Blocking histone deacetylase activity as a novel target for epithelial barrier defects in patients with allergic rhinitis

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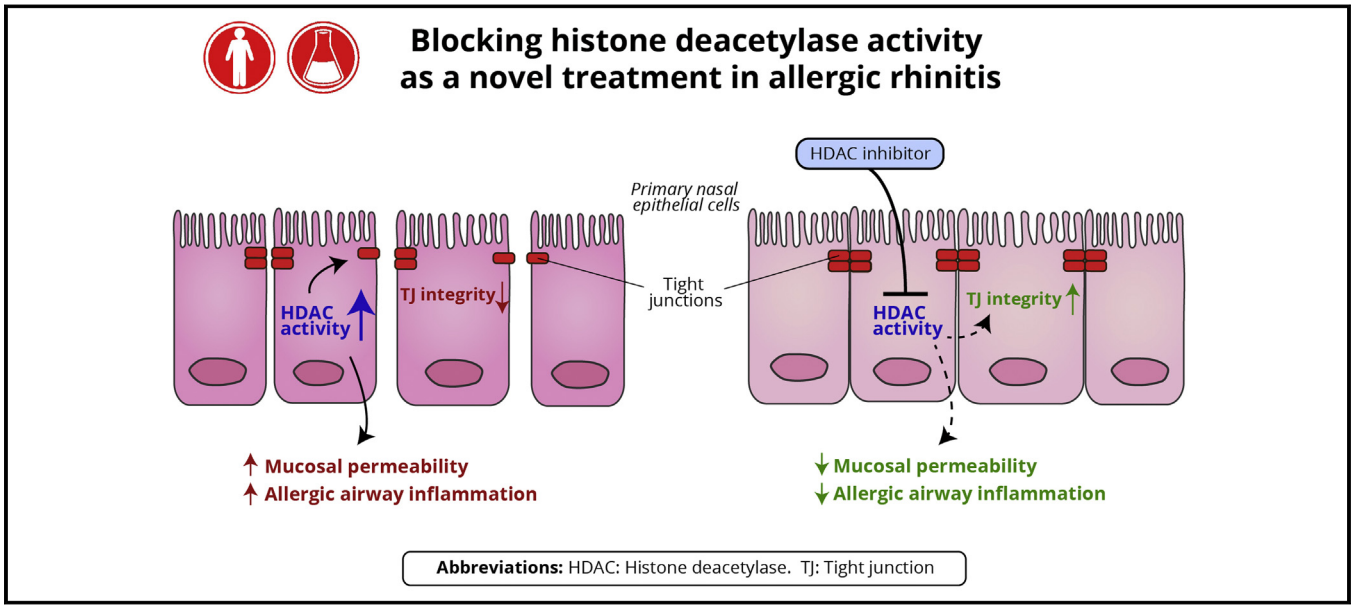
GRAPHICAL ABSTRACT



Background: A defective epithelial barrier is found in patients with allergic rhinitis (AR) and asthma; however, the underlying mechanisms remain poorly understood. Histone deacetylase (HDAC) activity has been identified as a crucial driver of allergic inflammation and tight junction dysfunction.
Objective: We investigated whether HDAC activity has been altered in patients with AR and in a mouse model of house dust mite (HDM)–induced allergic asthma and whether it contributed to epithelial barrier dysfunction.
Methods: Primary nasal epithelial cells of control subjects and patients with AR were cultured at the air-liquid interface to study transepithelial electrical resistance and paracellular flux of fluorescein isothiocyanate–dextran (4 kDa) together with mRNA expression and immunofluorescence staining of tight junctions. Air-liquid interface cultures were stimulated with different concentrations of JNJ-26481585, a broad-spectrum HDAC inhibitor. *In vivo* the effect of JNJ-26481585 on mucosal permeability and tight junction function was evaluated in a mouse model of HDM-induced allergic airway inflammation.
Results: General HDAC activity was greater in nasal epithelial cells of patients with AR and correlated inversely with epithelial integrity. Treatment of nasal epithelial cells with JNJ-26481585 restored epithelial integrity by promoting tight junction

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expression and protein reorganization. HDM-sensitized mice were treated with JNJ-26481585 to demonstrate the *in vivo* role of HDACs. Treated mice did not have allergic airway inflammation and had no bronchial hyperreactivity. Moreover, JNJ-26481585 treatment restored nasal mucosal function by promoting tight junction expression.

Conclusion: Our findings identify increased HDAC activity as a potential tissue-injury mechanism responsible for dysregulated epithelial cell repair, leading to defective epithelial barriers in AR. Blocking HDAC activity is a promising novel target for therapeutic intervention in patients with airway diseases. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: *Allergic rhinitis, tight junctions, primary nasal epithelial cells, epigenetic changes, histone deacetylase*

Abbreviations used
ALI: Air-liquid interface
AR: Allergic rhinitis
BAL: Bronchoalveolar lavage
DAPI: 4′-6-Diamidino-2-phenylindole dihydrochloride
HDAC: Histone deacetylase
HDM: House dust mite
FP: Fluticasone propionate
TER: Transepithelial electrical resistance
TSLP: Thymic stromal lymphopoietin
ZO-1: Zonula occludens 1

The nasal mucosa is the first line of defense, forming a physical barrier against external particles, such as allergens, pathogens, and irritants. This epithelial barrier is predominantly formed by tight junctions, the most apically located intercellular junctional complexes between adjacent epithelial cells.[1,2] Disturbed tight junction expression and/or function hampers the formation of a physical barrier, rendering the submucosal region more accessible to foreign molecules. Several studies reported impairment of the epithelial barrier in patients with a variety of chronic inflammatory diseases.

De Benedetto et al[3] observed impairment in bioelectric barrier function in patients with atopic dermatitis caused by reduced expression of claudin-1 and claudin-23. In patients with asthma and chronic rhinosinusitis, occludin and zonula occludens 1 (ZO-1) formation and transepithelial electrical resistance (TER) of primary airway epithelial cell cultures were significantly decreased compared with those in healthy subjects.[4-6] Our research group recently demonstrated decreased expression of occludin and ZO-1 in patients with allergic rhinitis (AR), which resulted in decreased TER *in vitro* on primary nasal epithelial cells.[7] In addition, we found that histamine, T_H2 cells,[8] and type 2 innate lymphoid cells[9] are key factors in initiating and maintaining a leaky barrier in patients with allergic airway diseases, although in-depth insight into the underlying mechanisms was lacking.

Epigenetic regulation has been postulated to mediate the effect of environmental and inflammatory triggers on airway diseases.[10-12] Epigenetic modifications comprise multiple biochemical mechanisms, such as chromatin modifications on histones and DNA methylation among others,[13] and are crucial for the development and differentiation of various cell types.[14,15] Histone acetylation induced by histone acetyl transferases is associated with gene transcription, whereas histone deacetylases (HDACs) remove acetyl groups from hyperacetylated histones, suppressing gene transcription. Eighteen HDACs have been identified and are classified in 4 groups.[11] It has been shown that diesel exhaust particles induce pulmonary inflammation and asthma exacerbations *in vivo* by degrading HDAC1.[16] Furthermore, exposure of mice to house dust mite (HDM) resulted in an aberrant methylation status of genes involved in the development of allergic asthma.[17] Interestingly, a single exposure of bronchial epithelial cells to IL-13 altered global DNA methylation patterns and resulted in long-lasting epigenetic changes near asthma-associated genes.[18] Building further on this, our group reported that IL-4 and IL-13 increased HDAC activity in bronchial epithelial cells from patients with allergic asthma.[5]

Because epigenetic modifications, which contribute to disease development, are neither permanent nor transient, identifying disease-specific epigenetic alterations might hold promise for novel therapeutic interventions.[19,20] Several studies have demonstrated that a nonspecific HDAC inhibitor, JNJ-26481585, has potent antitumor activity in solid and hematologic malignancies in human subjects,[21,22] with acceptable side effects.[23] Accordingly, HDAC inhibitors are being evaluated in clinical trials as monotherapy or add-on therapy for cancer treatment.[24] In addition, their use were also evaluated in patients with various lung diseases.[19] Treatment of bronchial epithelial cells with JNJ-26481585 reconstituted defective epithelial barrier function and stimulated the expression of tight junctions.[5] Whether altered HDAC activity drives epithelial defects in patients with AR has not been elucidated thus far. AR is as an inflammatory disorder of the nasal mucosa, affecting 30% of the Western population, and causes symptoms, such as nasal obstruction, rhinorrhea, sneezing, and itchy nose.[25,26]

The objective of this study was to investigate the role of HDACs within nasal epithelial cells from patients with AR and control subjects to determine whether alterations in HDAC activity underlie epithelial barrier defects. Here we demonstrate that endogenous HDAC activity in nasal epithelial cells from patients with AR is increased and contributes to the development of an impaired barrier. Inhibition of HDAC activity reconstitutes tight junction expression and function and attenuates allergic airway inflammation in a mouse model, identifying HDAC activity as a potential novel target for therapeutic intervention.

METHODS

Isolation of primary nasal epithelial cells

Inferior turbinates from nonallergic, nonsmoking healthy control subjects and nonsmoking, therapy-negative patients with AR were used for isolation of primary nasal epithelial cells. Patient data can be found in the Methods section in this article's Online Repository at www.jacionline.org and were approved by the local ethics committee of UZ Leuven. Highly purified nasal epithelial cell populations were obtained, as previously reported.[8] More information is provided in the Methods section in this article's Online Repository.

Air-liquid interface cultures

Isolated primary nasal epithelial cells from healthy control subjects and patients with AR were grown in bronchial epithelial basal medium (Lonza, Basel, Switzerland) supplemented with the SingleQuot Kit (Lonza) and placed

in a T75 culture flask at 37°C. Once cells reached 75% to 80% confluency, cells were detached and seeded on polyester transwell inserts (Costar, Corning, NY) at a density of 110,000 cells per well. Cells were grown submerged for 5 to 7 days until a confluent monolayer was formed. Afterward, cells were placed in the air-liquid interface (ALI) for 21 days to differentiate the cells. Nasal epithelial cells were cultured in bronchial epithelial growth medium supplemented with the SingleQuot kit, except for retinoic acid and triiodothyronine, which were mixed at a ratio of 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.

TER and permeability measurements

TER of ALI cultures was measured by using the Millicell ERS Voltohm meter (Millipore, Temecula, Calif), and results were presented as $\Omega \times$ square centimeter values. Paracellular flux across ALI cultures was measured by applying fluorescein isothiocyanate–labeled dextran (4 kDa) (FD4, Sigma-Aldrich) (2 mg/mL) to the apical compartment. Permeability was determined at 24, 48, and 72 hours in basolateral medium with a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany).

In vitro stimulation experiments

ALI cultures were treated with different concentrations of the HDAC inhibitor JNJ-26481585 (Apex Bio, Delhi, India) diluted in culture medium. All experiments were performed in triplicates, and mean values of TER are given.

mRNA isolation and RT-PCR

The methodology of mRNA isolation and quantitative RT-PCR has been described elsewhere.[5] Detailed information can be found in the Methods section in this article's Online Repository.

Mouse model of HDM-induced allergic airway inflammation

Experimental procedures were approved by the Ethical Committee for Animal Research at the KU Leuven (P103/2013). This model induces eosinophilic lung and nose inflammation caused by a type 2 immune response to HDM.[7,27] Male C57Bl/6 mice (Envigo, Horst, The Netherlands) were endonasally sensitized with 50 μL of HDM extract (1 μg; Greer Laboratories, Lenoir, NC) or exposed to 50 μL of saline at day 1. From days 8 to 12, mice were endonasally challenged with 50 μL of HDM extract (10 μg/d) or saline. One hour before each challenge, mice were treated endonasally with either fluticasone propionate (FP; 6 μg/d; Sigma-Aldrich), JNJ-26481585 (6.25 μg/d), or sham treatment. Twenty-four hours after the last challenge, 20 μL of FD4 (1 mg) was applied in the nose to evaluate mucosal permeability. One hour after FD4 administration, mice were killed with an intraperitoneal injection of Nembutal (Ceva, Brussels, Belgium). The nasal mucosa was harvested for RNA isolation, immunofluorescence, and determination of HDAC activity. Bronchoalveolar lavage (BAL) was performed with 3×1 mL of saline. Lungs were snap-frozen in liquid nitrogen and kept at -80°C until further processing. Lung were homogenized for cytokine determination, as described previously.[28] Serum was collected to determine FD4 levels with a fluorescence reader. Lung function was performed 72 hours after the last challenge by using an invasive measurement of dynamic resistance (flexiVent 7; SCIREQ, Montreal, Quebec, Canada), as previously described.[29,30] Mice were anaesthetized by means of intraperitoneal injection of pentobarbital diluted in saline. Airway resistance was recorded by using the quick-prime 3 perturbation (5 repeats with 15 seconds between each perturbation; values with a coefficient of determination > 0.9 are included in the analysis) in response to increasing concentrations of methacholine (Sigma-Aldrich) solution (0, 1.25, 2.5, 5, 10, 20, and 40 mg/mL). Experiments were performed with 5 mice per group and repeated 2 times.

Immunofluorescence staining of tight junctions

ALI cultures were fixed with 4% paraformaldehyde (Fluka/Sigma-Aldrich, Buch, Switzerland) at 72 hours. Cultures were blocked with goat serum in 1% BSA/PBS and stained for occludin (mouse anti-occludin antibody, Alexa Fluor 488; Invitrogen, Carlsbad, Calif) and ZO-1 (rabbit; anti–ZO-1–unlabeled antibody; Invitrogen), as detected by using goat anti-rabbit antibody Alexa Fluor 546 (Invitrogen). Samples were mounted in the mounting medium ProLong Gold with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; Life Technologies, Grand Island, NY).

Tight junction staining in murine nasal mucosa was performed by means of deparaffinization of 5-μm paraffin-embedded sections. After antigen retrieval, mucosal sections were incubated overnight at 4°C with antibodies to occludin (rabbit, anti–occludin–unlabeled antibody), ZO-1 (rabbit, anti–ZO-1–unlabeled antibody), and claudin-7 (rabbit, anti–claudin-7, unlabeled antibody). The next day, mucosal sections were washed and incubated with anti-rabbit antibody Alexa Fluor 488 for 1 hour at room temperature in the dark. Mucosal sections were mounted in the mounting medium ProLong Gold with DAPI.

Samples were analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland). Microscope software was used for 3-dimensional visualization of tight junctions and calculation of expression intensity. The magnification used was ×50.

HDAC activity assay

Nasal epithelial cells from healthy control subjects and patients with AR were cultured on transwell inserts in the ALI. After 21 days, 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 was calculated according to the manufacturer's instructions. Murine nasal mucosa was isolated at the end and processed for HDAC activity, as described in the manufacturer's protocol.

Western blotting

Detailed information for Western blotting can be found in the Methods section in this article's Online Repository.

ELISAs

Levels of murine TNF-α, IL-4, IL-5, and IL-13 in BAL fluid were quantified by using an in-house protocol, as described previously.[8] IL-1β and IL-6 levels were measured in BAL fluid according to the manufacturer's protocol (IL-6: reference 88-7064-22; IL-1β: reference 88-8014-22; Thermo Fisher, Waltham, Mass). In addition, levels of IL-25 (88-7002-22; Thermo Fisher), IL-33 (88-7333-22; Thermo Fisher), and thymic stromal lymphopoietin (TSLP; 88-7490-22; Thermo Fisher) were measured in lung homogenates, according to the manufacturer's recommendations.

Statistical analysis

Data were analyzed with GraphPad Prism software (version 7; GraphPad Software, La Jolla, Calif). Differences between 2 groups were evaluated by using a Student *t* test or the Mann-Whitney *U* test, depending on normality. For differences between multiple groups, 1-way ANOVA or a Kruskal-Wallis test with *post hoc* analysis was used. Two-way ANOVA was used to evaluate the effect of JNJ-26481585 in function of time. The results were considered significant at a P value of less than .05.

RESULTS

Endogenous HDAC activity is increased in patients with AR

Primary nasal epithelial cells were isolated from 9 healthy control subjects and 9 patients with AR, and cells were cultured at the ALI for 21 days to assess the specific role of HDACs in

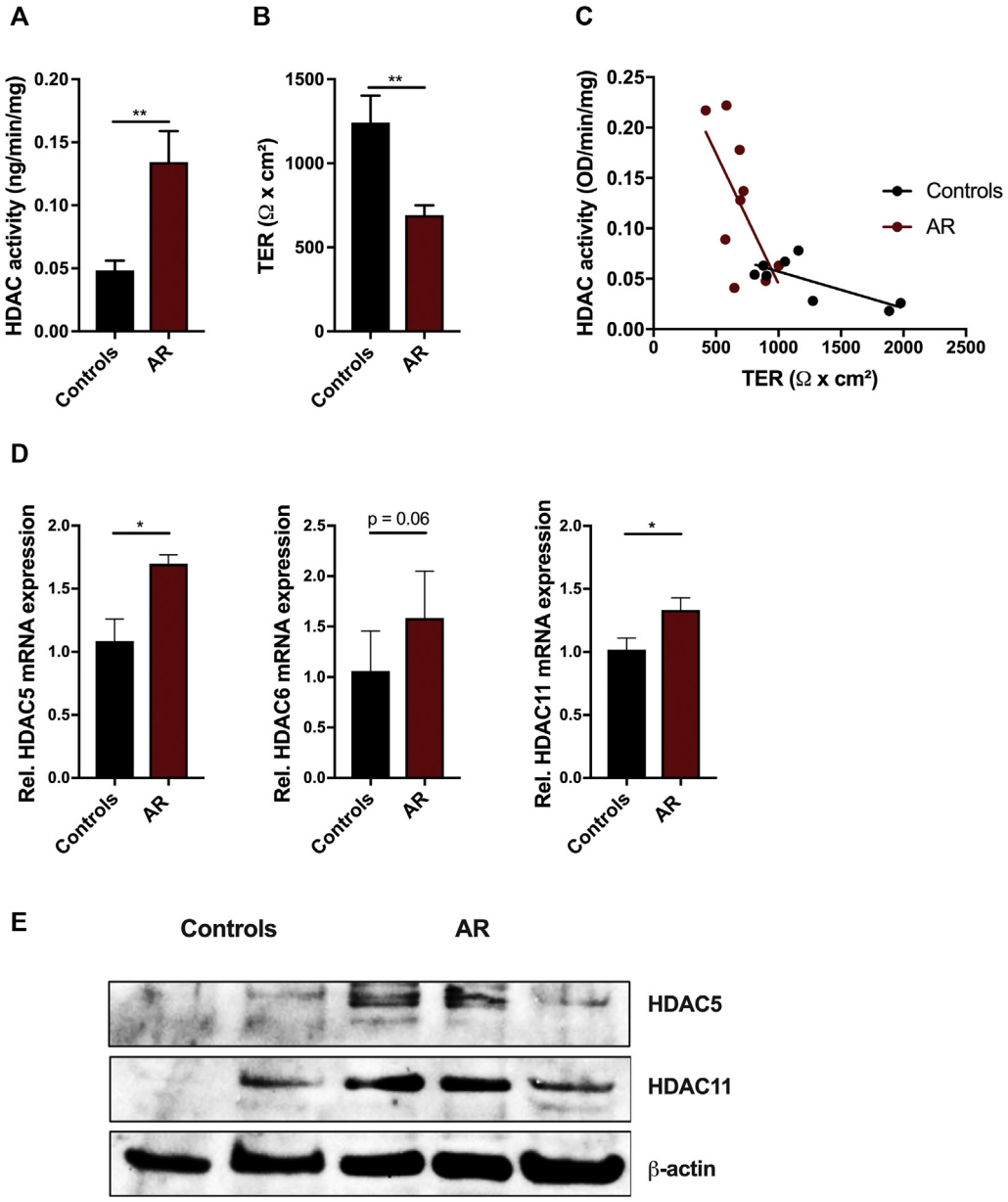


FIG 1. HDAC activity in primary nasal epithelial cells from patients with AR and healthy control subjects. **A,** Endogenous HDAC activity in primary nasal epithelial cells from patients with AR (n = 9) compared with healthy control subjects (n = 8). **B,** TER measurement of ALI cultures at day 21 of nasal epithelial cells from patients (n = 9) compared with healthy control subjects (n = 8). **C,** Correlation between HDAC activity and TER in ALI cultures of patients ($r = -0.65$, $P < .05$) and healthy control subjects ($r = -0.75$, $P < .05$). **D,** mRNA expression of HDAC5, HDAC6, and HDAC11 in ALI cultures of patients and control subjects was measured and normalized to the housekeeping gene elongation factor-2. **E,** Representative Western blot for HDAC5 and HDAC11 in ALI cultures of 2 control subjects and 3 patients. β-Actin was used as a loading control. *Error bars* are shown as means ± SEMs. Significance was calculated by using the unpaired Student *t* test. *$P < .05$ and **$P < .01$.

patients with AR and its relation to epithelial barrier dysfunction.[5,7,31] Patients' characteristics are depicted in Table E1 in this article's Online Repository at www.jacionline.org. Endogenous HDAC activity was significantly greater in ALI cultures of patients compared with control subjects (Fig 1, *A*). In line with previous publications,[7,32] we observed that TER of ALI cultures of patients was significantly decreased compared with that in healthy control subjects (Fig 1, *B*). Individual patient data demonstrated that endogenous HDAC activity negatively correlated with TER of both patients and healthy control subjects (Fig 1, *C*).

Next, the mRNA expression of the different HDAC molecules was evaluated in nasal epithelial cells.[33] We found that the mRNA expression of HDAC5 and HDAC11 was increased in nasal epithelial cells of patients compared with that in healthy control subjects, whereas the mRNA expression of other HDACs was

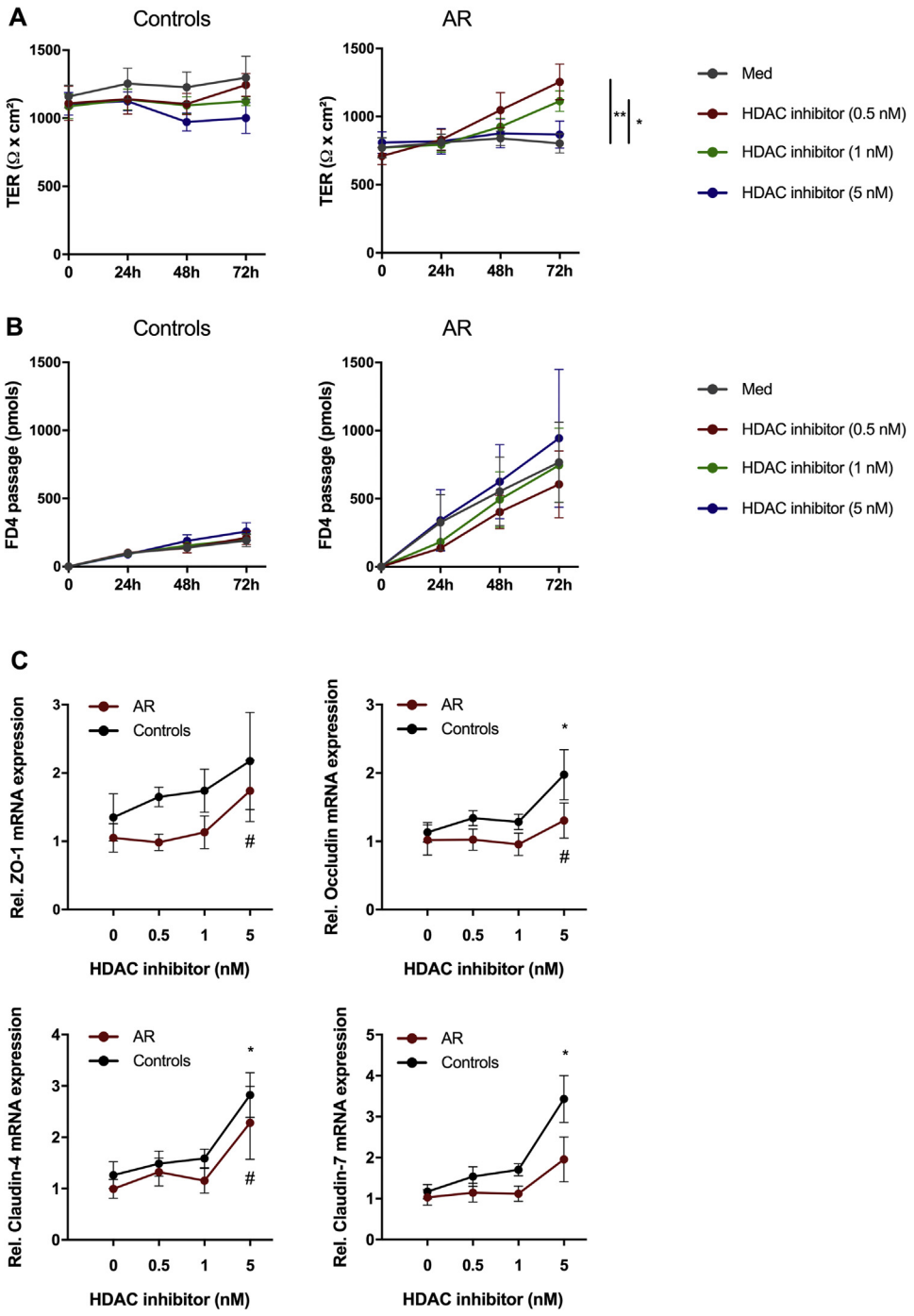
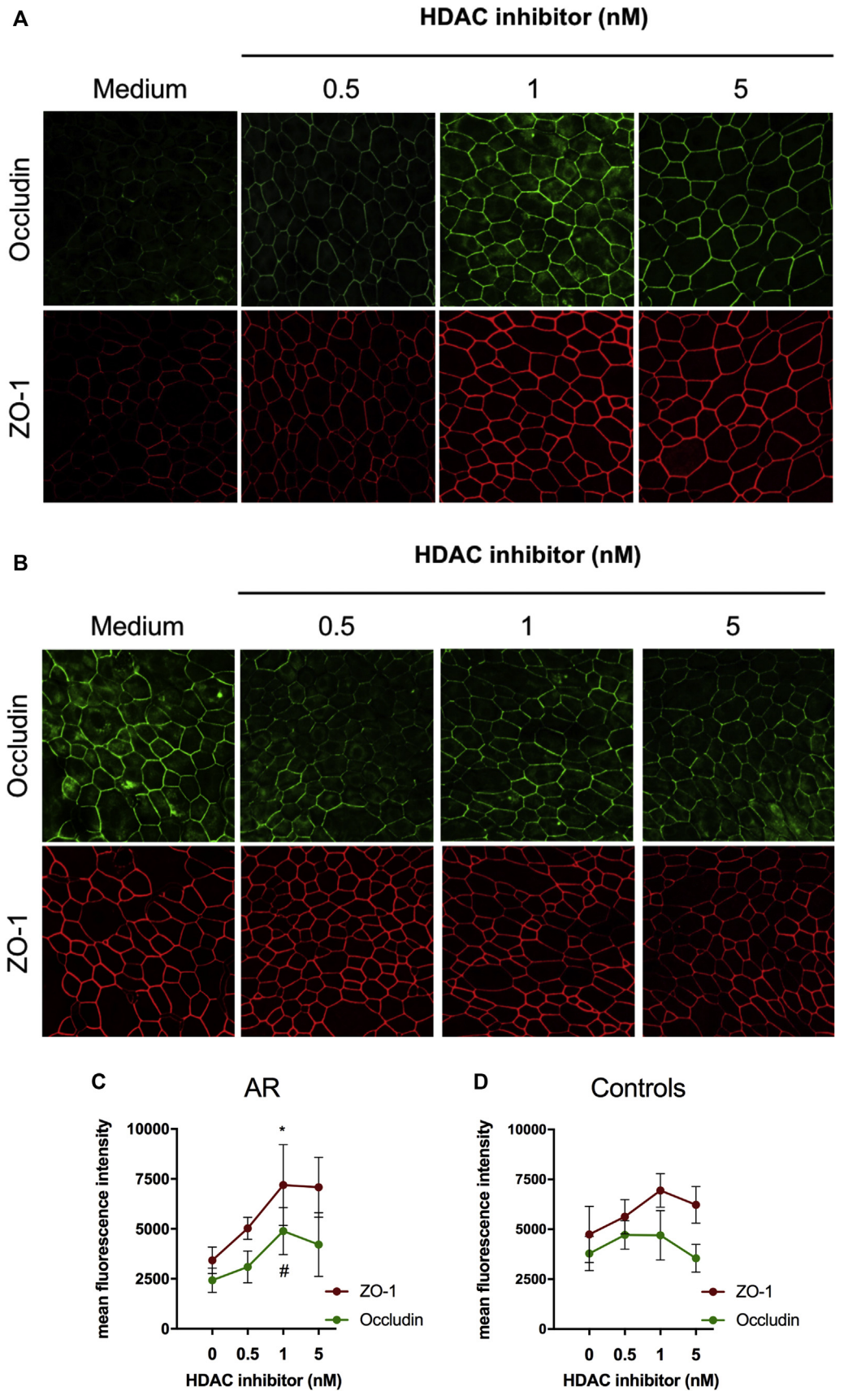


FIG 2. Effect of HDAC inhibition on epithelial barrier integrity in patients with AR *in vitro*. **A,** Effect of different concentrations of HDAC inhibitor (JNJ-26481585) on TER at different time points in ALI cultures of healthy control subjects and patients. Data are pooled from 5 healthy control subjects and 7 patients with AR. TER was measured in triplicates for the different concentrations for each donor. **B,** Effects of different concentrations of JNJ-26481585 on FD4 permeability in ALI cultures of healthy control subjects and patients. Results are pooled data from 4 healthy control subjects and 4 patients with AR. **C,** mRNA expression of different tight junction molecules in relation to JNJ-26481585 concentration in ALI cultures of nasal epithelial cells from healthy control subjects (n = 5) and patients with AR (n = 7). Expression is normalized to the housekeeping gene elongation factor-2. *Error bars* are shown as means ± SEMs. Significance was calculated with 2-way ANOVA with *post hoc* analysis for Fig 2, *A* and *B*, and with the Student *t* test for Fig 2, *C*. **P* < .05 and ***P* < .01.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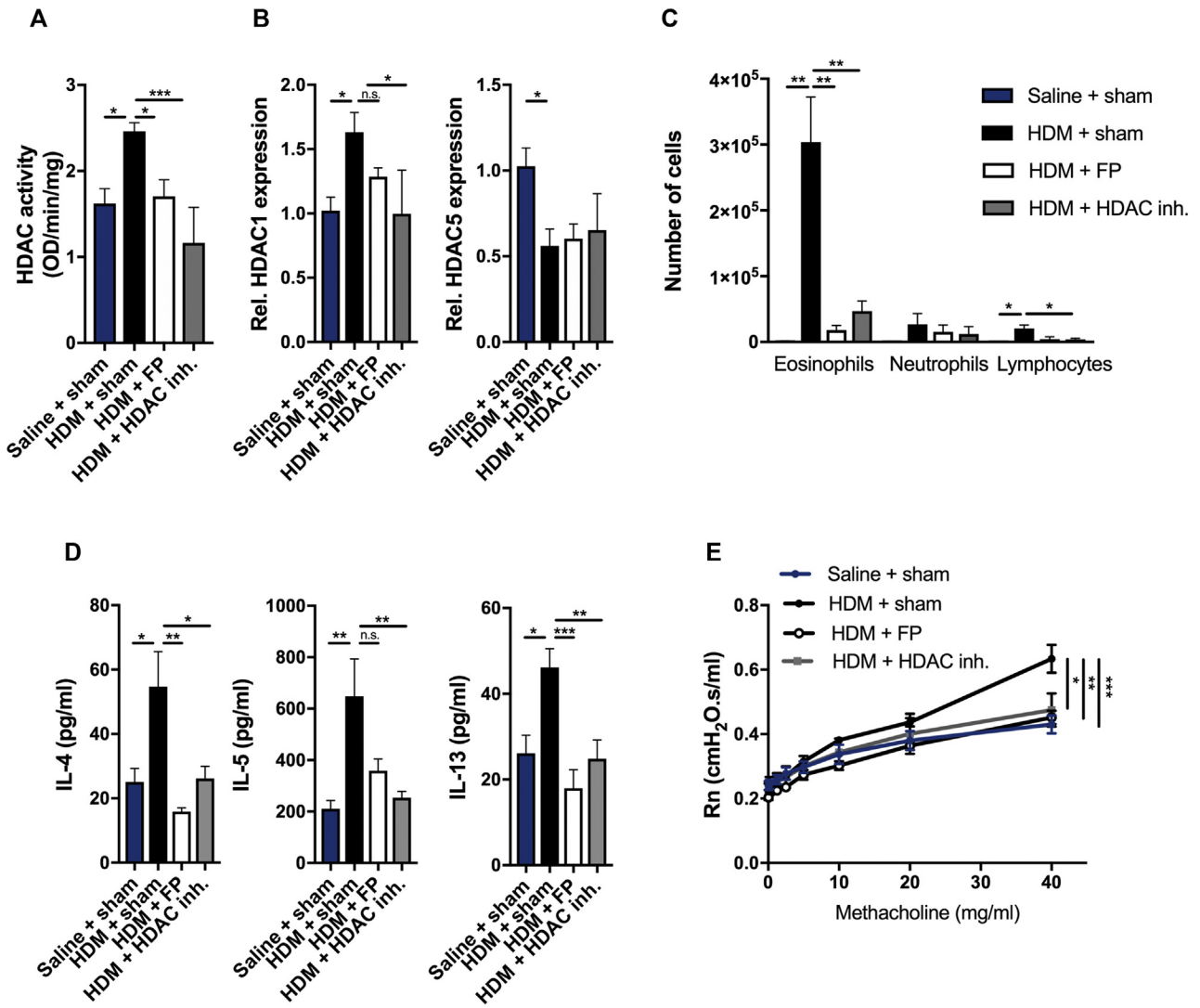
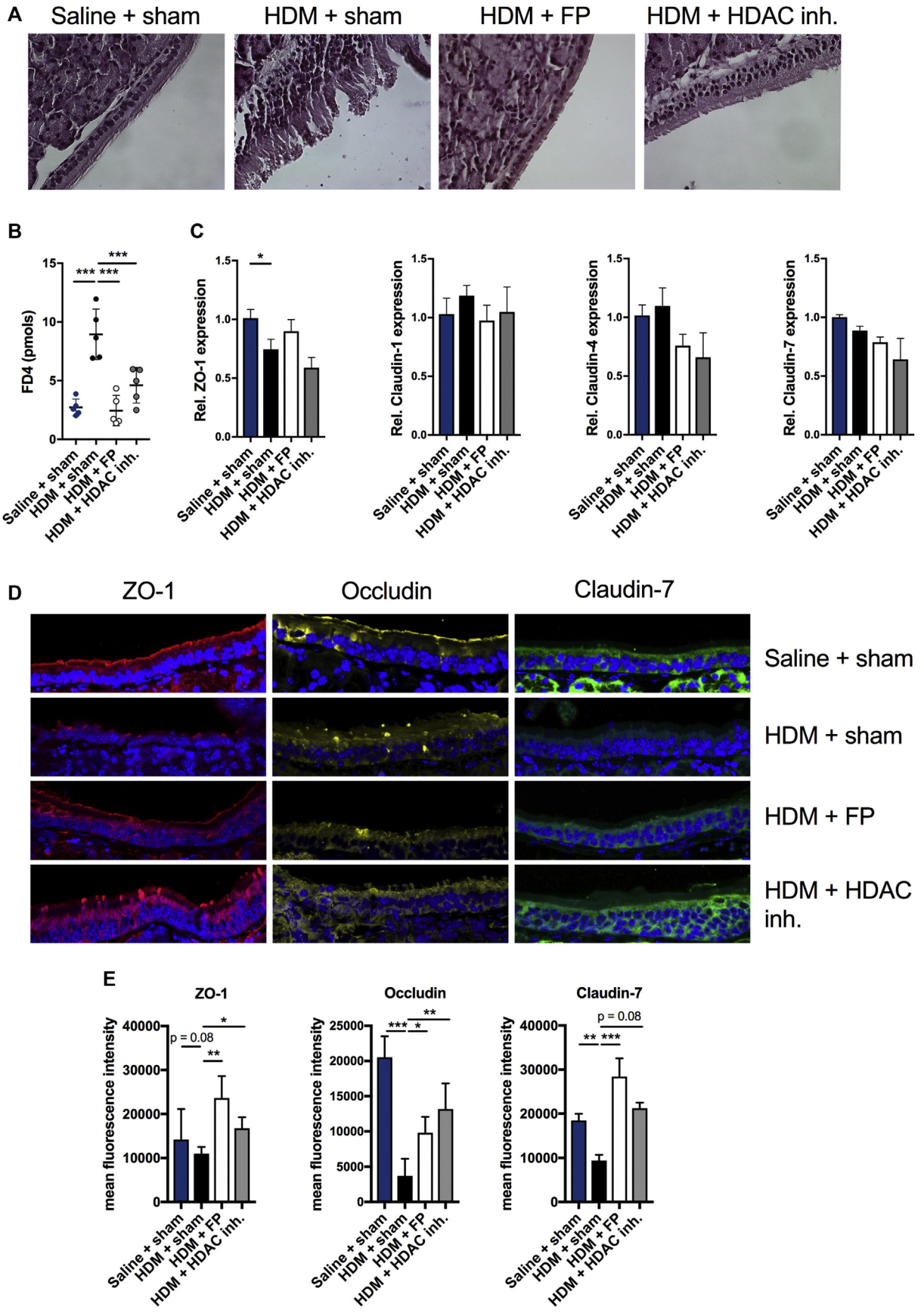


FIG 4. HDAC inhibition protects mice from asthmatic airway inflammation. Mice were endonasally sensitized with 1 μg of HDM extract, followed by 5 endonasal challenges with 10 μg of HDM extract. One hour before each challenge, mice were treated endonasally with either FP (6 μg/d), JNJ-26481585 (6.25 μg/d), or saline. **A,** Endogenous HDAC activity in the nasal mucosa of HDM-sensitized mice treated with sham, HDAC inhibitor (JNJ-26481585; 6.25 μg/d), or FP (6 μg/d; n = 5 mice per group). **B,** Relative mRNA expression of HDAC1 and HDAC5 in nasal mucosa normalized to the housekeeping gene elongation factor-2. **C,** BAL differential cell counts of HDM-sensitized mice that received treatment with JNJ-26481585, FP, or sham. **D,** Cytokine levels of IL-4, IL-5, and IL-13 measured in BAL fluid. **E,** Bronchial hyperreactivity was measured 24 hours after the last HDM exposure after exposure to increasing doses of methacholine using the flexiVent. All analyses in Fig 4, *A-D*, were performed on samples collected 24 hours after the last challenge with HDM. *Error bars* are shown as means ± SEMs. Significance was calculated by using 1-way ANOVA with *post hoc* analysis. **P* < .05, ***P* < .01, and ****P* < .001. *n.s.*, Not significant.

FIG 3. HDAC inhibition promotes tight junction rearrangement *in vitro.* **A,** ALI cultures of nasal epithelial cells from patients with AR were incubated with different concentrations of HDAC inhibitor (JNJ-26481585) and then stained for occludin (green) and ZO-1 (red). Images were made with ×50 magnification. **B,** ALI cultures of nasal epithelial cells of healthy control subjects were incubated with different concentrations of HDAC inhibitor (JNJ-26481585) and stained for occludin (green) and ZO-1 (red). Images were made at ×50 magnification. **C,** Quantitative fold change in mean fluorescence intensity of occludin and ZO-1 expression after JNJ-26481585 treatment in patients with AR (n = 5 donors per group). **D,** Quantitative fold change in mean fluorescence intensity of occludin and ZO-1 expression after JNJ-26481585 treatment in healthy control subjects (n = 4 donors). *Error bars* are shown as means ± SEMs. Significance was calculated by using the Student *t* test. **P* < .05 for ZO-1 comparison and #*P* < .05 for occludin comparison.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Rel. Claudin-7 expression
D
ZO-1
Occludin
Claudin-7
Saline + sham
HDM + sham
HDM + FP
HDM + HDAC inh.
E
ZO-1
Occludin
Claudin-7
mean fluorescence intensity
p = 0.08

not altered (Fig 1, *D*, and see Fig E1 in this article's Online Repository at www.jacionline.org). Western blotting revealed increased protein levels of HDAC5 and HDAC11 in epithelial cells of patients with AR compared with healthy control subjects (Fig 1, *E*). Overall, our data indicate increased endogenous HDAC activity in nasal epithelial cells from patients with AR, which correlated inversely with TER as a marker of barrier integrity.

Blocking HDAC activity reconstitutes tight junction expression and epithelial barrier function

Because increased HDAC activity is responsible for packed chromatin, leading to delayed cell division, fixed proinflammatory cytokine profile, delayed wound healing in patients with chronic inflammatory diseases, and a defect in correction of the barrier leakiness,[5] we investigated the possibility of restoring barrier defects by blocking HDAC activity. To test this, ALI cultures of nasal epithelial cells were incubated for 72 hours with different concentrations of JNJ-26481585.[21] We demonstrated that inhibition of HDACs with JNJ-26481585 dose-dependently restored TER of ALI cultures of patients with AR to the level of healthy control subjects without directly affecting control ALI cultures (Fig 2, *A*).

Baseline FD4 epithelial permeability, a surrogate marker for integrity, was significantly increased in ALI cultures of patients compared with control subjects (170 ± 14 vs 98 ± 11 pmol/L, $P < .05$). Treatment with JNJ-26481585 had no significant effect on FD4 permeability compared with medium control in ALI cultures of patients (Fig 2, *B*).

To further explore the role of HDAC inhibition on barrier integrity, we studied its effect on mRNA expression of different tight junction genes. Our data showed a dose-dependent effect of JNJ-26481585 on mRNA expression of ZO-1, occludin, claudin-4, and claudin-7 in ALI cultures of epithelial cells from both patients and healthy control subjects (Fig 2, *C*). At the protein level, immunofluorescence staining of ZO-1 and occludin in ALI cultures of patients with AR was very weak at baseline compared with that in healthy control subjects (Fig 3, *A* and *B*), and treatment with JNJ-26481585 dose-dependently promoted the expression of tight junctions in both patients with AR and control subjects (Fig 3, *A* and *B*). In addition, expression of Ki67, a marker of cell proliferation, was not altered after treatment with JNJ-26481585, suggesting that cell proliferation was not affected (see Fig E2 in this article's Online Repository at www.jacionline.org). Quantification of the immunofluorescence intensity of ZO-1 and occludin revealed the greatest mean intensity at 1 nmol/L JNJ-26481585 in patients with AR (Fig 3, *C* and *D*). In conclusion, our data demonstrate that blocking HDAC activity reconstitutes the defective epithelial barrier in patients with AR by increasing mRNA expression of tight junctions and reorganizing tight junction networks.

HDAC inhibition reduces allergic inflammation and promotes mucosal barrier function *in vivo*

To confirm the effect of HDAC inhibition on barrier function, we administered JNJ-26481585 in a mouse model of HDM-induced allergic asthma.[7,27] Mice with HDM allergy were endonasally instilled with JNJ-26481585 on 5 consecutive days (6.25 μg/d). The optimal concentration of JNJ-26481585 for *in vivo* barrier-restoring capacities was determined, as shown in Fig E3 in this article's Online Repository at www.jacionline.org. As a positive control, mice with HDM allergy were treated endonasally with FP (6 μg/d), whereas sham treatment was administered as a negative control. In accordance with our *in vitro* data, we observed a significantly greater endogenous HDAC activity in the nasal mucosa of mice with HDM allergy compared with control mice (Fig 4, *A*). A decrease in endogenous HDAC activity was seen in mice with HDM allergy on either JNJ-26481585 or FP treatment (Fig 4, *A*).

mRNA analysis revealed significantly increased HDAC1 expression, which was restored by JNJ-26481585, and a similar trend was observed after FP treatment (Fig 4, *B*). In contrast, mRNA expression of HDAC5 was significantly decreased in nasal mucosa of mice with HDM allergy compared with that of control animals, whereas the other subtypes were not altered (see Fig E4 in this article's Online Repository at www.jacionline.org).

We next studied whether JNJ-26481585 reduced allergic inflammation *in vivo*. We found that JNJ-26481585 treatment resulted in a strong reduction in eosinophil and lymphocyte counts in BAL fluid of mice with HDM allergy (Fig 4, *C*). HDM-induced allergic mice had significantly greater levels of IL-4, IL-5, and IL-13 in BAL fluid, which were all significantly decreased by HDAC inhibition with JNJ-26481585 to levels in nonallergic saline control mice (Fig 4, *D*). In addition, increased levels of IL-1β and TNF-α were found in BAL fluid of HDM-induced allergic mice. Treatment with JNJ-26481585 and FP significantly attenuated these levels (Fig E5, *A*, in this article's Online Repository at www.jacionline.org).

We also evaluated the effect of JNJ-26481585 and FP treatment on the epithelial cytokines IL-25, IL-33, and TSLP and found significantly increased levels of IL-25 and IL-33 in HDM-induced allergic mice. JNJ-26481585 and FP treatment of these mice decreased amounts of both cytokines to levels observed in nonallergic saline-treated control mice. TSLP levels did not differ between the groups (see Fig E5, *B*). Furthermore, we found that treatment with JNJ-26481585 significantly attenuated bronchial hyperreactivity in mice with HDM allergy, almost to a

FIG 5. HDAC inhibition reconstitutes mucosal barrier function in an HDM allergy mouse model. Mice were endonasally sensitized with 1 μg of HDM extract, followed by 5 endonasal challenges with 10 μg of HDM extract. One hour before each challenge, mice were treated endonasally with either FP (6 μg/d), JNJ-26481585 (6.25 μg/d), or saline. **A,** Representative hematoxylin and eosin staining of nasal mucosal sections harvested 24 hours after last HDM challenge. Images were made at ×40 magnification. **B,** Nasal mucosal permeability measured by FD4 leakage to serum 24 hours after the last challenge. **C,** Relative mRNA expression of ZO-1, claudin-1, claudin-4, and claudin-7 in nasal mucosa normalized to the housekeeping gene elongation factor-2. **D,** Nasal mucosal sections were immunolabeled for ZO-1 (red), occludin (yellow), claudin-7 (green), and DAPI (blue). Images were made with ×50 magnification. **E,** Quantitative fold change in mean fluorescence intensity of ZO-1, occludin, and claudin-7 expression after JNJ-26481585 treatment in nasal mucosa (n = 3 mice per group). *Error bars* are shown as means ± SEMs. Significance was calculated by means of 1-way ANOVA with *post hoc* analysis. *$P < .05$, **$P < .01$, and ***$P < .001$. *n.s.*, Not significant.

similar degree as FP treatment (Fig 4, *E*). Together, these findings suggest that HDAC inhibition might act as an anti-inflammatory agent for the treatment of allergic airway diseases.

Because JNJ-26481585 treatment attenuated allergic inflammation in mice with HDM allergy, we further analyzed the effect of JNJ-26481585 on nasal mucosal integrity. We found that inhibition of HDAC activity in mice with HDM allergy significantly decreased FD4 mucosal permeability (Fig 5, *B*). Histologic analysis of hematoxylin and eosin–stained nasal mucosal sections revealed a clear disrupted nasal mucosa, together with increased influx of inflammatory cells in mice with HDM allergy. Instillation of mice with HDM allergy with JNJ-26481585 or FP normalized the aberrant morphology of the nasal mucosa (Fig 5, *A*). The disrupted nasal mucosa of mice with HDM allergy was associated with a significantly decreased mRNA expression of ZO-1 (Fig 5, *C*). JNJ-26481585 and FP did not reconstitute mRNA expression levels of ZO-1. Immunofluorescence staining showed a clear expression of occludin, ZO-1, and claudin-7 in the nasal mucosa of saline control mice, whereas this was severely disrupted in mice with HDM allergy. Treatment with JNJ-26481585 and FP restored the expression of tight junction proteins (Fig 5, *D*). Quantification of the immunofluorescence intensity of ZO-1, occludin, and claudin-7 revealed a significantly decreased protein expression in asthmatic mice with HDM allergy compared with saline control mice. Treatment with JNJ-26481585 and FP reconstituted the expression of ZO-1 and occludin to the level of saline control mice (Fig 5, *E*). Taken together, our results indicate that blocking HDAC activity attenuates allergic inflammation and bronchial hyperreactivity, at least partially by promoting tight junction expression.

DISCUSSION

The importance of epithelial barrier defects in patients with chronic diseases is evidenced by various studies showing differences in tight junction function and expression in cells or tissues from subjects with and without specific diseases.[1,34] To date, however, these studies are generally limited in causal mechanisms responsible for onset and development of disease. Indeed, a major limitation of studying barrier function in subjects is the difficulty in differentiating changes in tight junctions related to disease onset from those changes resulting from disease progression and severity because these changes might not be permanent or transient. Epigenetic modifications and, more specifically, HDAC activity are crucial in mediating the effect of environmental and inflammatory triggers on onset, progression, and chronicity of airway diseases. Nonetheless, limited data are focused on HDACs and their influence on tight junction expression. Our study was designed to investigate the role of HDACs underlying epithelial barrier defects in patients with AR.

By using *in vitro* primary nasal epithelial cell cultures, we first identified increased endogenous HDAC activity in cells isolated from patients with AR compared with control subjects. HDAC activity correlated inversely with epithelial integrity, demonstrating for the first time a potential link between impaired epithelial barrier function and increased HDAC activity. We demonstrated that mRNA expression of HDAC5 and HDAC11 was significantly upregulated in diseased epithelial cells. The specific function of increased HDAC5 expression in patients with airway diseases is not known. However, in patients with systemic sclerosis, HDAC5 overexpression in endothelial cells inhibits proliferation and migration, contributing to a similar phenotype (ie, leaky endothelium/epithelium).[35] These findings need to be confirmed in the airways. Moreover, HDAC11 is a class IV enzyme without any clearly defined physiologic function, although there is some evidence that HDAC11 influences immune activation versus immunotolerance.[36]

In contrast to previous publications, there was no decreased HDAC2 expression found in epithelial cells of patients with AR. Reduced HDAC2 expression and activity have been demonstrated in patients with asthma and chronic obstructive pulmonary disease.[37,38] These patients were insensitive to the anti-inflammatory effects of corticosteroids, which is not the case in our population and might explain this difference. Additionally, reduced HDAC2 expression was not found in all patients with severe steroid–insensitive asthma, suggesting a possible role for different HDACs in different disease phenotypes.[39]

Because HDACs were associated with barrier disruption, we next explored the possibility of reconstituting a defective epithelial barrier by inhibiting HDAC activity. Currently, only a few HDAC inhibitors are US Food and Drug Administration approved, mostly for the treatment of cutaneous and peripheral T-cell lymphoma and myelomas.[40] Because HDAC activity is increased in multiple malignant cells, the potential of HDAC inhibitors either as a single therapy or in combination with other drugs is being tested in patients with breast cancer and glioblastoma, among others conditions.[41] In our study we used a second-generation HDAC inhibitor, JNJ-26481585, which inhibits both class I and II HDACs and has potent antitumor activity in preclinical *in vivo* models of human cancers.[21] In addition to its antitumor activity, JNJ-26481585 dose-dependently increased epithelial integrity in primary nasal epithelial cells of patients with AR. JNJ-26481585 has a high potency for HDAC1 (inhibitory concentration of 50%, 0.11 nmol/L) and a moderate potency for HDAC2, HDAC4, HDAC10, and HDAC11 (inhibitory concentration of 50% range, 0.33-0.64 nmol/L).[21] The selectivity for HDAC5, which was significantly upregulated in epithelial cells from patients with AR, is 10 times lower compared with that for HDAC11. Given that the greatest increase in TER in patients with AR was obtained with 0.5 nmol/L JNJ-26481585 and the differences in potency, this notion would suggest that barrier defects in patients with AR are presumably ascribed to increased HDAC11 and not to HDAC5, although this needs further confirmation.

In addition, we found that JNJ-26481585 directly increased the mRNA expression of different tight junctions, which is expected because blocking HDAC activity promotes gene expression in general.[13] Surprisingly, Ki67 mRNA expression was decreased after blocking HDAC activity. Because Ki67 is merely a marker of cell proliferation at a given time point, we hypothesize that decreased Ki67 expression indicates the presence of a confluent epithelial monolayer rather than exhaustion of airway basal stem cells to regenerate a defective epithelial barrier in case of epithelial shedding. Aside from this intrinsic effect, JNJ-26481585 also promoted tight junction protein reorganization and thus recovery of barrier integrity.[42,43]

We also showed the importance of HDACs and their inhibition *in vivo* in a mouse model of HDM-induced allergic asthma because no mouse model exists solely for upper airway disease. Despite the differences in embryologic origin of the upper and lower airways, we did not observe any differences in barrier function between the upper and lower airways (data not shown). Given

that the same tight junction proteins (ie, occludin and ZO-1) are affected in patients with asthma and AR, we believe that the HDM-asthma mouse model is a suitable model to study both upper and lower airway function.

Mice with HDM allergy showed increased endogenous HDAC activity compared with saline control mice. HDAC1 expression was significantly increased at the mRNA level, which is contradictory with a previous publication showing decreased HDAC1 levels in asthmatic patients.[44] We believe that differences in tissue, human lung biopsy specimens versus murine nasal biopsy specimens, or methodology (ie, protein vs mRNA levels) might explain this. Another explanation might be that barrier function is differentially regulated in mice compared with human subjects. Indeed, expression of HDAC5 was significantly decreased in the nasal mucosa of mice with HDM allergy, which was upregulated in human epithelial cells. Beyond the role of HDAC5 in angiogenesis,[45] its function in allergic inflammation *in vivo* remains unknown. Mice with HDM allergy had classical features of type 2 inflammation with increased eosinophils counts and IL-4, IL-5, IL-13, IL-1β, and TNF-α *levels* in BAL fluid; IL-25 and IL-33 levels in lung homogenates; and bronchial hyperreactivity. HDAC inhibition in these mice significantly decreased type 2 inflammation and bronchial hyperreactivity.[46-49] In addition to the effect of JNJ-26481585 on features of type 2 inflammation, we also found that JNJ-26481585 promoted tight junction expression to restore defective epithelial barrier in mice with HDM allergy. Although no differences could be observed in mRNA levels of tight junctions, JNJ-26481585–treated mice with HDM allergy had significantly increased tight junction protein expression compared with mice receiving sham control. Interestingly, mice with HDM allergy treated with corticosteroids had a similar improvement in barrier function. It is likely that these findings represent a possible similar mechanism through which corticosteroids and JNJ-26481585 exert their effect, but this remains to be elucidated in future studies.

Of note, we did not observe any off-target effects of HDAC inhibition in our models. Because HDAC inhibition promotes chromatin remodeling, one can assume that transcription of different proinflammatory genes can be promoted, which might contribute to disease development. We suggest that the absence of any side effects might be due to several reasons.

First, we studied the rather fast effects of JNJ-26481585 on gene transcription (within 72 hours). It is possible that transcription of proinflammatory genes takes more time and therefore was missed in our *in vivo* setting.

Second, we studied the effect on isolated primary nasal epithelial cells. We did not investigate the effect of JNJ-26481585 on other cells, such as mast cells, T cells, and dendritic cells, which are normally in close proximity with epithelial cells.

Third, JNJ-26481585 has a rather short half-life of approximately 8 hours. As such, in our mouse model we did not study the effect of continuous administration of JNJ-26481585 on expected and unexpected effects. Indeed, a possible limitation of HDAC inhibitors is the relatively narrow therapeutic window. Many HDAC inhibitor effects on biological targets are reversible on drug removal, and sustained effects often require continuous dosing. Further research is warranted to determine optimal dosage and timing to limit off-target effects of HDAC inhibitors.

Overall, the present study provides new insights into the underlying mechanisms of epithelial barrier defects in patients with AR and asthma. These results indicate that HDAC inhibition can reconstitute epithelial barrier defects by promoting expression of tight junction proteins and reorganizing tight junction networks. In addition, the data demonstrate that HDAC-targeting agents can be considered novel treatment options in patients with allergic airway diseases.

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Key messages

- **Increased HDAC activity is a potential key factor underlying a defective epithelial barrier in patients with AR.**
- **Targeting HDACs might hold potential therapeutic interventions in patients with chronic airway diseases.**

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METHODS

Isolation of primary nasal epithelial cells

Inferior turbinates from nonallergic, nonsmoking healthy control subjects and nonsmoking, therapy-negative patients with AR were used for isolation of primary nasal epithelial cells. Inferior turbinates were enzymatically digested in 0.1% Pronase (protease XIV; Sigma) solution in DMEM-F12 culture medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2% Ultroser G (Pall Life Sciences, Leuven, Belgium). After overnight incubation at 4°C while shaking, the protease reaction was stopped by adding FCS (10%). Cells were washed in culture medium and pelleted by means of centrifugation for 5 minutes at 100*g*. Cells were then resuspended in 10 mL of culture medium and incubated in a plastic culture flask for 1 hour at 37°C to remove fibroblasts. The cell suspension was mixed with 2×10^7 prewashed CD45 and CD15 magnetic beads (Dynabeads; Invitrogen, Merelbeke, Belgium), and epithelial cells were purified by means of negative selection according to the manufacturer's instructions.

mRNA isolation and RT-PCR

The methodology of mRNA isolation and quantitative RT-PCR has been described elsewhere.[E1] Briefly, RNA was isolated by using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Reverse transcription of samples was performed with reverse transcription reagents (Fermentas, Thermo Fisher, Waltham, Mass) with random hexamers, according to the manufacturer's protocol. cDNA was used for quantitative RT-PCR. Expression levels were normalized to expression of the housekeeping gene elongation factor-2.

Western blot

Epithelial cells at day 21 in the ALI were harvested, and proteins were isolated (Tissue Protein Extraction Reagent; Life Technologies, Grand Island, NY). Equal amounts of total protein (25 μg) were separated by using SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were incubated overnight with primary antibodies: rabbit polyclonal anti-HDAC5 (1:25; Abcam, Cambridge, United Kingdom) and rabbit polyclonal anti-HDAC11 (1:40; Abcam). As a protein-loading control, all blots were stained with mouse monoclonal anti–β-actin (1:5000; Abcam). Secondary antibody was peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (both 1:5000; Dako, Glostrup, Denmark). Visualization was performed by using chemiluminescence (Western Lightening; PerkinElmer, Waltham, Mass). Western blotting was performed in 3 control subjects and 3 patients with AR.

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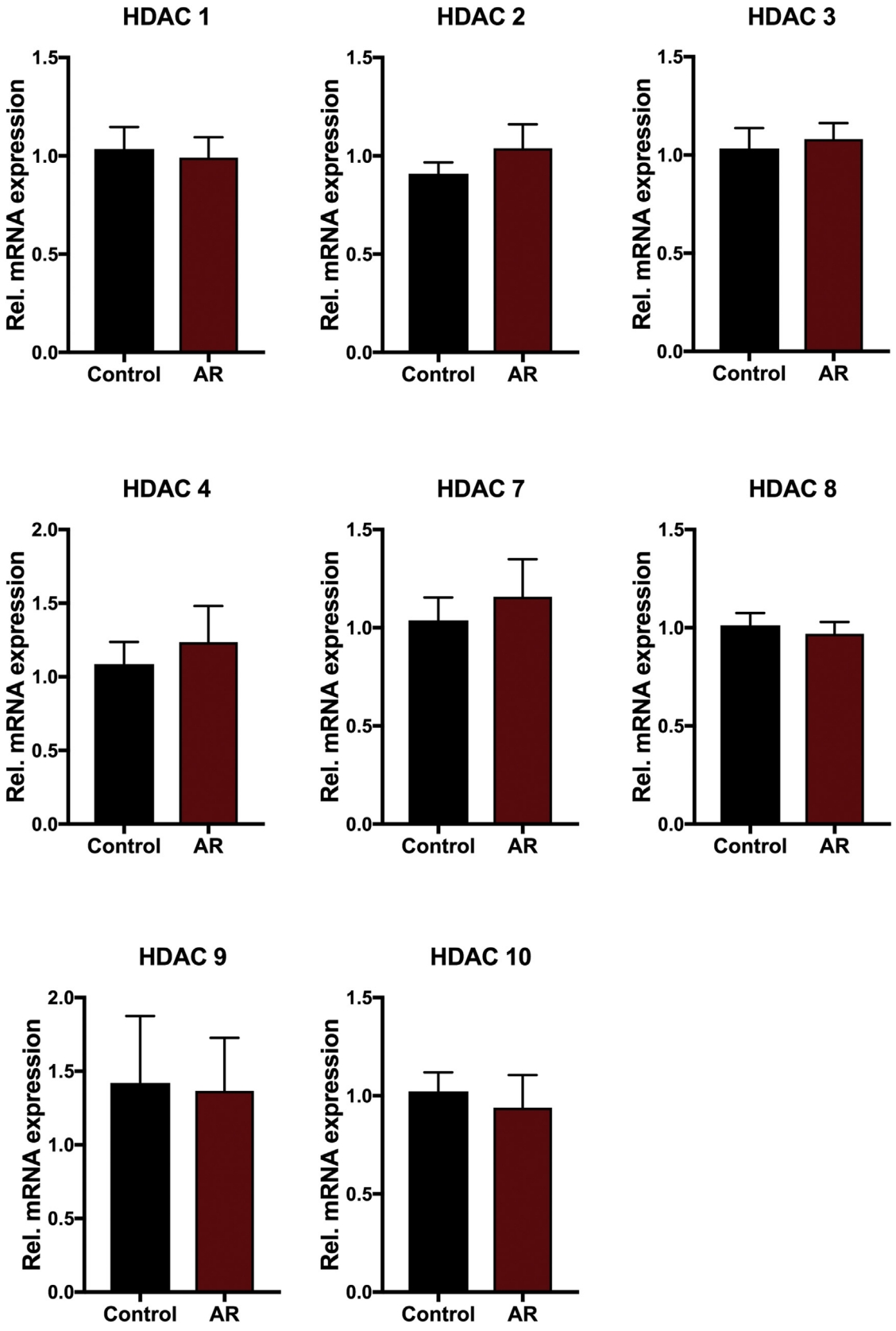

FIG E1. Expression of different HDACs in nasal epithelial cells from healthy control subjects and patients with AR. mRNA expression of HDACs in ALI cultures of patients and control subjects were measured and normalized to the housekeeping gene elongation factor-2. *Error bars* are shown as means ± SEMs.

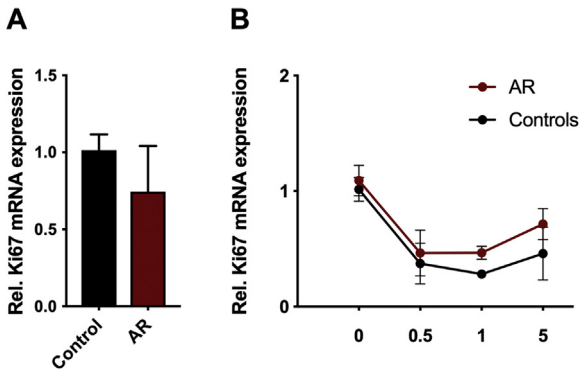

FIG E2. Effect of HDAC inhibition on Ki67 expression. **A,** Relative mRNA expression of Ki67 in ALI cultures of control subjects and patients with AR. **B,** mRNA expression of Ki67 in relation to JNJ-26481585 concentration in ALI cultures of nasal epithelial cells from healthy control subjects (n = 4) and patients with AR (n = 6). Expression is normalized to the housekeeping gene elongation factor-2. *Error bars* are shown as means ± SEMs.

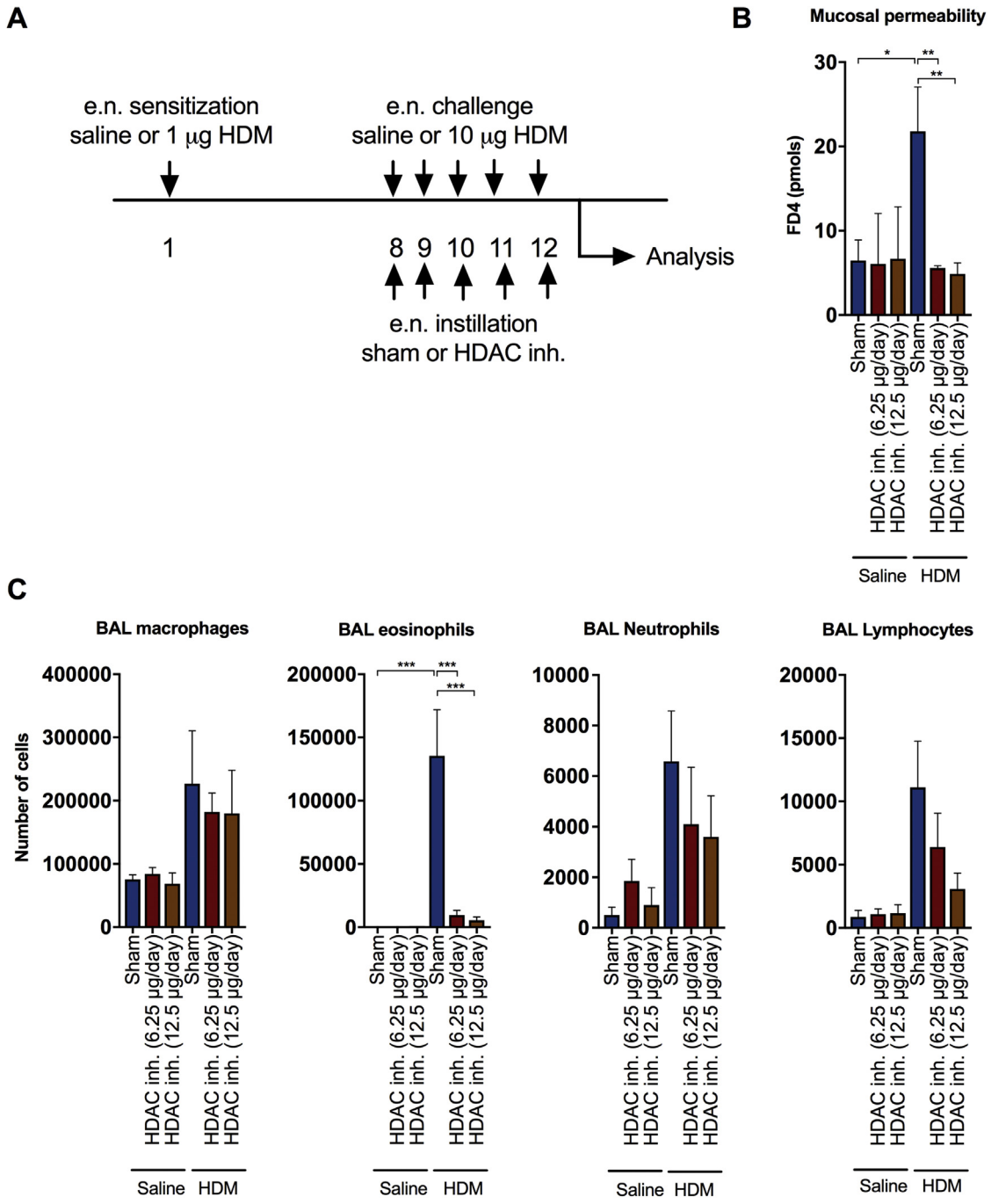

FIG E3. Effect of different concentrations of the HDAC inhibitor JNJ-26481585 in a mouse model of HDM-induced allergic asthma. **A,** Mice were endonasally *(e.n.)* sensitized with 1 μg of HDM extract, followed by 5 endonasal challenges with 10 μg HDM extract. One hour before each challenge, mice were treated endonasally with JNJ-26481585 (6.25 or 12.5 μg/d) or saline. **B,** Nasal mucosal permeability measured based on FD4 leakage to serum 24 hours after the last challenge. **C,** BAL differential cell counts of HDM-sensitized mice that received treatment with JNJ-26481585 or sham. *Error bars* are shown as means ± SEMs. Significance was calculated by using 1-way ANOVA with *post hoc* analysis. *$P < .05$, **$P < .01$, and ***$P < .001$.

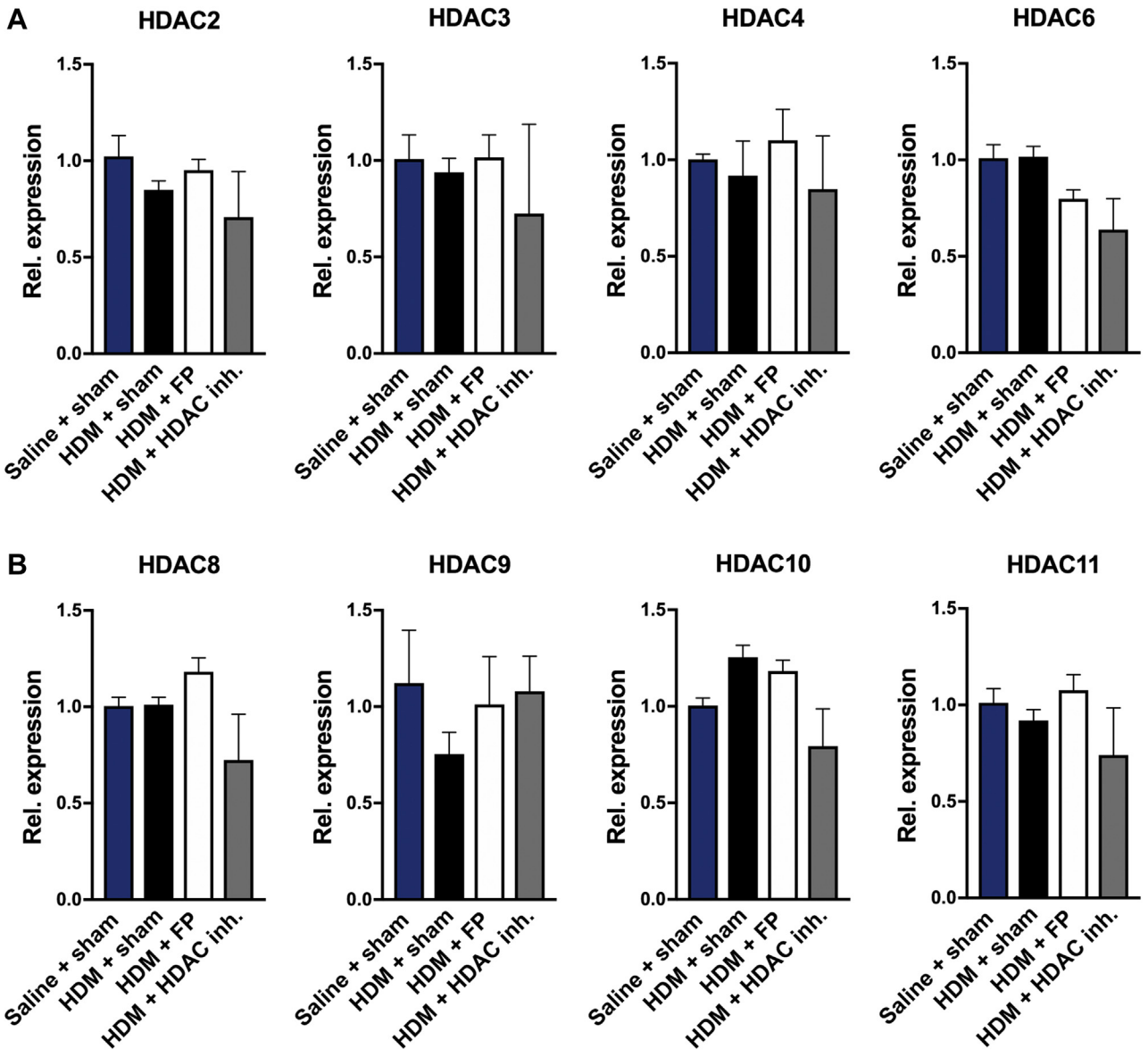

FIG E4. Effect of HDAC inhibition on inflammatory markers in an HDM allergy mouse model. **A,** Levels of TNF-α, IL-1β, and IL-6 measured in BAL fluid. **B,** Levels of IL-25, IL-33, and TSLP in lung homogenates (n = 5 mice per group). *Error bars* show medians and interquartile ranges. Significance was calculated by using the Kruskal-Wallis test with *post hoc* analysis. **P* < .05, ***P* < .01, and ****P* < .001. *n.s.*, Not significant.

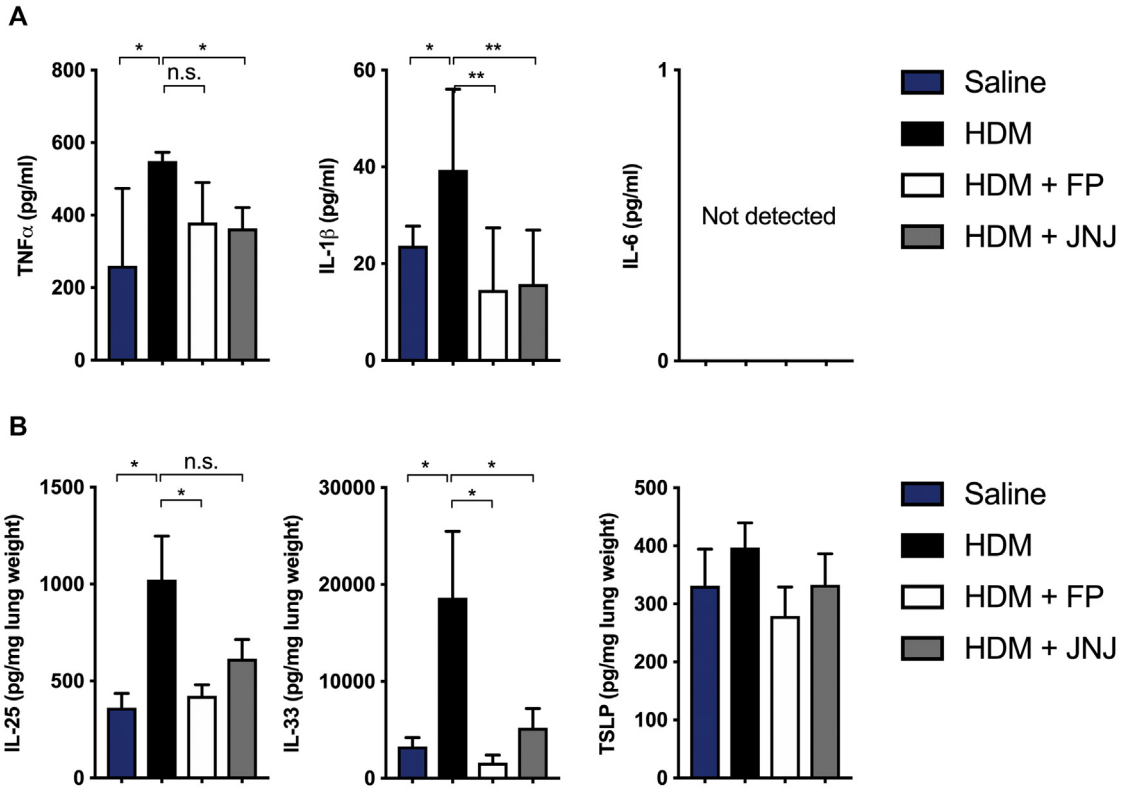

FIG E5. **A** and **B**, Expression of different HDACs in nasal mucosa of mice with HDM-induced allergic asthma. mRNA expression of HDACs in nasal mucosa of mice with HDM allergy is shown and was normalized to the housekeeping gene elongation factor-2. *Error bars* are shown as means ± SEMs.

TABLE E1. Patients' characteristics

	Control subjects	Patients with AR	*P* value
No.	9	9	
Age (y), mean ± SD	33 ± 12	41 ± 14	.18
Sex (male/female)	6/3	8/2	
Allergy (%)	0	100	
HDM (%)	0	100	
Intranasal steroids (%)	0	0	