Letter to the Editor

Claudin 5 in a murine model of allergic asthma: Its implication and response to steroid treatment

To the Editor:

Allergic inflammation develops in tissues with large epithelial surface areas exposed to the environment, such as the lung, skin, and gut.[1] In the lung allergens can activate cells in the epithelium to produce cytokines that induce T_H2 responses.[2] In addition, allergens (eg, the house dust mite *Dermatophagoides pteronyssinus* proteinase Der p 1) can disrupt epithelial cell tight junctions (TJs), suggesting a role for epithelial barrier function in allergic sensitization and asthma.[2] However, less is known about endothelial barrier function in asthmatic patients.[3] Increased angiogenesis, vasodilatation, vascular congestion, and edema contribute to thickening of the airway wall and reduction of the airway lumen in asthmatic patients.[3]

Endothelial cell-cell junctions communicate cell position, limit growth and apoptosis, and regulate vascular homeostasis.[3] A component of endothelial TJs, claudins are tetra-membrane–spanning proteins oriented with amino- and carboxy-termini in the cytosol, resulting in the formation of 2 extracellular loops that control TJ adhesion.[4] In airway endothelial cells claudin 5 (CLDN5) plays a critical role in controlling endothelial cell polarity and pericellular permeability.[4] Previously, altered lung CLDN5 expression was found to be associated with increased susceptibility to acute lung injury in mice.[5] In spite of the important role of claudins as regulators of TJ complexes and cell permeability, the role of CLDN5 in the increased vascular permeability seen in asthmatic patients remains poorly characterized.

To address this issue, 50 asthmatic patients were recruited and followed for more than 6 years (see Table E1 in this article's Online Repository at www.jacionline.org), and plasma CLDN5 levels were determined. The study of patients and animals was approved by Soonchunhyang University's Institutional Review Board and Animal Care and Use Committee, respectively. Plasma CLDN5 levels decreased in patients with stable asthma compared with those in control subjects (Fig 1), suggesting that asthma therapy can decrease plasma CLDN5 levels. During exacerbation, CLDN5 levels increased in patients compared with levels seen in those with stable asthma, but in most cases, levels were similar to those in control subjects.

In a mouse model of ovalbumin (OVA) allergic sensitization/challenge, OVA challenge induced lung CLDN5 transcript and protein, and this increase was reduced by dexamethasone (Fig 2, *A*). Membrane CLDN5 levels increased, and cytosolic CLDN5 levels decreased (Fig 2, *B*). In addition, CLDN5 immunostaining increased in lung microvascular endothelial cells of OVA-sensitized/challenged mice (Fig 2, *C*). The CLDN5 staining was quantified as vessel density and increased after OVA. This response was diminished in mice treated with dexamethasone. The increase in vascularity (as measured based on vessel density) is similar to the findings of Sun et al,[6] who showed that budesonide diminished allergen-induced angiogenesis in a mouse model. In addition, confocal image analysis revealed a significant increase in the density of CLDN5 staining.

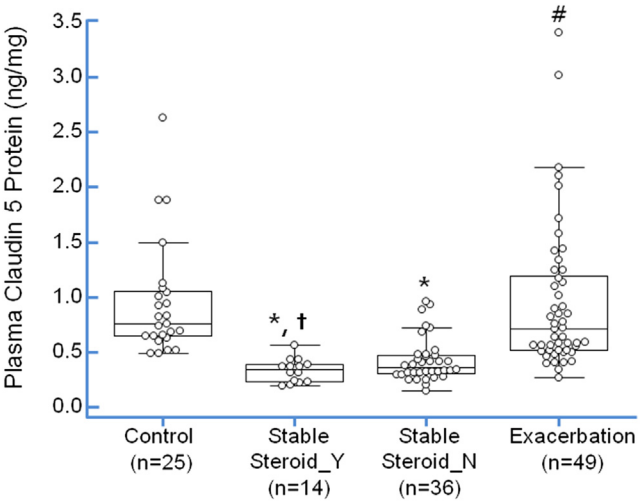

FIG 1. Plasma CLDN5 levels increased during asthma exacerbations. Patients with stable asthma were divided into those who were currently using corticosteroids *(Stable Steriod_Y)* and those who were not *(Stable Steroid_N)*. *$P < .001$, control subjects versus patients with stable asthma. †$P < .001$, Stable Steroid_Y versus Stable Steroid_N. #$P < .001$, stable versus exacerbation.

Normally, TJs form a continuous ring that circumscribes each cell, and these rings were disrupted in OVA-sensitized/challenged mice. Dexamethasone partially restores the TJ continuous pattern.

Forkhead box O1 (FOXO1) decreases CLDN5 expression, and phosphorylation of FOXO1 (p-FOXO1) relieves this decrease and increases CLDN5 gene expression.[5,7] Lung p-FOXO1 protein levels increased in OVA-sensitized/challenged compared with those in saline-treated mice, whereas phosphorylated catenin (cadherin-associated protein) β1 (p-CTNNB1) and phophorylated V-akt murine thymoma viral oncogene homolog 1 (p-AKT1) protein levels decreased (see Fig E1 in this article's Online Repository at www.jacionline.org). This was accompanied by increased p-FOXO1/FOXO1 expression and decreased p-AKT1/AKT1 and p-CTNNB1/CTNNB1 expression in OVA-sensitized/challenged mice, which are consistent with the observed alteration of CLDN5 expression.

Normal human bronchial epithelial (NHBE) and human lung microvascular endothelial (HMVEC-L) cells were treated with Der p 1 and CLDN5 transcript levels were measured to examine whether Der p 1 could alter CLDN5 levels in airway cells. CLDN5 transcripts increased in HMVEC-L cells after 24-hour exposures to Der p 1 but were unchanged in NHBE cells (see Fig E2 in this article's Online Repository at www.jacionline.org). Der p 1 treatment also led to a modest decrease in transendothelial electrical resistance (TEER). IL-4, which is released during airway allergen challenge, previously has been found to decrease CLDN5 and increase TEER levels in porcine aorta endothelial cells.[7] In contrast to aorta cells,[7] IL-4 increased CLDN5 levels and decreased TEER levels in HMVEC-L cells. Dexamethasone inhibited the IL-4–induced CLDN5 level increase and restored TEER levels. Thus in the airways increased CLDN5 levels are associated with decreased TEER levels, probably through

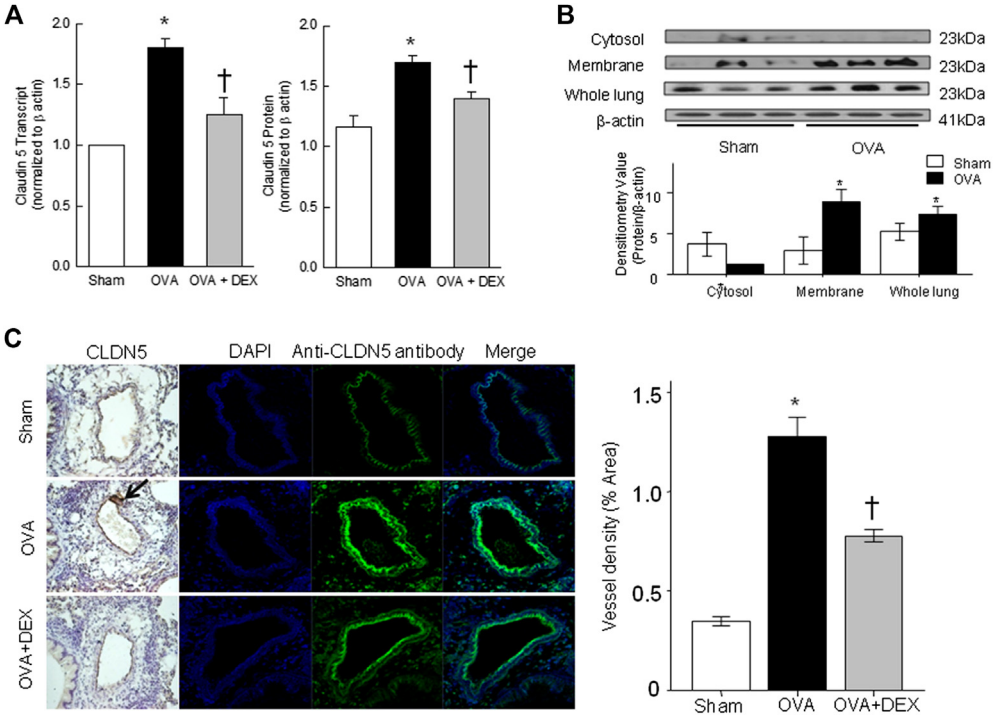

FIG 2. **A,** Lung CLDN5 transcript and protein expression increased in OVA-sensitized/challenged mice, and dexamethasone *(DEX)* diminished these responses. **B,** Increased CLDN5 expression is associated with the membrane, whereas cytosolic CLDN5 expression decreased. **C,** Increased CLDN5 expression was associated with disrupted vascular integrity. TJs form a continuous ring that circumscribes each cell and is more evident but appears disrupted. Values are means ± SEs (n = 8 mice per group). *$P < .05$, OVA versus sham. †$P < .05$, OVA versus OVA + DEX. *DAPI*, 4′-6-Diamidino-2-phenylindole dihydrochloride.

disruption of pulmonary endothelial cell TJs, which is in accordance with the pulmonary epithelial cell responses noted previously.[4]

Endothelial cells function as gatekeepers, controlling the infiltration of blood proteins and cells into the vessel wall. This unique characteristic is achieved by the coordinated opening and closing of cell-cell junctions and through specialized transcellular systems of transport vesicles. Junctional proteins maintain endothelial integrity, promote cell-cell adhesion, and transmit intracellular signals that regulate contact-induced inhibition of cell growth, apoptosis, gene expression, and new vessel formation. In this study CLDN5 transcript and protein expression increased in mouse lungs after OVA sensitization/challenge. The increased CLDN5 content was associated with a disruption of TJ rings. These responses were diminished by dexamethasone treatment.

Inhaled corticosteroids are currently the most effective anti-inflammatory therapy for persistent asthma.[7] Exacerbations often result from bronchospasm, mucus production, and airway edema. The efficacies of corticosteroids include reductions of airway inflammation, airway responsiveness, asthma symptoms, exacerbation frequency, and mortality. These effects are accompanied by improvement of lung function and quality of life. Corticosteroid therapy also might diminish the increase in bronchial vascularity and edema observed in asthmatic patients.[3,8,9] In our mouse model airway inflammation, airway responsiveness, and cytokine levels were reduced by corticosteroid treatment (see Fig E1), and CLDN5 expression recovered in terms of transcript, protein, and endothelial integration, supporting that regulation of endothelial TJ can be a therapeutic target for decreasing airway inflammation and responsiveness. These findings support the possibility that regulation of lung endothelial barrier function might constitute a novel therapeutic approach for asthma.

Kuk-Young Moon, MS[a]*
Pureun-Haneul Lee, BS[a]*
Byeong-Gon Kim, BS[a]
Choon-Sik Park, MD, PhD[a]
George D. Leikauf, PhD[b]
An-Soo Jang, MD, PhD[a]*

From [a]the Genome Research Center for Allergy and Respiratory Diseases, Soonchunhyang University Bucheon Hospital, Bucheon, Korea, and [b]the Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pa. E-mail: jas877@schmc.ac.kr.

*These authors contributed equally to this work.

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METHODS

Experimental design

Fifty asthmatic patients were recruited and followed for 6.6 ± 3.6 years, and plasma CLDN5 levels were determined during both stable conditions and in an exacerbated state (Table E1). This study was approved by Soonchunhyang University's Institutional Review Board. Eight BALB/c mice were exposed to saline (control), OVA, or OVA plus dexamethasone (3 mg/kg). This study was approved by Soonchunhyang University's Animal Care and Use Committee. Detailed analyses included measurement of lung CLDN5 mRNA and protein levels, lung histology, and bronchoalveolar lavage. Additional tests were performed with a cell line derived from primary HMVEC-L and NHBE cells.

Asthma exacerbation

Asthma exacerbation was defined based on Global Initiative for Asthma guidelines as episodes of progressive increase in shortness of breath, cough, wheezing, or chest tightness or some combination of these symptoms accompanied by decreases in expiratory airflow and use of systemic corticosteroids (tablets, suspension, or injection) or an increase from a stable maintenance dose for at least 3 days and a hospitalization or emergency department visit because of asthma requiring systemic corticosteroids.

Cell culture

Primary NHBE cells (catalog no. CC-2540; Lonza, Basel, Switzerland; seeding density, 3000 cells/cm^2) were grown (37°C in a 5% CO_2 atmosphere) in T-flasks with the BEGM BulletKit (catalog no. CC-3170, Lonza). Primary HMVEC-L cells (catalog no. CC-2527, Lonza; seeding density, 5000 cells/cm^2) were grown (37°C in a 5% CO_2 atmosphere) in the EGM-2 MV BulletKit (catalog nos. CC-3156 and CC-4176, Lonza). Medium was replaced every 48 hours until cells reached 90% confluence (37°C in a 5% CO_2 atmosphere). Cells are seeded in 6-well plates. Twenty-four hours before the experiment, the medium was changed to EBM-2 basal medium with 0.1% FBS, which was followed by treatment with 10 μg/mL house dust mite *Dermatophagoides pteronyssinus* product 1 (Der p 1; Arthropods of Medical Importance Resource Bank, Institute of Tropical Medicine, Yonsei University), 10 ng/mL recombinant human IL-4 (catalog no. 204-IL-050; R&D Systems, Yeongdeungpo-gu, Seoul, Korea), 10 μmol/L dexamethasone, or IL-4 and dexamethasone for 4, 8, or 24 hours.

TEER measurements

TEER was used as a measure of TJ formation in HMVEC-L cells seeded initially at 8×10^3 cells/mL. These cells were treated with Der p 1, IL-4, or IL-4 and DEX for 4, 8, or 24 hours. TEER was measured with an EVOM meter (World Precision Instruments, Sarasota, Fla).

Animals

Female 6-week-old BALB/c mice (n = 8 mice per group) were sensitized by means of intraperitoneal injection at days 0 and 14 with 50 μg of grade V chicken egg OVA (Sigma-Aldrich, St Louis, Mo) that was emulsified in 10 mg of hydroxyl aluminum plus 100 μL of Dulbecco PBS. At days 21 to 23, all mice received intranasal challenges with 150 μg of grade III OVA (Sigma-Aldrich) in 50 μL of Dulbecco PBS. Control mice were sensitized and challenged with saline. Additional groups of mice were treated with 3 mg/kg intraperitoneal dexamethasone (water soluble, Sigma-Aldrich) 1 hour before each OVA challenge daily for 3 days. On day 23, airway hyperresponsiveness (AHR) was measured, bronchoalveolar lavage fluid (BALF) was collected, and lung tissue was processed for protein, RNA, and hematoxylin and eosin (H&E) staining and confocal imaging.

AHR, BALF, and morphologic analysis

Mice were anesthetized with 2.5 mg/kg tiletamine and xylazine (Zoletil and Lumpum; Bayer Korea, Seoul, Korea), and AHR was assessed after challenges with 0, 5, 20, or 100 mg/mL methacholine (Sigma-Aldrich). The following day, BALF was obtained and centrifuged, and the supernatant was stored (−20°C). The cell pellet was resuspended for cell counting, and cytospin slides were made for cell differentials (500 cells per mouse, Diff-Quick-staining). A portion of the lung was fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, sectioned (4 μm), and stained (H&E stain).

RNA extraction and quantitative RT-PCR

RNA was isolated with a TRI Reagent (Molecular Research Center, Cincinnati, Ohio). For human cell line RNA, cDNA was prepared from 3 μg of RNA by using oligo dT, RNaseOUT, and Superscript II reverse transcriptase (42°C for 50 minutes; Invitrogen, Carlsbad, Calif), followed by heating inactivation (70°C for 15 minutes). Quantitative RT-PCR (qRT-PCR) was performed in a 20-μL reaction with 3 μg of cDNA, 1 μL of primer, and 4 μL of Taq DNA polymerase (Life Technologies, Grand Island, NY). For human cell lines, PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles with denaturation at 95°C for 30 seconds, 60°C for 30 seconds, and annealing for 72°C for 7 minutes. The primers were as follows: human CLDN5 (forward: GATGGGGTGCGGGCAGTA, reverse: CACGGATTGGCTGCTTCG) and human β-actin (forward: CATCCGCAAAGACCTGTACG, reverse: CCTGCTTGCTGATCCACATC).

For mouse lung RNA, cDNA was prepared from 3 μg of RNA with Superscript III and First-Strand Synthesis SuperMix (Invitrogen). qRT-PCR was conducted with Power SYBR Green PCR Master Mix with a qRT-PCR system (StepOne; Life Technologies, Grand Island, NY). Primers used for amplification were as follows: mouse CLDN5 (forward: CAAGGTGTATGAATCTGTGCT, reverse: GTCAAGGTAACAAAGAGTGCCA) and mouse β-actin (forward: ACGTTGACATCCGTAAAGA, reverse: GCCACGTTGACATCCGTAAAGA). The relative CLDN5 transcript level was calculated according to the $2^{-\Delta\Delta Ct}$ method normalized to β-actin.

Immunofluorescence staining

Mouse lung sections were deparaffinized and rehydrated in an ethanol series. The sections were blocked for nonspecific binding with 1.5% normal horse serum and incubated with CLDN5 (1:400; Abcam, Cambridge, Mass) with Alexa Fluor 488–conjugated donkey polyclonal anti-rabbit IgG (1:1000, Abcam), and nuclei were counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride (1:1000, Invitrogen). Confocal laser microscopy was performed with a fluorescence microscope at 6400 (Carl Zeiss Microsystems, Thornwood, NY) at × 20 magnification and LSM 510 META with a × 40 objective by using a confocal laser scanning microscope camera. Sections were observed by using confocal laser scanning microscopy (LSM 510 META), and images were generated with the Zeiss LSM image browser.

Immunohistochemistry

Mouse lung sections were deparaffinized and rehydrated in an ethanol series. The sections were treated with 1.4% H_2O_2 in methanol for 30 minutes to block endogenous peroxidase and then blocked for nonspecific binding with 1.5% horse serum and incubated with the anti-rabbit CLDN5 (1:100, Abcam). The next day, sections were incubated with the ABC Kit (Vector Laboratories, Burlingame, Calif). Color reaction was developed by staining with the Liquid DAB + Substrate Kit (Golden Bridge International, Mukilteo, Wash). After immunohistochemical staining, the slides were counterstained with Harris hematoxylin for 1 minute. Images were analyzed with the ImageJ program

(National Institutes of Health, Bethesda, Md). Vessel density in each case was quantified with an average of CLDN5 arbitrary density numbers from 6 to 8 fields.

Western blotting

Extracted lung tissue were homogenized in a protein lysis solution containing 50 mmol/L Tris-HCL (pH 7.4), 50 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 0.5 mmol/L EDTA, and 100 mmol/L phenylmethylsulfonyl fluoride in distilled water and centrifuged at 14,000 rpm for 30 minutes at 4°C, and insoluble materials were collected. Lung tissue plasma membrane protein was extracted with the MEM kit (Thermo Fisher, Rockford, Ill). Mouse lung proteins were separated by means of SDS-PAGE and transferred to polyvinyl difluoride membranes. The membranes were blocked for 5% BSA in 0.1% Tween 20 and Tris-buffered saline for 2 hours at room temperature, and the membranes were incubated with rabbit anti-CLDN5 (1:1000, Abcam), rabbit anti-AKT1 (1:1000, Cell Signaling, Beverly, Mass), rabbit anti–p-AKT1 (1:1000, Cell Signaling), rabbit anti-FOXO1 (1:1000, Cell Signaling), rabbit anti–phosphorylated FOXO1 (anti–p-FOXO1; 1:1000, Cell Signaling), rabbit anti-CTNNB1 (1:1000; BD Bioscience, Bedford, Mass), rabbit anti–phosphorylated CTNNB1 (anti–p-CTNNB1; 1:1000, Cell Signaling; overnight at 4°C). The membranes incubated with horseradish peroxidase–conjugated secondary antibodies. Detection was performed with the WEST-ZOL plus Western Blot Detection System (iNtRon, Sungnam, Korea). The relative abundance of protein determined by using quantitative densitometric data was normalized to β-actin (Sigma-Aldrich).

ELISA

Levels of CLDN5 and the inflammatory mediators, IL-4 and IL-5 in BALF were measured by means of ELISA (R&D Systems, Minneapolis, Minn). Test sample ODs were adjusted relative to the positive and negative control samples supplied in each kit to compare results from different plates. The mean OD of duplicate wells was calculated. The index value of each tested serum was defined by using the following formula:

Index = (OD of tested serum − OD of negative control)/(OD of positive control − OD of negative control) × 100. Low detection limits were 2 or 7 pg/mL and 0.156 to 10 ng/mL for IL-4 or IL-5 and CLDN5, respectively, based on the manufacturer's recommendations.

Statistical analysis

Data are expressed as means ± SEMs, and the Mann-Whitney *U* test was used to determine significant differences between groups. *P* values of less than .05 were deemed to indicate statistical significance.

RESULTS

Dexamethasone decreases OVA-induced inflammation, cytokine levels, and AHR in mice

OVA-sensitized/challenged mice had increased inflammatory cell counts in BALF compared with control mice. Dexamethasone reduced the OVA-induced increase in numbers of neutrophils, eosinophils, and macrophages in BALF to levels similar to those in saline-treated animals. Cell counts (in millions) were as follows: total cells, 5 ± 3, 304 ± 29, and 24 ± 3; macrophages, 5 ± 2, 90 ± 11, and 5 ± 1; eosinophils, 0.5 ± 3, 208 ± 27, and 16 ± 3; neutrophils, 0.5 ± 1, 11 ± 2, and 0.5 ± 2; lymphocytes, 0.1 ± 0.1, 1 ± 1, and 0.5 ± 0.1 for the control, OVA, and OVA plus dexamethasone groups, respectively. IL-4 (36 ± 3 pg/mL) and IL-5 (20 ± 2 pg/mL) increased in BALF of OVA-sensitized/challenged mice compared with those of control mice (less than the limits of detection = 2 and 7 pg/mL, respectively). Dexamethasone reduced IL-4 (30 ± 3 pg/mL) and IL-5 (13 ± 1 pg/mL) levels in BALF induced by OVA sensitization/challenge. OVA-sensitized/challenged mice had increased AHR compared with control mice. Dexamethasone treatment reduced the OVA-induced increase in AHR to a level slightly greater than that of saline-treated control mice. On histologic examination, OVA-sensitized/challenged mice had numerous focal regions with inflammatory cell infiltrates and peribronchial and intraluminal areas of exudation. Dexamethasone treatment reduced the cellular infiltration induced by OVA sensitization/challenge. The semiquantitative value of the inflammatory index from H&E-stained images was attenuated by dexamethasone in the OVA-sensitized/challenged mice. Increased CLDN5 immunohistochemical staining was noted in mononuclear inflammatory cells and endothelial cells and epithelial cells in the OVA group.

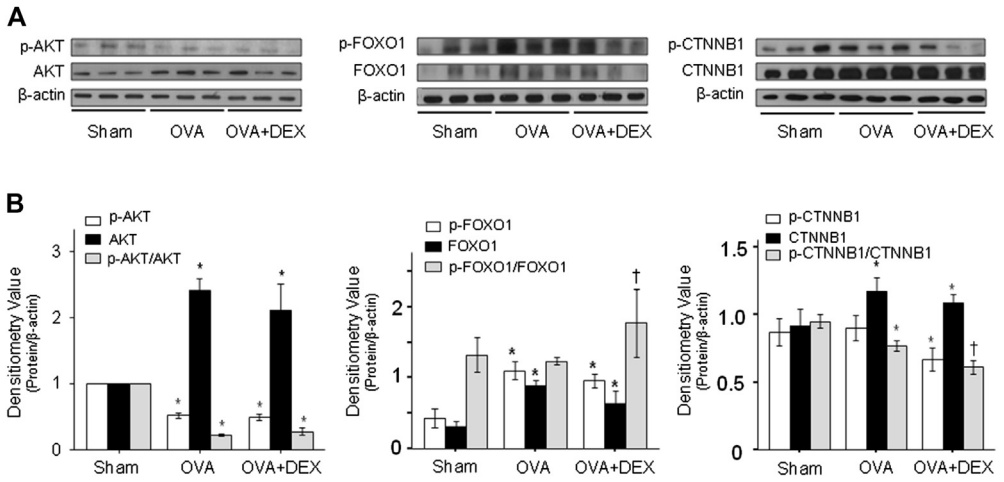

FIG E1. Immunoblot stain of mouse lung protein (n = 6) using antibodies directed to AKT, p-AKT, FOXO1, pFOXO1, CTNNB1, and p-CTNNB1. **A,** Immunoblot of AKT, p-AKT, FOXO1, p-FOXO1, CTNNB1 and p-CTNNB1. **B,** Densitometric analysis of immunoblots. Lung p-FOXO1 protein levels increased in OVA-sensitized/challenged *(OVA)* mice compared with those in saline-treated *(Sham)* mice, whereas p-CTNNB1 and p-AKT1 protein levels decreased. This was accompanied by an increase in p-FOXO1/FOXO1 expression and decreases in p-AKT1/AKT1 and p-CTNNB1/CTNNB1 expression in OVA-sensitized/challenged mice consistent with phosphorylation of FOXO1 as a contributing factor to the increase in CLDN5 protein levels. Values indicate means ± SEMs normalized to β-actin. **P* < .05, OVA versus sham. †*P* < .05, OVA versus OVA plus dexamethasone. *DEX*, Dexamethasone.

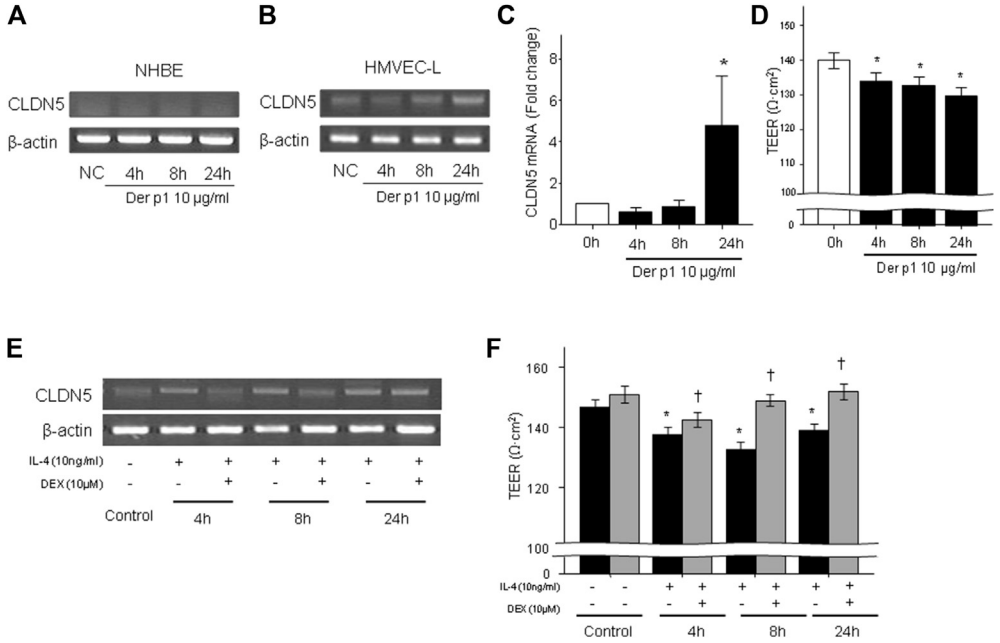

FIG E2. CLDN5 transcript in HMVEC-L and NHBE cells exposed to the house dust mite peptidase Der p 1 or IL-4. **A,** CLDN5 transcripts in NHBE cells treated with control or Der p 1, as determined by using RT-PCR. No change was detected. **B,** CLDN5 mRNA expression in HMVEC-L cells treated with controls and Der p 1 determined by using qRT-PCR. Der p 1 increased CLDN5 transcript levels at 24 hours. **C,** Densitometric analysis of CLDN5 mRNA of Der p 1–treated HMVEC-L cells. Der p 1 increased CLDN5 transcript levels at 24 hours. **D,** Der p 1 reduced TEER levels in HMVEC-L cells compared with control values. **E,** IL-4 increased CLDN5 transcript levels in HMVEC-L cells. This response was inhibited by dexamethasone treatment. **F,** TEER change in HMVEC-L cells treated with control, IL-4, or IL-4 and dexamethasone. Values are means ± SEMs (n = 6). **P* < .05, IL-4 versus control. †*P* < .05, IL-4 versus IL-4 and dexamethasone. *DEX*, Dexamethasone.

TABLE E1. Clinical characteristics in control subjects and patients with asthma

Characteristic	Control subjects	Asthmatic patients	
		Stable	Exacerbation
No. of subjects	25	50	
Sex (male/female)	2/23	20/30	
Age of initial visit (y)	58.3 ± 6.2	54.9 ± 14.1	
Age of asthma onset (y)		47.06 ± 17.25	
Asthma duration (y)		6.63 ± 3.60	
Smoking status (NS/ES/CS)	25/0/0	32/12/6	
Cigarettes smoked, pack years		9.0 ± 15.7	
Body mass index (kg/m^2)	24.8 ± 2.61	25.4 ± 3.3.1	
Initial lung function			
FEV_1 (% predicted)	115.36 ± 16.59	82.42 ± 21.94*	
FVC (% predicted)	96.56 ± 14.51	83.24 ± 17.73*	
FEV_1/FVC ratio	84.24 ± 6.05	73.02 ± 10.88*	
Stable and exacerbate lung function			
FEV_1 (% predicted)		85.43 ± 19.82	62.60 ± 18.14†
FVC (% predicted)		84.83 ± 16.68	66.51 ± 16.76†
FEV_1/FVC ratio		74.53 ± 9.72	68.07 ± 11.84†
PC_{20} (mg/mL)		9.18 ± 10.37	
Total IgE (kU/L)	106.63 ± 188.7	421.5 ± 699.19*	
Atopy	1 (4%)	21 (42%)*	
Attack average/y		3.38 ± 3.24	
Duration of exacerbation during follow-up		6.63 ± 3.61	
Blood WBC/μL	5587.2 ± 1268.0	7768.8 ± 448.9*	9873.0 ± 4917.1
Blood eosinophils (%)	2.73 ± 2.26	5.09 ± 4.97*	3.83 ± 5.42
Blood neutrophils (%)		54.7 ± 12.9	64.6 ± 19.3†

Data are expressed as means ± SDs.

BMI, Body mass index; *CS*, current smoker; *ES*, exsmoker; *FVC*, forced vital capacity; *NS*, nonsmoker.

*$P < .01$ compared with control subjects.

†$P < .05$ compared with patients with stable asthma.