a role in asthma pathogenesis by enabling the paracellular influx of allergens, toxins, and microbes to the submucosal tissue.

Objective: The regulation of bronchial epithelial TJs by T_H2 cells and their cytokines and their involvement in epigenetic regulation of barrier function were investigated.

Methods: The expression, regulation, and function of TJs were determined in air-liquid interface (ALI) cultures of control and asthmatic primary human bronchial epithelial cells (HBECs) by means of analysis of transepithelial electrical resistance, paracellular flux, mRNA expression, Western blotting, and immunofluorescence staining.

Results: HBECs from asthmatic patients showed a significantly low TJ integrity in ALI cultures compared with HBECs from healthy subjects. T_H2 cell numbers and levels of their cytokines, IL-4 and IL-13, decreased barrier integrity in ALI cultures of HBECs from control subjects but not in HBECs from asthmatic patients. They induced a physical separation of the TJs of adjacent cells in immunofluorescence staining of the TJ molecules occludin and zonula occludens-1. We observed that expression of histone deacetylases (HDACs) 1 and 9, and Silent information regulator genes (sirtuins [SIRTs]) 6 and 7 were significantly high in HBECs from asthmatic patients. IL-4 and IL-13 significantly increased the expression of HDACs and SIRTs. The role of HDAC activation on epithelial barrier leakiness was confirmed by HDAC inhibition, which improved barrier integrity through increased synthesis of TJ molecules in epithelium from asthmatic patients to the level seen in HBECs from control subjects.

Conclusion: Our data demonstrate that barrier leakiness in asthmatic patients is induced by T_H2 cells, IL-4, and IL-13 and HDAC activity. The inhibition of endogenous HDAC activity reconstitutes defective barrier by increasing TJ expression. (J Allergy Clin Immunol 2017;139:93-103.)

Key words: *Asthma, bronchial epithelial cells, epigenetic, tight junctions, T_H2 cells, IL-4, IL-13, histone deacetylase*

Asthma is a chronic inflammatory disorder of the airways characterized by reversible obstruction.[1,2] Type 2 inflammation, with the major role of T_H2 cells and their cytokines, is considered a major pathophysiologic mechanism of asthma.[3,4] T_H2 cells produce a pattern of cytokines, such as IL-4, IL-5, and IL-13, which cooperate in T_H2 cell differentiation, IgE isotype switching and production, tissue and blood eosinophilia, T-cell and eosinophil homing to tissues, and airway hyperresponsiveness.[4]

Airway epithelial cells constitute the first-line barrier to prevent penetration of environmental agents, such as allergens and pollutants, into the inner tissues, and they mount an innate immune response against infectious agents.[5-7] The epithelial barrier is predominantly formed by tight junctions (TJs) located at the most apical part of the intracellular junctional complexes between the neighboring epithelial cells.[8,9] They consist of transmembrane proteins: claudins, occludin, junctional adhesion molecules, peripheral membrane proteins (eg, zonula occludens [ZO-1, ZO-2, and ZO-3] and cingulin), and gap and adherens junctions, which allow the transmembrane proteins to organize in the membrane and attach to the cytoskeleton.[8,9]

The first observation of differences in TJ structures in asthmatic patients was performed by using electron microscopy in 1988, showing that varying degrees of TJ abnormalities were observed

From [a]the Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Davos, Switzerland, Christine Kühne-Center for Allergy Research and Education (CK-CARE); [b]the Department of Medicine, Jagiellonian University Medical College, Krakow; [c]the Department of Pneumology, University Hospital Freiburg; and [d]the Institute of Immunology, Zagreb.

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Corresponding author: Cezmi A. Akdis, MD, Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Davos, Switzerland, Obere Strasse 22, 7270 Davos, Switzerland. E-mail: akdiasac@siaf.uzh.ch.

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Abbreviations used
AIA: Aspirin-induced asthma
ALI: Air-liquid interface
ATA: Aspirin-tolerant asthma
BEGM: Bronchial epithelial growth medium
COPD: Chronic obstructive pulmonary disease
FITC: Fluorescein isothiocyanate
HBEC: Human bronchial epithelial cell
HDAC: Histone deacetylase
SIRT: Silent information regulator genes (sirtuins)
TER: Transepithelial electric resistance
TJ: Tight junction
TSLP: Thymic stromal lymphopoietin
ZO: Zonula occludens

in the bronchial epithelium of healthy, bronchitic, and asthmatic subjects.[10] Recent studies have shown that barrier function of the epithelium is impaired in patients with various inflammatory diseases, including asthma, atopic dermatitis, and chronic rhinosinusitis.[11-16] Disruption of bronchial epithelial TJ integrity leads to loss of barrier function, enabling passage of proinflammatory and tissue-damaging agents, such as allergens and toxins, from the lumen into the airway parenchyma.[17]

Epigenetic changes are crucial for the development and differentiation of various cell types and can regulate essential genes, which are known to initiate and maintain asthmatic inflammation.[18-20] The mechanisms of epigenetic regulation comprise the involvement of several families of enzymes, an umbrella of biochemical reactions, such as chromatin modifications, on histones and other structures and DNA methylation.[21,22] The balance between the actions of these enzymes is a very crucial mechanism for the regulation of gene expression by controlling numerous developmental processes and disease states.[23,24] Increased expression of proinflammatory genes is regulated by acetylation of core histones, particularly by histone acetyltransferases. In contrast, histone deacetylases (HDACs) remove the acetyl groups from hyperacetylated histones, which leads to suppression of gene transcription.[25,26] They are classified in 5 groups: class I includes HDACs 1, 2, 3, and 8; class IIa contains HDACs 4, 5, 7, and 9; class IIb consists of HDACs 6 and 10; class III comprises Silent information regulator genes (sirtuins [SIRTs]) 1 to 7; and class IV includes only HDAC11.[27,28]

The aim of the present study was to investigate the regulation, expression, and function of TJs of bronchial epithelial cells in asthmatic patients in response to T_H2 cells and their related cytokines and those present in the epithelial cell microenvironment, such as IL-4, IL-5, IL-13, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). We hypothesized that there is a relationship between leakiness in the epithelium and increased activity of HDACs in asthmatic patients. Our data demonstrated a mechanism for leakiness in the epithelium in response to T_H2 stimuli and a novel role for IL-4 and IL-13 in increased HDAC expression. The inhibition of HDAC activity reconstitutes barrier integrity in bronchial epithelial cells from asthmatic patients.

METHODS

Study subjects

Bronchoscopy with epithelial cell brushings was performed in 27 subjects (9 control subjects and 18 asthmatic patients). Clinical characteristics of the study participants are presented in Table E1 in this article's Online Repository at www.jacionline.org. Samples were collected at the University Hospital, Jagiellonian University Medical College, Krakow, Poland, and the University Hospital Freiburg, Department of Pneumology, Freiburg, Germany. The diagnosis of asthma was mad by a pulmonologist or allergologist according to Global Initiative for Asthma guidelines. All asthmatic patients and control subjects were nonsmokers. The study was approved by the University Bioethics Committee (KBET/209/B/2011), and the study subjects provided written consent to participate.

Air-liquid interface cultures

Primary human bronchial epithelial cells (HBECs) from bronchial brushings of asthmatic patients and control subjects were grown in bronchial epithelial basal medium (Lonza, Basel, Switzerland) supplemented with the SingleQuot Kit placed in a 75-cm^2 T-flask in a humidified incubator at 37°C in 5% CO_2. HBECs obtained from control subjects and asthmatic patients in passages 2 or 3 were seeded in air-liquid interface (ALI) cultures onto 6.5-mm-diameter polyester membranes with a pore size of 0.4 μm (growth area, 0.33 cm^2; Costar; Corning, Corning, NY) at a density of 1.5×10^5 cells/well. Bronchial epithelial growth medium (BEGM) supplemented with the SingleQuot kit, except for retinoic acid and triiodothyronine (Lonza), was mixed 1:1 with Dulbecco modified Eagle medium (Gibco-BRL, Invitrogen, Carlsbad, Calif). Fresh all-trans retinoic acid (Sigma-Aldrich, Saint Louis, Mo) was supplemented at a concentration of 10 ng/mL, and medium was changed every other day. Cells were grown submerged for 5 days to complete confluence, apical medium was removed, and BEGM-DMEM at a ratio of 1:1 with all-trans retinoic acid was added basolaterally to differentiate these cells. Experiments were performed between 21 and 28 days of ALI culture. Six of 12 primary cell lines each for asthmatic patients and healthy control subjects were used for T_H2 cells and ALI coculture experiments, and the other 6 were used for stimulation with T_H2-derived cytokines.

Cytokine stimulation and cocultures

ALI cultures were stimulated with different cytokines to the basolateral compartment in the above-mentioned BEGM. IL-4 (Novartis, Basel, Switzerland), IL-5 (PeproTech, Rocky Hill, NJ), IL-13 (eBioscience, San Diego, Calif), IL-25, IL-33, and TSLP (R&D Systems, Minneapolis, Minn) were all used at concentrations of 10 and 50 ng/mL. For the blocking experiments, we used anti–IL-4 (2 μg/mL) and anti–IL-13 (3 μg/mL) antibodies (both from R&D Systems) at the optimum dose, according to the manufacturer's instructions. These doses were sufficient to neutralize the transepithelial electrical resistance (TER)–decreasing effect of exogenously added IL-4 and IL-13 in ALI cultures of control HBECs. T_H2 cells were *in vitro* differentiated from naive T cells into T_H2 cells, as described previously.[29,30] BEGM was mixed 1:1 with complete RPMI-1640 (Sigma-Aldrich) containing 10% FCS and antibiotics for cocultured HBECs and T_H2 cells. HBECs were cocultured with T_H2 cells at a concentration of 1×10^5 and 5×10^5 cells/well added to the basolateral compartment in the same total volume of 0.6 mL. T_H2 cells were stimulated with anti-CD28 (125 ng/mL), anti-CD3 (125 ng/mL), anti-CD2 (125 ng/mL), and IL-2 (100 U/mL). As a control, epithelial cells alone were cultured by using the same conditions.

Inhibition of HDACs

HBECs from control subjects and asthmatic patients cultured at the ALI were treated with the HDAC inhibitor JNJ-26481585 (Apex Bio, Delhi, India) diluted in BEGM-DMEM at different concentrations, including 5, 10, 50, and 100 nmol/L in the basolateral compartment.

Transepithelial electric resistance and paracellular flux measurement

TER of the HBECs grown in ALI cultures was measured daily by using the Millicell ERS Voltohmmeter (Millipore, Temecula, Calif), and results were presented as $\Omega \times cm^2$. Samples from each ALI culture donor were measured

in duplicates in multiple well systems. Paracellular flux across the epithelium was measured when fluorescein isothiocyanate (FITC)–labeled 4-kDa dextran (2 mg/mL, Sigma-Aldrich) was added apically. Two and 24 hours after addition, 2- and 24-hour paracellular flux experiments produced similar results. FITC fluorescence in basolateral medium was assessed with a fluorescence reader (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) at 480 nm.

mRNA isolation and RT-PCR

mRNA expression of bronchial epithelial cells of ALI cultures was analyzed, as described earlier.[12] TaqMan Low Density Array Micro Fluidic Card PCR (Applied Biosystems, Foster City, Calif) was performed, as previously described.[31] In the fluidic card PCR elongation factor 1α was used as the housekeeping reference gene. The use of elongation factor 1α and glyceraldehyde-3-phosphate dehydrogenase as a control did not change the results.

Relative quantification was done on a ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the $2^{-\Delta\Delta CT}$ formula, as described previously.[31]

Immunofluorescence staining of TJs

ALI cultures were fixed with 4% paraformaldehyde (Fluka/Sigma Aldrich, Buch, Switzerland) at 48 or 72 hours, as described in the Results section. ALI samples were blocked with goat serum in 1% BSA/PBS and stained for TJs: occludin (mouse anti-occludin antibody, Alexa Fluor 488, 100 μg/mL, Invitrogen); ZO-1 (rabbit, anti–ZO-1 unlabeled antibody, Invitrogen) detected by using goat anti-rabbit antibody Alexa Fluor 546 or Alexa Fluor 633 (Invitrogen); claudin-4 (mouse anti-claudin-4 antibody, Invitrogen) detected by using goat anti-mouse antibody Alexa Fluor 546; E-cadherin (rabbit anti–e-cadherin antibody, Alexa Fluor 555, Cell Signaling, Danvers, Mass); and vimentin (monoclonal mouse anti-vimentin antibody; Dako, Glostrup, Denmark) detected by using goat anti-mouse antibody Alexa Fluor 488 (Invitrogen). They were also stained for HDACs: HDAC1 (rabbit, polyclonal anti-HDAC1 antibody; Abcam, Cambridge, United Kingdom); HDAC2 (mouse monoclonal anti-HDAC2 antibody, Abcam); HDAC7 (rabbit polyclonal anti-HDAC7 antibody, Abcam); and HDAC9 (rabbit polyclonal anti-HDAC9 antibody, Abcam). Finally, they were stained for SIRTs: SIRT1 (mouse monoclonal anti-SIRT1 antibody, Abcam); SIRT6 (mouse monoclonal anti-SIRT6 antibody, Abcam) detected by using goat anti-mouse antibody Alexa Fluor 488 (Invitrogen); and SIRT7 (rabbit polyclonal anti-SIRT7 antibody, Abcam) detected by using goat anti-rabbit antibody Alexa Fluor 546 (Invitrogen). Samples were mounted in the mounting medium ProLong Gold with 4′-6-diamisino-2-phenylindole dihydrochloride (DAPI; Life Technologies, Grand Island, NY). Samples were analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland). IMARIS software (Bitplane, Zurich, Switzerland) was used for 3-dimensional visualization of TJs in HBECs and calculation of expression intensity.

HDAC activity assay

HBECs from control and asthmatic donors were cultured in a 75-cm^2 T-flask or a 6-well plate, and after 7 days, the cells were collected and processed according to the manufacturer's instructions for the EpiQuik Nuclear Extraction Kit I (EpiGentek, Farmingdale, NY) and Epigenase HDAC Activity Direct Assay Kit (colorimetric, EpiGentek). HDAC activity and the percentage of its inhibition was calculated, according to the manufacturer's instructions.

Western blotting

Western blotting was performed, as previously described.[32] Briefly, equal amounts of lysate proteins (20 μg) were resolved on a 4-20% Mini-PROTEAN TGX Gel (Bio-Rad Laboratories, Hercules, Calif) by using 10× Tris/glycine/SDS and deionized H_2O running buffer (Bio-Rad Laboratories) and then transblotted to the nitrocellulose membranes by using the Trans-Blot Turbo Blotting System (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat skimmed milk in PBS containing 0.1% Tween 20 for 1 hour and then incubated with the primary antibody overnight at 4°C. Primary antibodies were the same as in immunofluorescence staining. The membranes were subsequently washed and then incubated with a specific secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hour (anti-rabbit HRP and anti-mouse HRP; Jackson ImmunoResearch Laboratories, West Grove, Pa). The blots were developed with the SuperSignal West Femto Kit (Thermo Scientific, Waltham, Mass) and visualized on the Luminescent Image Analyzer LAS-1000 (Fujifilm, Tokyo, Japan).

Statistical analysis

Data were analyzed with GraphPad Prism software (Version 5; GraphPad Software, La Jolla, Calif). Differences between independent variables were evaluated by using the Mann-Whitney *U* test. For differences between paired values, the Wilcoxon rank sum test and 2-way ANOVA were used. The results were considered as significant at *P* values of less than .05, less than .01, less than .001, and less than .0001.

RESULTS

Decreased bronchial epithelial cell TJ integrity in asthmatic patients and the role of T_H2 cells

We first investigated whether there was a difference in the TJ barrier integrity of HBECs isolated from patients with asthma and control subjects. The bronchial epithelial cells from asthmatic patients showed a significantly lower TER compared with those of control subjects during the first 7 days of development of ALI cultures, as well as after long-term cultures (Fig 1, *A* and *B*). Because asthma is a predominantly T_H2 cytokine–dominated disease, we then decided to coculture T_H2 cells with HBECs to investigate the effect of the T_H2 cells. Control HBECs showed decreased TER after 48 and 72 hours in an ALI cocultured with T_H2 cells. In contrast, ALI cultures of epithelium from asthmatic patients had an initial low TER, which did not further decrease in response to T_H2 cells (Fig 1, *C*). Measurement of paracellular passage of FITC-labeled 4-kDa dextran confirmed the link between barrier integrity and TER results and was significantly increased by T_H2 cells in HBECs from control subjects (Fig 1, *D*). T_H2 cells cocultured with control HBECs showed separation and opening of the TJs between neighboring cells, which was illustrated in a video of immunofluorescence staining (see Videos E1 and E2 in this article's Online Repository at www.jacionline.org). HBECs from asthmatic patients already showed reduced expression of occludin and ZO-1 compared with control subjects and did not respond to T_H2 cells (Fig 1, *E*).

Next, we profiled the mRNA expression of the whole epithelial junction–related complex (TJ molecules, adaptor proteins, gap, and adherens junction) in ALI cultures of HBECs from asthmatic patients and control subjects without any stimulation (see Fig E1 in this article's Online Repository at www.jacionline.org). We found that levels of claudin-4 and adaptor proteins and gap and adherens junctions, such as plactoglobin, nectin-1, nectin-2, and connexin-26, were expressed at significantly low levels in HBECs from asthmatic patients compared with levels seen in control epithelium.

IL-4 and IL-13 decrease the TJ integrity of bronchial epithelial cells

During our investigations to identify the responsible factor of T_H2 cells that decreases barrier integrity, we demonstrated that IL-4 and IL-13, the 2 main T_H2 cytokines, induced a significant decrease in TER in HBECs from control subjects but not those

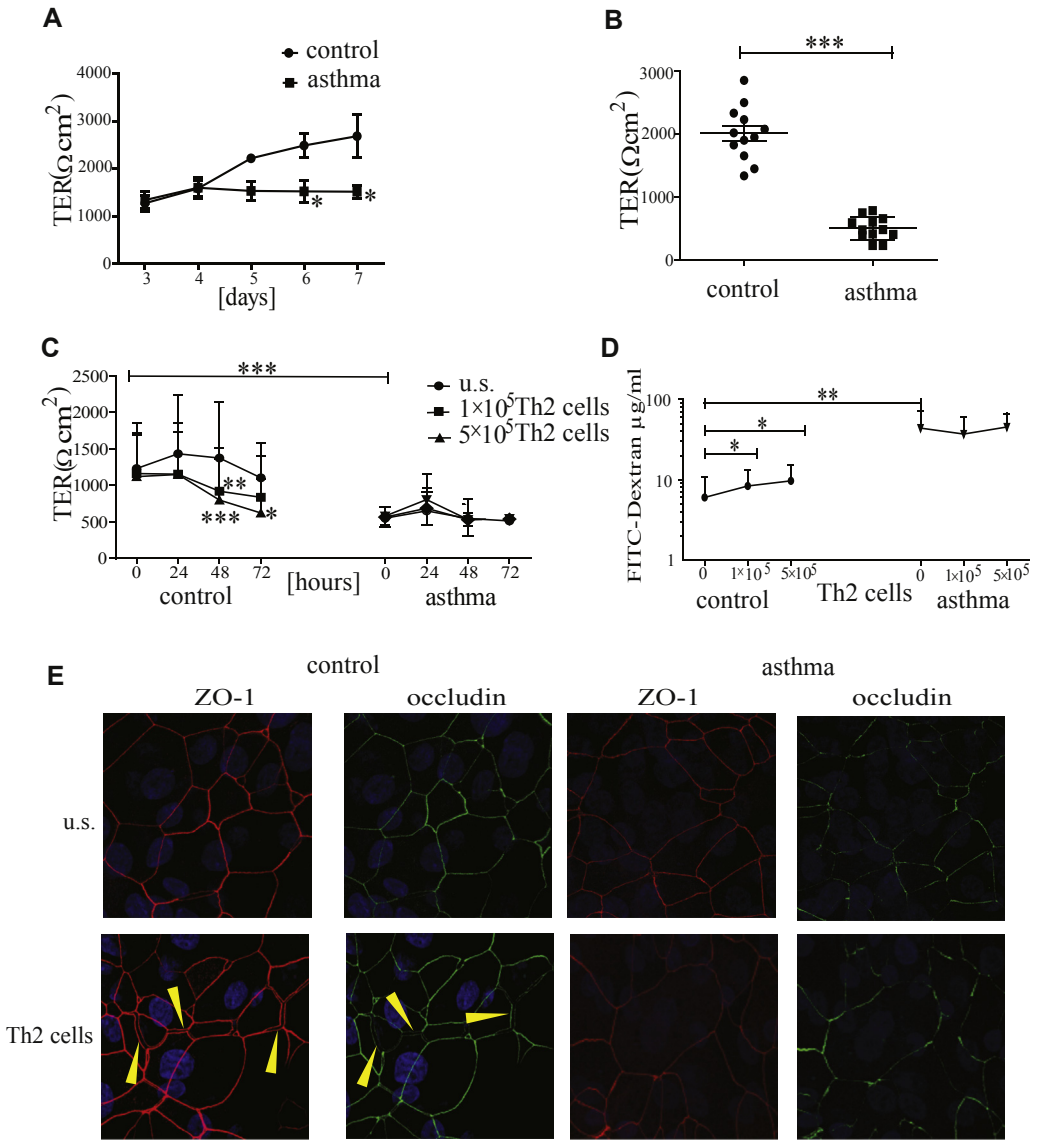

FIG 1. Barrier defect in ALI cultures of HBECs from asthmatic patients and their response to T_H2 cells. **A,** TER during development of ALI cultures from days 3 to 7 in HBECs from control subjects and asthmatic patients. **B,** TER from HBECs after differentiation on day 21. **C,** TER in response to T_H2 cells in ALI cultures of control subjects and asthmatic patients. **D,** Paracellular flux of 4-kDa FITC-dextran in response to T_H2 cells in ALI cultures from control subjects and asthmatic patients. **E,** Representative immunofluorescence staining of occludin and ZO-1 in ALI cocultured with T_H2 cells (5×10^5 cells, 48 hours) in HBECs from control subjects and ALI cultures of asthmatic patients. *Arrows* point out stratification of TJs. Data are presented as means ± SDs. *$P < .05$, **$P < .01$, and ***$P < .001$, Wilcoxon rank sum test. N = 6 per group in duplicates. *u.s.*, Unstimulated.

from asthmatic patients (Fig 2, *A*). Measurement of paracellular flux was significantly increased by IL-4 and IL-13 in parallel cultures of healthy control cells, confirming the TER results (Fig 2, *B*). The effects of IL-4 and IL-13 on TJs were also verified by using immunofluorescence staining, as presented also in a video, which demonstrated that ALI cultures displayed openings of the TJs between neighboring cells (see Video E3 in this article's Online Repository at www.jacionline.org). There was already a weak expression of occludin and ZO-1 in ALI cultures of HBECs of asthmatic origin without any further change by IL-4 and IL-13 levels (Fig 2, *C*). Removal of IL-4 and IL-13 from culture leads to recovery of TER in bronchial epithelial cells of control subjects (see Fig E2 in this article's Online Repository at www.jacionline.org). Blocking of IL-4 and IL-13 did not show any significant difference in TER measurement in bronchial epithelial cells from asthmatic patients (see Fig E3 in this article's Online Repository at www.jacionline.org). Other T_H2 milieu cytokines, such as IL-5, IL-25, IL-33, and TSLP, did not induce any change in TER in HBECs of control subjects and asthmatic patients (see Fig E4 in this article's Online Repository at www.jacionline.org).

HDACs and SIRTs are upregulated by IL-4 and IL-13

It was tempting to investigate whether the barrier defect in bronchial epithelial cells induced by T_H2 cells and cytokines in

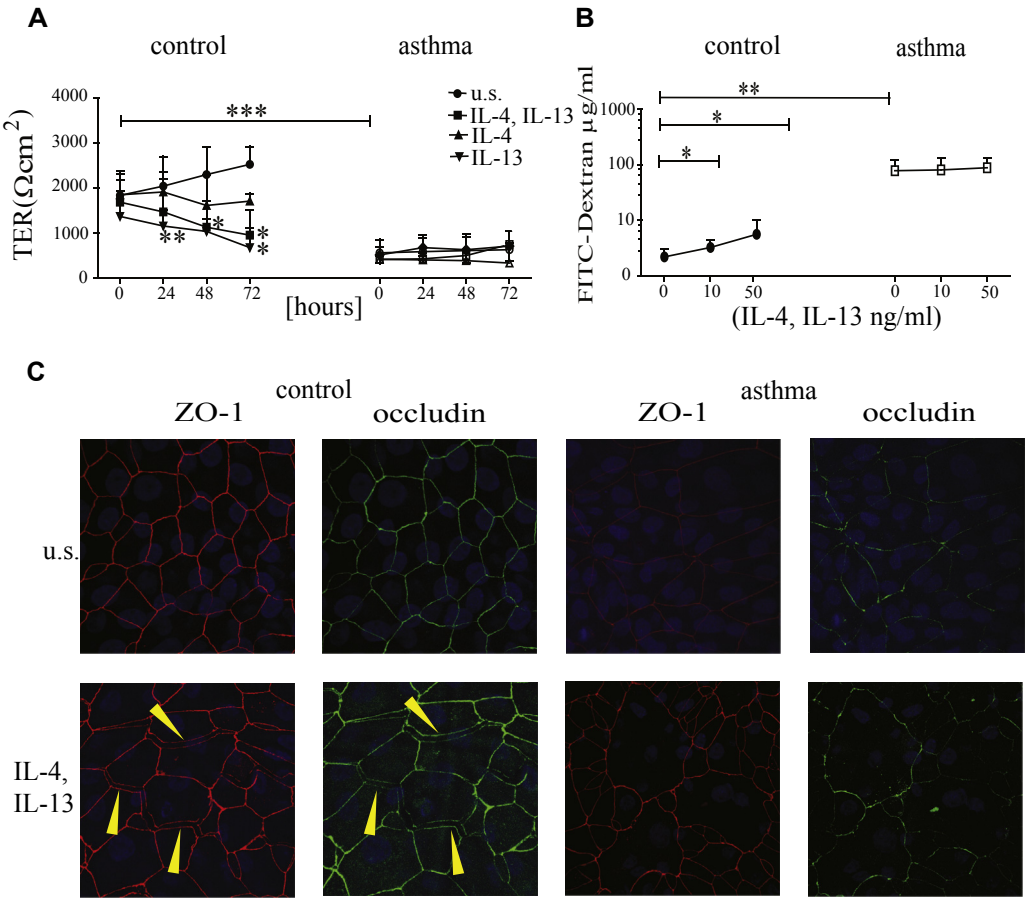

FIG 2. Decreased epithelial barrier by IL-4 and IL-13 in ALI cultures. **A,** TER from IL-4 and IL-13 (50 ng/mL)–stimulated HBECs from control subjects and asthmatic patients in ALI cultures. **B,** Paracellular flux of 4-kDa FITC-dextran after stimulation with IL-4 and IL-13 (50 ng/mL) in HBECs from control subjects and asthmatic patients. **C,** Representative immunofluorescence staining of occludin and ZO-1 in ALI cultures stimulated with IL-4 and IL-13 (50 ng/mL, 48 hours) in control and asthmatic donors. *Arrows* point out stratification of TJs. Data are presented as means ± SDs. **P* < .05, ***P* < .01, and ****P* < .001, Wilcoxon rank sum test. N = 6 per group in duplicates. *u.s.*, Unstimulated.

asthmatic patients is associated with epigenetic processes. Accordingly, HDAC expression in bronchial epithelial cells showed potent regulation patterns in response to IL-4 and IL-13. Most of the HDACs were significantly upregulated both in control subjects and asthmatic patients on IL-4 and IL-13, which was confirmed on mRNA (Fig 3, *A*) and protein (Fig 3, *B*) expression levels. After demonstration of their effects on HDACs, we focused more on regulation of HDAC expression and activity in the present study. Accordingly, we investigated the cellular localization of HDACs and SIRTs in ALI cultures and observed that HDAC1, HDAC2, SIRT1, SIRT6, and SIRT7 were localized in the nucleus and HDAC7 and HDAC9 in the cytoplasm of HBECs (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). As an important finding, higher mRNA expression of HDAC1 and HDAC9 was demonstrated in bronchial epithelial cells from asthmatic donors compared with those from control subjects (Fig 3, *A*, and see Fig E5, *B*). Western blotting of the ALI cultures showed a significant increase in HDAC1 and HDAC2 protein expression in ALI cultures from control subjects but not those from asthmatic patients, and HDAC7 expression was observed in epithelium from both control subjects and asthmatic patients either by IL-4 or IL-13.

Next, we investigated expression of the SIRT family of HDACs, which are considered regulatory genes of different biological functions, such as aging, metabolism, and stress resistance.[33] Similar to HDAC1 and HDAC9, higher expression of SIRT6 and SIRT7 was found in resting conditions of HBECs in asthmatic patients (see Figs E5 and E6 in this article's Online Repository at www.jacionline.org). SIRTs 1, 2, 4, 5, 6, and 7 were upregulated by IL-4 and IL-13. Additionally, expression of SIRT1 and SIRT5 was significantly higher on IL-13 stimulation in control subjects. These data demonstrate an essential role for IL-4 and IL-13 in the regulation of the HDAC and SIRT family of enzymes with epigenetic properties.

Reconstitution of asthmatic bronchial epithelial barrier integrity through HDAC inhibition

We analyzed further whether there is functional relevance of increased mRNA and protein expression of HDACs in asthmatic patients. As initial evidence suggesting this relationship, we observed a significantly increased HDAC activity in bronchial epithelial cells from asthmatic patients (Fig 4, *A*). This was followed by investigation of the effects of inhibition of HDAC

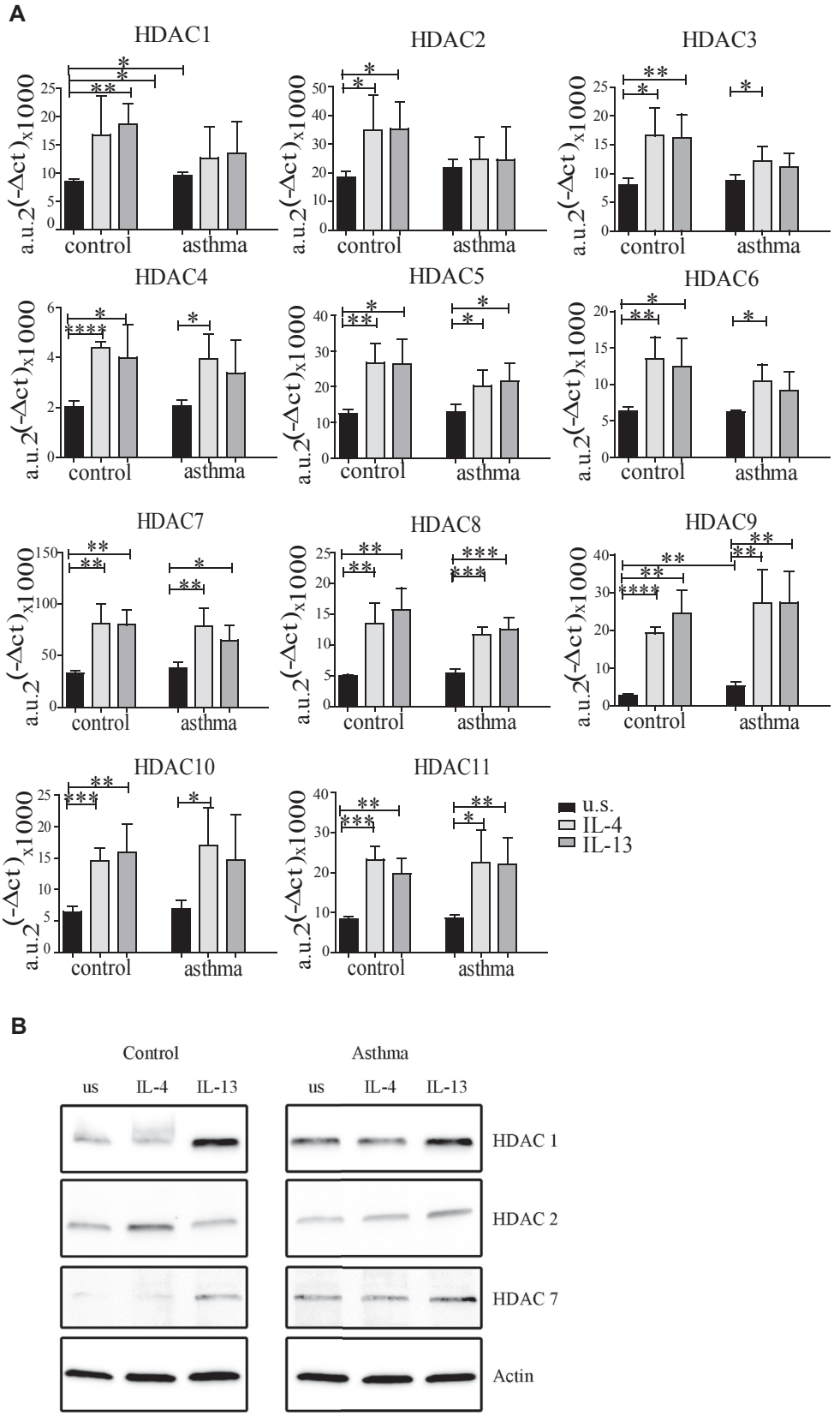

FIG 3. Increased expression of HDACs by IL-4 and IL-13. **A,** Expression of 11 members of the HDAC family of ALI cultures in HBECs from control subjects and asthmatic patients with IL-4 (50 ng/mL) and IL-13 (50 ng/mL) for 48 hours. Measurement was performed with the TaqMan Micro Fluidic Card system. Data represent means ± SDs. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, Student *t* test. N = 5 per group. *u.s.*, Unstimulated. **B,** Representative Western blots of HDACs 1, 2, and 7 in HBECs from control subjects and asthmatic patients after stimulation with IL-4 and IL-13 (n = 3 per group).

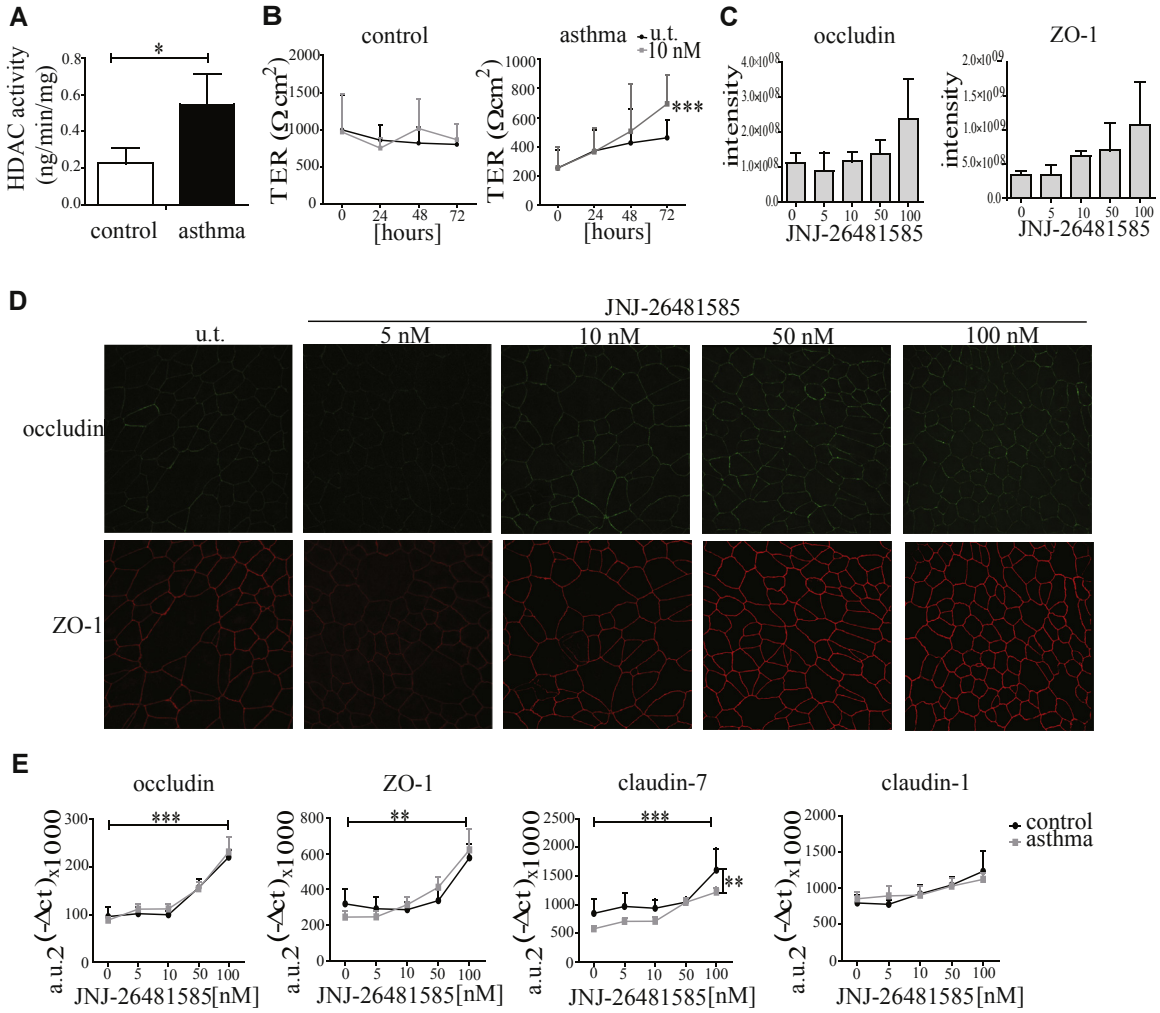

FIG 4. HDAC inhibition increased TJ expression in epithelial cells. **A,** HDAC activity in control and asthmatic donors (n = 4 per group). **B,** TER in ALI cultures of HBECs from control subjects and asthmatic patients treated with 10 nmol/L HDAC inhibitor (JNJ-26481585). **C,** Fluorescence intensity for occludin and ZO-1 measured from immunofluorescence staining of HBECs from asthmatic patients treated with indicated doses of JNJ-26481585 for 72 hours. **D,** Representative immunofluorescence staining of occludin and ZO-1 in HBECs from asthmatic patients after treatment with HDAC inhibitor (Fig 4, *C* and *D*, n = 3 per group). **E,** mRNA expression of occludin, ZO-1, claudin-7, and claudin-1 in ALI cultures of HBECs from control and asthmatic donors after HDAC inhibition for 72 hours. Data represent means ± SDs. **P* < .05, ***P* < .01, and ****P* < .001, 2-way ANOVA. N = 8 per group. *u.t.*, Untreated.

activity in asthmatic patients. After these experiments, ALI cultures were treated with a global HDAC inhibitor, JNJ-26481585, to demonstrate the role of HDAC regulation through the T_H2 response on barrier function. The HDAC inhibitory activity of this compound was confirmed in epithelial cells in comparison with another widely known HDAC inhibitor, trichostatin A (see Fig E7 in this article's Online Repository at www.jacionline.org). HDAC inhibition significantly increased TER in bronchial epithelial cells, particularly in ALI cultures from asthmatic donors (Fig 4, *B*), but we did not observe any difference between patients with aspirin-tolerant asthma (ATA) and those with aspirin-induced asthma (AIA; see Fig E8 in this article's Online Repository at www.jacionline.org). Reconstitution of TER in ALI cultures from asthmatic patients was associated with an increased expression of occludin and ZO-1 proteins with an intact and honeycomb-like structure in a dose-dependent manner by using immunofluorescence staining (Fig 4, *C* and *D*).

During recovery of epithelial barrier function through HDAC inhibition, expression of E-cadherin (see Fig E9, *A*, in this article's Online Repository at www.jacionline.org), which plays a role in cell binding and tissue formation, and the major intermediate filament-vimentin was also upregulated (see Fig E10, *A*, in this article's Online Repository at www.jacionline.org). In parallel, expression of the TJs occludin, claudin-1, claudin-4, claudin-7, and ZO-1 increased in a dose-dependent manner through HDAC inhibition (Fig 4, *E*, and see Fig E9, *B*). In addition, genes, such as epidermal growth factor receptor, amphiregulin, and Ki67, which are linked to the growth and regeneration of epithelial cells, showed increased expression after HDAC inhibition, suggesting increased activation and proliferation of epithelial cells (see Fig E10, *B*).

Then the question was posed whether HDAC inhibition induces stable barrier integrity in HBECs from asthmatic patients to protect against IL-4– and IL-13–induced damage. HDAC

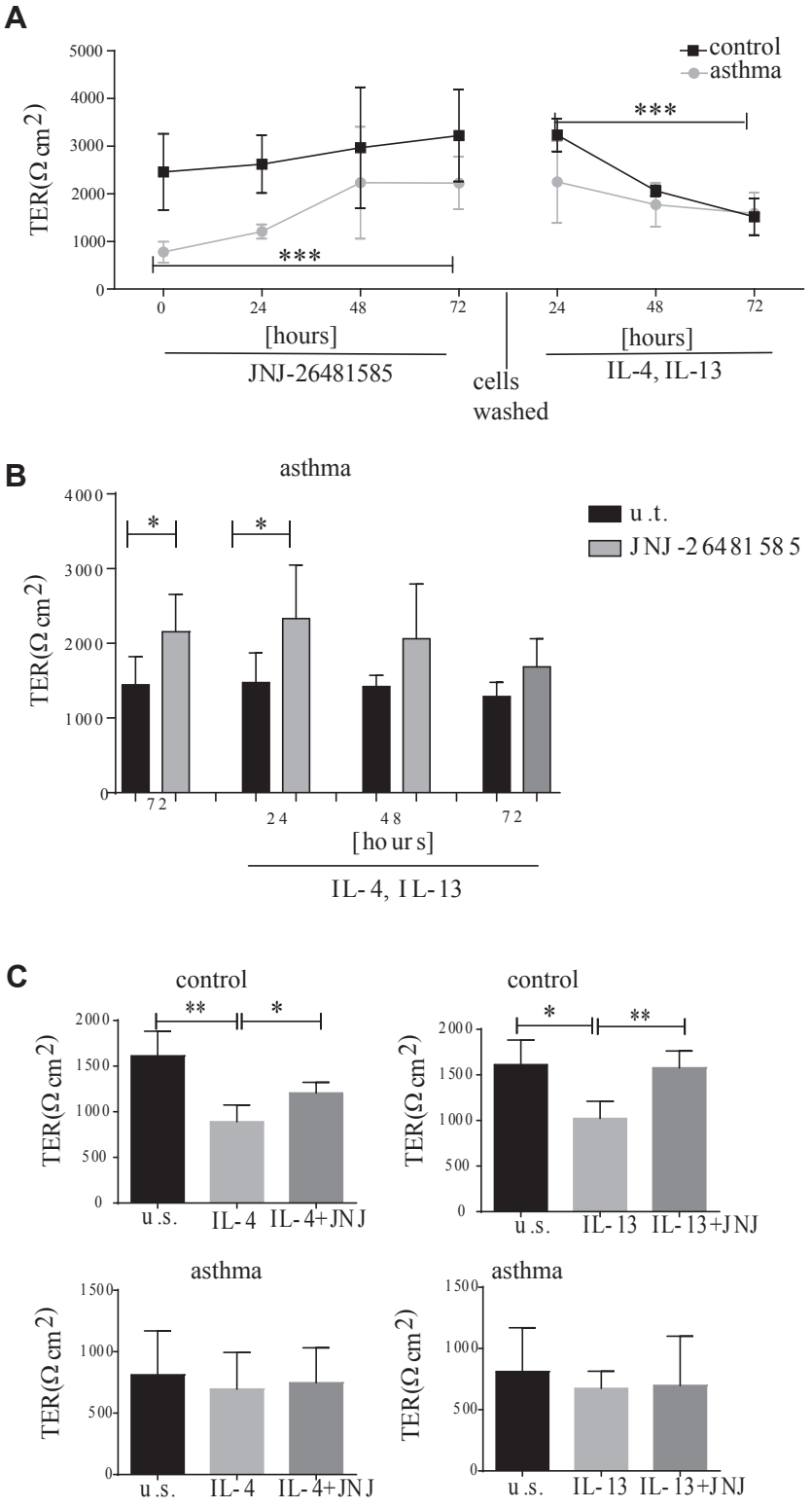

FIG 5. HDAC inhibition protects bronchial epithelial cell barrier damage caused by IL-4 and IL-13. **A,** TER HBECs from control subjects and asthmatic patients pretreated with 10 nmol/L HDAC inhibitor (JNJ-26481585). After 72 hours of treatment, HDAC inhibitor was washed away, and all epithelial cells were stimulated with IL-4 (50 ng/mL) and IL-13 (50 ng/mL) for 72 hours. TER measurement was performed. Data represent means ± SDs. ***$P < .001$, 2-way ANOVA test. N = 3 per group. **B,** TER measurement of bronchial epithelial cells from control and asthmatic donors stimulated with IL-4 (10 ng/mL) and IL-13 (10 ng/mL) and with the HDAC inhibitor JNJ-26481585 (100 nmol/L) for 72 hours (n = 3). Data are presented as means ± SDs. *$P < .05$ and **$P < .01$, unpaired *t* test. N = 3 per group. *u.s.*, Unstimulated. **C,** TER measurement of bronchial epithelial cells from control and asthmatic donors stimulated with IL-4 (10 ng/mL) and IL-13 (10 ng/mL) and together with the HDAC inhibitor JNJ-26481585 (100 nmol/L) for 72 hours (n = 3). Data are presented as means ± SDs. *$P < .05$ and **$P < .01$, unpaired *t* test. N = 3 per group. *u.s.*, Unstimulated.

inhibition from the start of cultures significantly increased TER in ALI cultures of HBECs from asthmatic patients (Fig 5). Normal HBECs did not show any further increase. Afterward, the HDAC inhibitor was washed away, and the ALI cultures were stimulated with IL-4 and IL-13. Stimulation with these T_H2 cytokines significantly decreased TER in control epithelial cells, but epithelial cells from asthmatic patients stayed at the same level and did not decrease to initial low TER levels. Interestingly, the initial effect of TER increase in epithelial cells of asthmatic patients after treatment with the HDAC inhibitor JNJ-26481585 remained significantly high in the first 24 hours. Although statistical significance disappeared after 48 and 72 hours, the IL-4 and IL-13 combination did not decrease TER fully to initial levels. These data suggest that increased TER through HDAC inhibition is partially sustained and cannot be rapidly and fully decreased by T_H2 cytokines. These data demonstrate that a protective effect against T_H2 cytokines is observed on the TJ barrier of asthmatic epithelium by HDAC inhibition.

TER was decreased in epithelial cells from control subjects on IL-4 or IL-13 stimulation, but this effect was reversed when cytokines were added together with the HDAC inhibitor JNJ-26481585. In contrast, IL-4 and IL-13 alone or in combination with HDAC inhibition did not influence TER measurement in epithelial cells form asthmatic patients.

DISCUSSION

Although it is a heterogeneous disease with several phenotypes and endotypes, the majority of asthma cases have been linked to type 2 inflammation,[34,35] with a strong evidence that T_H2 cells can initiate and maintain key pathophysiologic features through production of cytokines, such as IL-4, IL-5, and IL-13.[4,36] The barrier integrity of bronchial epithelial cells is a very important component of the defense mechanisms of the airways.[17] Reduced epithelial barrier function might increase the susceptibility to allergen sensitization and decrease the threshold of exposure to a certain allergen dose, which is required to drive local antigen-dependent inflammation.[37,38]

Impairment of this protective function of TJs was suggested in asthmatic patients as a potential causative factor in asthma development. These studies were reviewed by Georas and Rezaee.[7] In some of these studies, the authors did not see any differences in TER between asthmatic patients and nonasthmatic subjects.[39,40] The reasons for this discrepancy can be listed as a growth factor dependency and the use of cadaveric samples. However, there is substantial evidence consistent with our data showing reduced TER and decreased protein expression of TJs (particularly, ZO-1 was analyzed) in asthmatic patients.[16,41-43]

These findings were not only confined to asthmatic patients because irregular, patchy, and decreased expression of the TJ molecules, occludin, and ZO-1 was described in the epithelial layer of biopsy specimens of patients with chronic rhinosinusitis with nasal polyps and patients with atopic dermatitis.[11,31] Although T_H2 cells and cytokines induced a gap between 2 adjacent epithelial cells, epithelium from asthmatic patients did not typically show this gap. This difference can be due to chronic inflammation and exposure to multiple inflammatory factors in asthmatic patients and acute and short-term exposure to T_H2 cells and type 2 cytokines and remains to be further investigated. It can be hypothesized that the cellular composition between ALI cultures from asthmatic patients and control subjects might play role in TER differences. However, it has been studied in detail that ALI cultures from HBECs of healthy subjects and asthmatic patients do not show any difference in cellular composition, such as goblet cells and ciliated and nonciliated epithelial cells.[39-41] In addition, our experiments were performed in a group of patients, including those with AIA, those with ATA, and control subjects. Although AIA and ATA are suggested as different endotypes, both groups show sputum eosinophilia,[34] and our data demonstrate that there is no difference in barrier leakiness.

The present study investigates the function and expression of TJs in bronchial epithelial cells obtained from asthmatic patients and control subjects and their link to the T_H2 response and epigenetic regulation. Our data suggest a mechanism for defective barrier function in asthmatic patients, which was observed when normal epithelial cells were exposed to cytokines produced by effector T_H2 cells. Epithelium from asthmatic patients showed lower barrier integrity already at the start of ALI development, as observed with low TER measurements. The protein expression of TJs was significantly reduced and disassembled. In contrast, the mRNA expression of TJs did not show any difference between bronchial epithelial cells from asthmatic patients and control subjects, which is in agreement with the findings of Xiao et al.[41]

To elucidate the mechanisms underlying the impaired integrity of epithelium from asthmatic patients, we measured gene expression of 62 TJs and associated proteins performed in ALI cultures.[31] It appeared that the mRNA expression of TJs, adaptor proteins, and gap and adherens junctions, particularly claudin-4, plactoglobin, nectin-1, nectin-2, and connexin-26, were expressed at low levels in asthmatic patients. Claudins 1, 4 and 7 have been reported to be the most expressed junctional molecules in the lungs.[41]

Interestingly, the leaky barrier function of asthmatic epithelium with decreased TER and increased paracellular flux in human ALI cultures of bronchial epithelium was observed even after 2 to the 3 passages of the primary epithelial cells. These findings have led us to investigate to role of epigenetics in barrier function and the regulation of TJs in asthmatic patients. Here we demonstrate 2 essential findings of epigenetic regulation in asthmatic patients showing that there is an increased gene expression of HDAC1 and HDAC9 in asthmatic epithelium and that IL-4 and IL-13 are potent upregulators of HDAC mRNA and protein expression. All HDACs were upregulated by IL-4 and IL-13, except for HDAC1 and HDAC2 in asthmatic patients, suggesting that increased HDAC activity is a hallmark of T_H2-related asthma. Expression of HDACs has been reported in several gene array studies, and in one of them, HDAC1 and HDAC9 expression was upregulated in bronchial epithelial cells of asthmatic patients similar to our findings.[44-46] Similar to our findings in asthmatic patients, HDAC regulation has been reported with decreased expression of HDACs 2, 3, 5, and 8 in peripheral lung tissue in comparison with that seen in nonsmokers and in patients with different stages of chronic obstructive pulmonary disease (COPD).[47] In addition, HDAC6-mediated selective autophagy was observed in patients with COPD associated with cilia dysfunction.[48]

In agreement with the data for HDACs, SIRT expression showed regulation patterns in response to IL-4 and IL-13 in bronchial epithelial cells. These enzymes play an important role in the cellular response to certain types of stress and toxicity. We showed increased expression of SIRT6 and SIRT7 in asthmatic patients. The regulation of SIRTs 2, 4, 6, and 7 showed a similar response pattern to that of HDACs by IL-4 and IL-13 stimulation

in ALI cultures from asthmatic patients and control subjects. SIRT1 and SIRT5 were highly expressed after IL-13 stimulation in bronchial epithelial cells from control subjects and also together showed a stronger response to IL-13 compared with that seen in asthmatic patients. It was also shown that filaggrin is regulated by SIRT1, which is critical for skin barrier integrity.[49] The SIRT family is involved in gene silencing, and several studies have shown that expression of SIRT1, an anti-inflammatory and antiaging protein, is decreased in the lungs of patients with COPD. They also control the resistance to oxidative stress and DNA repair.[50] SIRT6 was associated with the redox state, inhibited cellular senescence and fibrosis, and was reported to have protective effects against COPD.[51]

To complement the mRNA expression, we also observed higher general activity of HDACs in bronchial epithelial cells from asthmatic donors. The treatment of bronchial epithelial cells of asthmatic patients with a new "second-generation" HDAC inhibitor significantly increased barrier integrity, which can be used for the prevention and treatment of asthma by correcting the leaky epithelial barrier. This HDAC inhibitor, JNJ-26481585, shows high potency for HDAC1; moderate potency for HDACs 2, 4, 10, and 11; and low potency to HDACs 6 and 7. It has greater than 30-fold selectivity against HDACs 3, 5, 8, and 9.[52,53] On HDAC inhibition, increased mRNA and protein expression of the junctional molecules occludin, ZO-1, claudin-4, claudin-7, and E-cadherin, as well as improved TJ staining of epithelial cells, which formed honeycomb-like structures, was observed in bronchial epithelial cells from both asthmatic patients and control subjects. Together, these data suggest that chronic type 2 cytokine exposure and increased HDAC activity might be one of the key reasons for decreased barrier integrity in asthmatic patients.

HDAC inhibition was previously shown to lead to greater expression of TJs, such as ZO-1, ZO-2, and cingulin, in fibroblasts and Hela cell lines.[54] It was also shown recently that CpG-DNA can enhance the barrier function of bronchial epithelial cells in asthmatic patients.[55]

Taken together, the present study showed decreased bronchial epithelial barrier integrity in HBECs from asthmatic patients and epigenetic regulation of the epithelial HDACs and SIRTs by IL-4 and IL-13, 2 of the most potent T_H2 cytokines. The increased expression of particular HDACs and SIRTs in asthmatic epithelium, the correction of leakiness by inhibition of HDAC activity, and the protective effect of HDAC inhibition on barrier integrity opens a new window for the prevention and treatment of asthma.

Clinical implications: Decreased TJ integrity and altered bronchial epithelial barrier by T_H2 cells and their cytokines in asthmatic patients might play a role in increased exposure to environmental factors, such as allergens, to the inner tissues and contribute asthma pathogenesis.

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