CpG-DNA enhances the tight junction integrity of the bronchial epithelial cell barrier

To the Editor:

Asthma is an inflammatory airway disorder profoundly involving immune system and bronchial tissue function. The

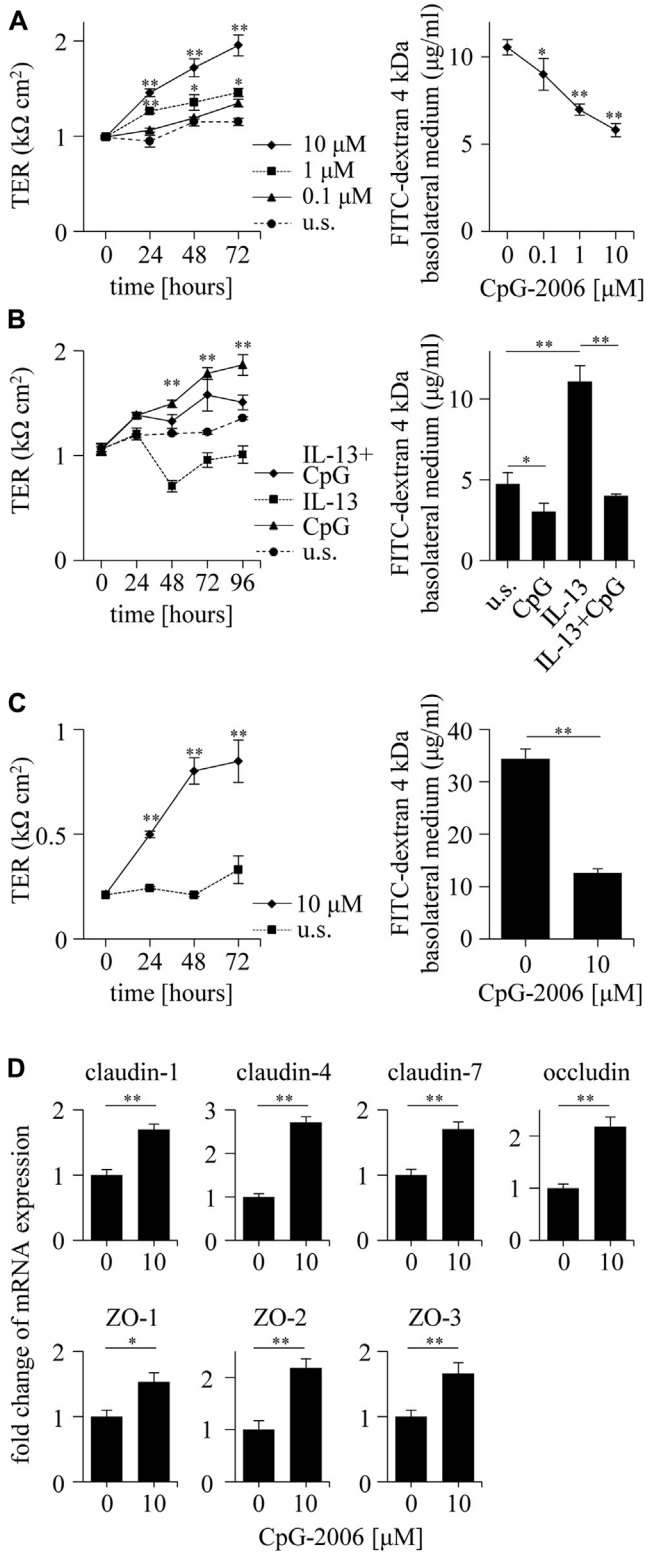

FIG 1. Effect of CpG-2006 stimulation on ALI-cultured bronchial epithelial cells. **A,** *Left panel,* Increased TER over time in healthy donor–derived bronchial epithelial cells at different concentrations of CpG-2006 treatment. *Right panel,* Dose-dependent decrease in FITC-dextran paracellular permeability across nonasthmatic epithelium treated with CpG-2006. CpG-2006 was added to cultures in the beginning of the experiment. **B,** TER *(left panel)* and paracellular FITC-dextran permeability *(right panel)* of epithelial cells from nonasthmatic subjects stimulated by IL-13 with or without pretreatment of CpG-2006. CpG-2006 or IL-13 was added at t = 0 or t = 24, respectively. TER is compared between the IL-13 plus 10 μmol/L CpG-2006– and IL-13–treated groups. **C,** TER kinetics *(left panel)* and paracellular permeability of FITC-dextran *(right panel)* in bronchial epithelial cells from asthmatic patients in response to 10 μmol/L CpG-2006. **D,** Alteration of gene expression of TJ-related molecules in response to 10 μmol/L CpG-2006. Representative data are shown in Fig 1, *A-D* (n = 3). *u.s.,* Unstimulated. **P* < .05 and ***P* < .01.

bronchial epithelial layer serves as the first site of exposure to inhaled allergens, dust particles, pollutants, or microorganisms. Consequently, bronchial epithelial cells are at the forefront of tissue defense and the innate immune response, preventing invasion of tissues as a physical barrier. Tight junctions (TJs) located at the most apical region of the lateral cell membrane seal the epithelium and form an essential part of the barrier between inner tissues and the external environment.[1,2]

Accumulating evidence has revealed dysfunction of the bronchial epithelial barrier in asthmatic patients, as well as in patients with atopic dermatitis and chronic rhinosinusitis.[1,3,4] An impaired barrier allows entry of inhaled substances into submucosal tissues in higher quantities, which might lead to allergen sensitization and induction of innate and adaptive immune responses. In addition, activated immune cells release proinflammatory cytokines that can further disrupt the epithelial barrier and result in airway remodeling.[1] It has been demonstrated that stimulation with different proinflammatory factors, cytokines, and dust mite or pollen extracts compromises the bronchial epithelial barrier.[1,5,6] Therefore barrier dysfunction appears to be a crucial event in the initiation and chronicity of asthma.

We report that CpG-DNA, which is recognized by Toll-like receptor (TLR) 9, enhances barrier function of bronchial epithelial cells by increasing TJ molecule expression. First, we investigated whether CpG-DNA (CpG-2006) could affect the barrier integrity of bronchial epithelial cells. We stimulated air-liquid interface (ALI) cultures of primary human bronchial epithelial cells from nonasthmatic subjects with various concentrations of CpG-2006. Stimulation with 1 or 10 μmol/L CpG-2006 constitutively increased transepithelial electric resistance (TER; Fig 1, *A*, left panel). In parallel experiments paracellular permeability, which was measured as diffusion of fluorescein isothiocyanate (FITC)–conjugated 4-kDa dextran across the epithelial layers, was decreased in a dose-dependent manner (Fig 1, *A*, right panel). There was no difference observed between apical and basolateral CpG administration (see Fig E1 in this article's Online Repository at www.jacionline.org). In addition, other TLR9 ligands, CpG-K3 and CpG-K23, were also able to increase barrier function (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). Control DNA, which cannot activate TLR9, changed neither TER nor paracellular permeability (see Fig E2, *C*). Collectively, these data demonstrate that CpG-DNA can enhance the barrier integrity of healthy bronchial epithelial cells.

It was demonstrated previously that the T_H2 response, particularly when mediated by IL-4 and IL-13, can compromise the bronchial and sinus epithelial barrier.[1,4] Accordingly, we investigated whether CpG-2006 can rescue IL-13–induced barrier impairment. Consistent with previous studies, IL-13 decreased and CpG-2006 increased barrier integrity in ALI cultures (Fig 1, *B*). Of interest, CpG-2006 treatment rescued the decrease in IL-13–stimulated ALI-cultured bronchial epithelial cells (Fig 1, *B*). However, when bronchial epithelial cells were pretreated

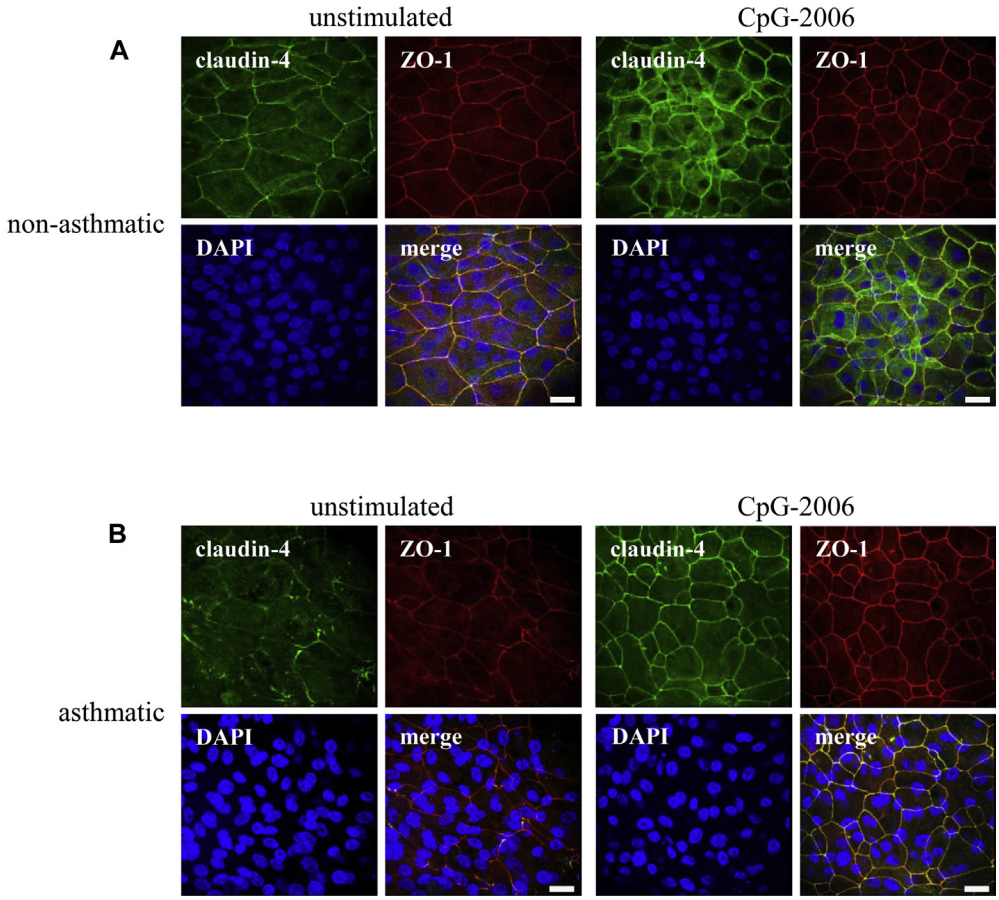

FIG 2. Immunofluorescence detection of claudin-4 *(green)* and ZO-1 *(red)* in ALI-cultured bronchial epithelial cells from nonasthmatic **(A)** and asthmatic **(B)** subjects. Representative pictures from 3 donors each are shown. Cells were fixed after 48 hours of stimulation with 10 μmol/L CpG-2006. 4′,6-diamidino-2-phenylindole *(DAPI)* was used to visualize nuclei. *Bar* = 20 μm.

with IL-13, CpG-2006 could not overcome the barrier impairment (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting that the chronic status or severity of the type 2 immune response during inflammation can influence the efficacy of CpG. Although detailed mechanisms remain to be examined, IL-13 receptor and TLR9 signals might modulate each other for barrier integrity.

These findings focused our interest on investigation of whether CpG-2006 could restore the already impaired barrier of bronchial epithelial cells from asthmatic patients. We observed that CpG-2006 stimulation significantly increased the initially low TER and decreased FITC-dextran flux of bronchial epithelial ALI cultures from asthmatic patients (Fig 1, *C*). These results suggest that the disrupted epithelial barrier in asthmatic patients can be recovered by TLR9-specific ligands.

Next, we evaluated the expression of TJ-related genes using quantitative PCR. Expression of claudin-1, claudin-4, claudin-7, occludin, zonula occludens (ZO)-1, ZO-2, and ZO-3 increased after CpG-2006 stimulation of ALI-cultured bronchial epithelial cells from nonasthmatic donors (Fig 1, *D*, and see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). In contrast, control DNA did not affect the expression of these genes (see Fig E4, *B*).

Because TJ proteins must be correctly assembled into TJ structures to efficiently contribute to the barrier function of the cells, we investigated the distribution of the TJ-associated molecule ZO-1 and claudin-4 protein, which is known as a sealing claudin. CpG-2006–stimulated bronchial epithelial cells from nonasthmatic donors had higher ZO-1 and claudin-4 immunofluorescence at the cell boundary compared with that in unstimulated control cells (Fig 2, *A*, and see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). Bronchial epithelial cells from asthmatic patients showed a constitutive decrease in claudin-4 and ZO-1 immunofluorescence. CpG-2006 stimulation remarkably restored the impaired TJ immunostaining of bronchial epithelial ALI cultures from asthmatic patients (Fig 2, *B*, and see Fig E5, *B*), which is consistent with the TER and FITC-dextran flux measurements shown above. Therefore improvement of the barrier in bronchial epithelial cells by CpG-2006 seems to be mediated by enhanced expression of TJ-related molecules and their appropriate distribution on cell-cell borders.

Rapid increase in the prevalence of asthma over the past several decades was predominantly attributed to alteration of environmental factors rather than genetic changes within a few generations. The hygiene hypothesis explains the increasing morbidity of allergic inflammation ascribed to less exposure to bacteria and their products.[7] Recent reports revealed the protective effect of the airway microbiome, a putative source of CpG-DNA, against allergic airway inflammation.[8] Furthermore, it was

reported that CpG-DNA treatment is a promising therapeutic candidate for asthma.[9] The induction of T_H1 and regulatory T- and B-cell responses were suggested as immunoprotective mechanisms.[7,10,11] In the present study we demonstrated that CpG-DNA improved and restored human bronchial barrier integrity through increased expression of TJ-related molecules and their proper allocation.

In conclusion, these data suggest that administration of CpG-DNA could be a useful intervention and demonstrate an additional explanation for the hygiene hypothesis in both the prevention and treatment of asthma by restoring impaired epithelial barrier.

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New wheat allergens related to baker's asthma

To the Editor:

Baker's asthma is a serious occupational obstructive airway disease affecting 4% to 25% of bakery workers worldwide.[1] Wheat flour is the primary cereal used in bread baking, and wheat flour allergy is the leading cause of baker's asthma.[2,3] The diagnosis of wheat allergy requires the following criteria to be fulfilled: a clinical history with typical exposure-related symptoms and confirmation of type I sensitization by skin prick tests, ImmunoCAP tests, or both and specific inhalation challenge tests (SICTs) when indicated. The natural origin of the native flour extracts used for the commercially available skin prick and ImmunoCAP tests results in batch-to-batch variation, a limited allergen spectrum, and contamination by other components. Therefore the test reliability is limited, and a considerable number of false-negative results with commercial extracts have been shown.[4]

Because of these limitations, the gold standard for the diagnosis of baker's asthma is still the SICT. However, this test is invasive and not free of risk. Therefore there is a need to reduce the number of false-negative results in the diagnosis of wheat sensitization.

Many different wheat allergens are responsible for development of wheat flour allergy, as demonstrated by immunoblotting.[4] To date, some wheat flour allergens have been described at the molecular level, such as allergens belonging to the α-amylase inhibitor family; acyl-CoA oxidase and fructose-bisphosphate aldolase, a wheat glycoprotein with peroxidase activity; triosephosphate isomerase; thioredoxin; glutathione transferase, 1-Cys-peroxiredoxin; dehydrin; and profilin.[4,5] In our previous work we also identified the relevant wheat allergen Tri a 21, a water-insoluble αβ-gliadin.[6] There are 21 wheat allergens (9 of them are food allergens) registered in the International Union of Immunological Societies nomenclature, although none of them have been identified as major allergens. With this lack of major allergens, there is a need to expand the baker's allergen panel by identifying further allergens that can be added to available diagnostics (ie, by spiking commercial extracts with recombinant proteins) to improve their sensitivity and specificity.

With the objective of improving the diagnostic tests by completing the baker's allergen panel, we aimed to identify and characterize further wheat allergens associated with baker's allergic asthma. For this purpose, we produced recombinant allergens because they are of great purity and show higher specificity than native extracts. Sera from 151 bakers with allergic bronchial asthma caused by a type I IgE–mediated response to flour dust were used for IgE immunodetection. The diagnosis of baker's asthma was made based on a clinical history and

METHODS

Cell cultures and stimulations

Primary human bronchial epithelial cells were purchased from Lonza (Basel, Switzerland) or obtained from Dr Marek Sanak and Dr Bogdan Jakiela (Jagiellonian University, Krakow, Poland). All cells lines from Jagiellonian University were collected after obtaining written informed consent, and the protocol was approved by the University Bioethical Committee. Donor information is shown in Table E1. Cells were cultured as monolayers in bronchial epithelial cell basal medium supplemented with the SingleQuots Kit (Lonza) at 37°C in a humidified atmosphere at 5% CO_2. Medium was changed every second day. Cells were seeded at a density of 1.5×10^5 cells in a 6.5-mm diameter polyester membrane with a pore size of 0.4 μm (Corning Costar, Corning, NY) in medium, as previously reported, to develop ALI cultures.[E1] After the cells grew to complete confluence, medium in the apical compartment was removed to allow the cells to differentiate. Stimulation experiments were initiated when TER reached a plateau. For cell stimulation, culture media were supplemented with the following reagents: 0.1 to 10 μmol/L CpG-2006 (5′-TCGTCGTTTGTCGTTTTGTCGTT-3′; Microsynth GmbH, Balgach, Switzerland), 10 μmol/L CpG-K3 (5′-ATCGACTCTCGAGCGTTCTC-3′), 10 μmol/L CpG-K23 (5′-TCGAGCGTTCTC-3′; a kind gift from Dr Ihsan Gursel, Bilkent University, Ankara, Turkey), 1 to 100 μg/mL salmon sperm DNA (InvivoGen, San Diego, Calif), and 10 ng/mL IL-13 (PeproTech, London, United Kingdom). CpG-2006 at 10 μmol/L is equal to 77 μg/mL DNA.

TER and paracellular flux measurement

TER and paracellular diffusion of FITC-labeled 4-kDa dextran (Sigma-Aldrich, St Louis, Mo) were determined as surrogate markers of barrier integrity. TER was measured at indicated time points before and after stimulation with a Millicell-ERS Volt-Ohm Meter (Millipore, Temecula, Calif). Paracellular flux measurements were performed after TER experiments. Paracellular permeability was evaluated 24 hours after apical addition of 2 mg/mL FITC-dextran based on the intensity of FITC in the basolateral medium, as determined with an ELISA reader (Mithra LB 940; Berthold Technologies, Bad Wildbad, Germany) at 480 nm. Each measurement was carried out in triplicates.

mRNA isolation and RT-PCR

Total RNA was extracted and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNase-free DNase (Qiagen), according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by using a RevertAid RT kit containing random hexamers (Thermo Fisher Scientific, Woburn, Mass). Quantitative PCR was performed with target gene–specific primers (Microsynth) and SYBR Green PCR Master Mix (Thermo Fisher Scientific) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif), as described in the manufacturer's protocol. Sequences of the primers for detecting target gene expression are listed in Table E2. The amount of elongation factor 1α mRNA was used to standardize the quantities of each transcript according to the $-2^{\Delta\Delta CT}$ value.[E2]

Immunofluorescence staining of TJs

ALI-cultured cells were fixed with 4% paraformaldehyde (Fluka, Sigma-Aldrich) and permeabilized with PBS containing 0.1% Triton-X (Acros Organics, Gell, Belgium) and 0.02% SDS (Sigma-Aldrich). After rinsing these specimens with PBS, they were incubated with optimally diluted polyclonal rabbit anti–ZO-1 (Invitrogen, Carlsbad, Calif) at room temperature for 45 minutes and Alexa Flour 546–labeled goat anti-rabbit antibody (Invitrogen) and Alexa Flour 488–labeled anti–claudin-4 mAb (clone 3E2C1, Invitrogen) under the same conditions for another 45 minutes. Then slides were mounted with ProLong-Gold Antifade Reagent (Invitrogen) containing 4′,6-diamidino-2-phenylindole for counterstaining of the cell nuclei. Specimens were examined under a confocal microscope (Leica TCS SPE, Leica Microsystems, Heerbrugg, Switzerland). Data were analyzed and fluorescent intensities were quantified with ImageJ software (National Institutes of Health, Bethesda, Md).

Statistics

Data analysis was performed with Prism Version 6 software (GraphPad Software, La Jolla, Calif). Statistical significance was evaluated by using the 2-tailed unpaired Student *t* test or ANOVA with Tukey *post hoc* tests. Graph bars in the figures present means ± SD.

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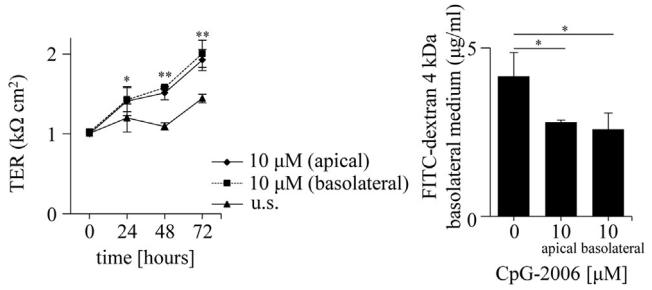

FIG E1. There is no difference in the efficacy of CpG-2006 treatment between apical and basolateral administration in nonasthmatic bronchial epithelial cells. TER time kinetics *(left panel)* and paracellular flux *(right panel)* were measured. CpG-2006 stimulation was started at t = 0 and maintained until finishing the experiment. CpG-2006 treatment of the ALI from apical and basolateral administration versus unstimulated control *(u.s.)*: $*P < .05$ and $**P < .01$.

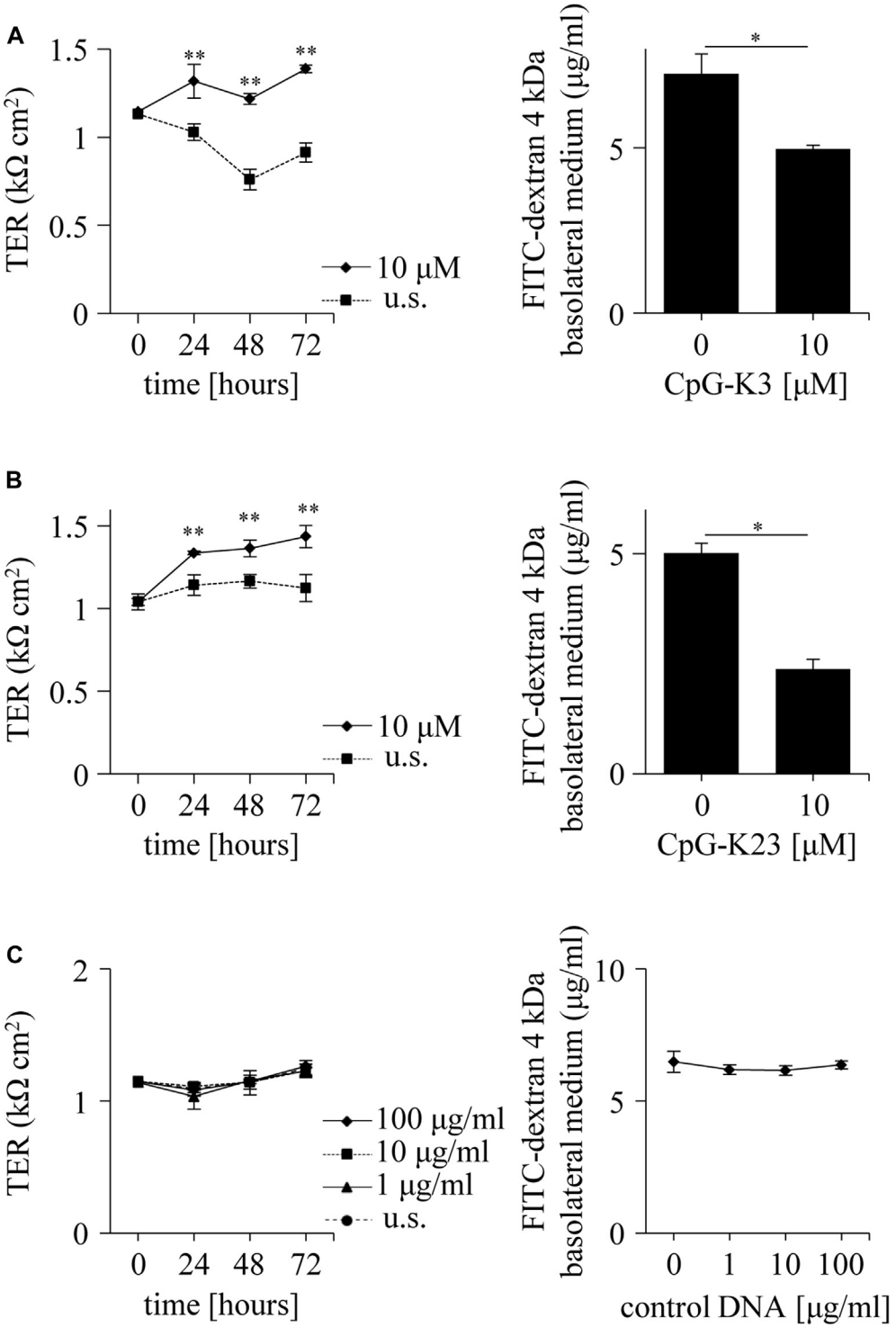

FIG E2. Role of different CpGs and control DNA in the improvement of barrier integrity. Barrier integrity as determined by using TER and paracellular flux in response to CpG-K3 **(A)**, CpG-K23 **(B)**, and control DNA **(C)** stimulation were determined based on TER *(left panels)* and FITC-dextran 4-kDa paracelluar flux *(right panels)*, is shown. Stimulations with CpGs or control DNA were started at t = 0 and maintained until finishing the experiments. CpG-K3 and CpG-K23 also enhance barrier integrity. Control DNA does not affect TER or paracellular permeability of FITC-dextran. *u.s.*, Unstimulated. $*P < .05$ and $**P < .01$.

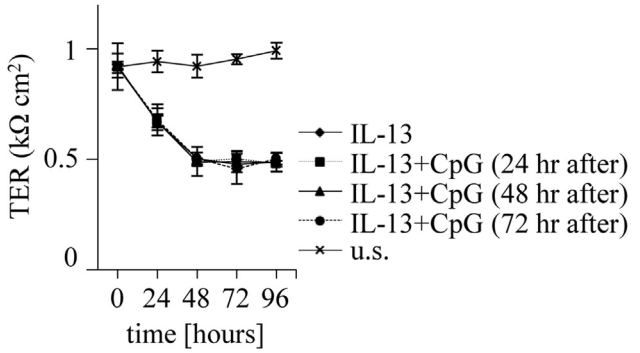

FIG E3. TER kinetics of nonasthmatic bronchial epithelial cells treated with IL-13 and CpG-2006. IL-13 treatment was started at t = 0, and CpG-2006 was added at 10 μmol/L after 24, 48, or 72 hours of IL-13 stimulation. All stimulations were maintained until the end of the experiment. Extended time exposure to IL-13 abolishes reactivity to CpG. *u.s.*, Unstimulated.

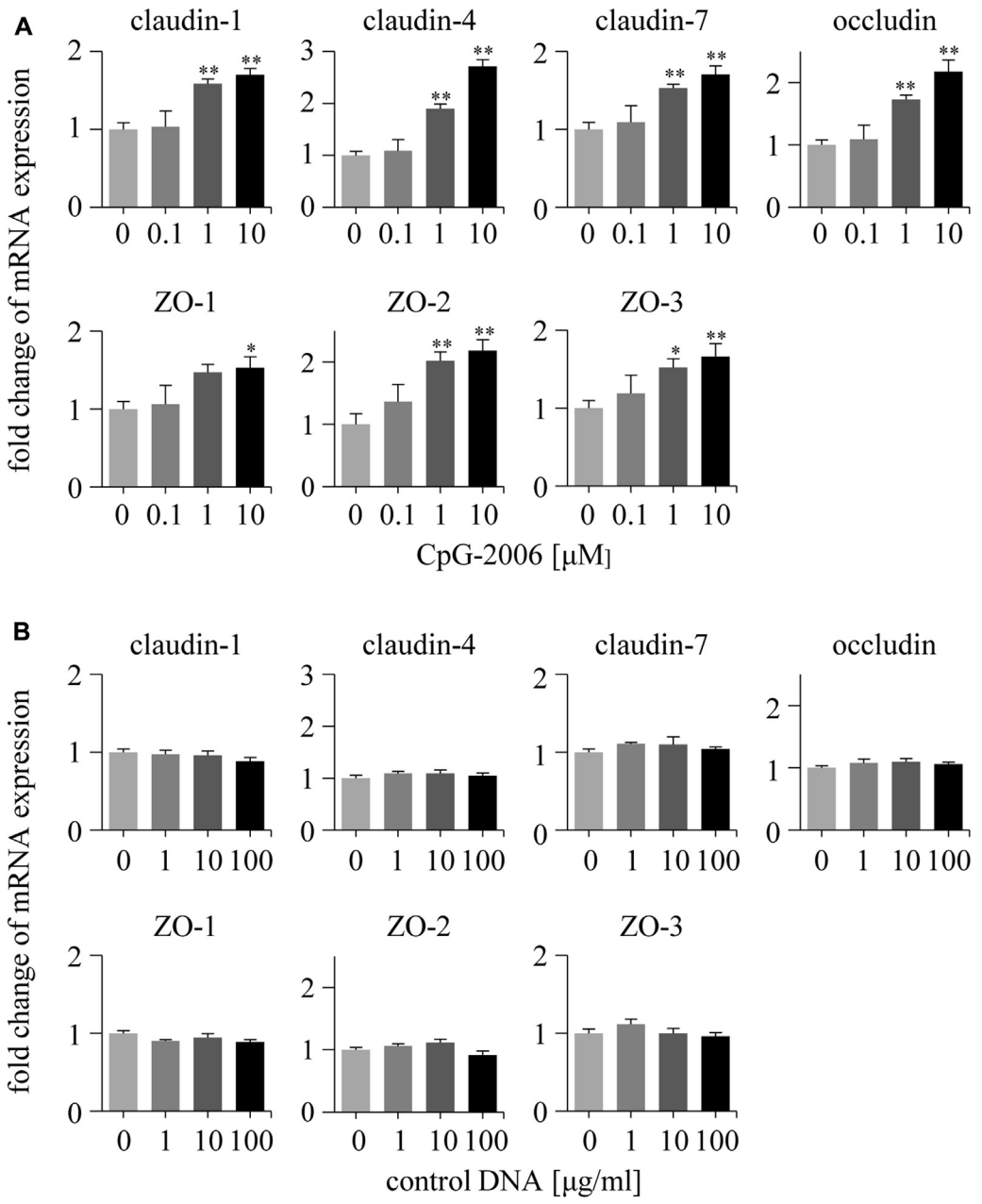

FIG E4. CpG-DNA enhances mRNA expression of TJs, claudin-1, claudin-4, claudin-7, and occludin, as well as that of the TJ-associated molecules ZO-1, ZO-2, and ZO-3. The dose-dependent response of mRNA expression of TJ-related molecules is shown. Bronchial epithelial cells from nonasthmatic subjects were treated with CpG-2006 **(A)**. Control DNA did not show any effect **(B)**. $*P < .05$ and $**P < .01$.

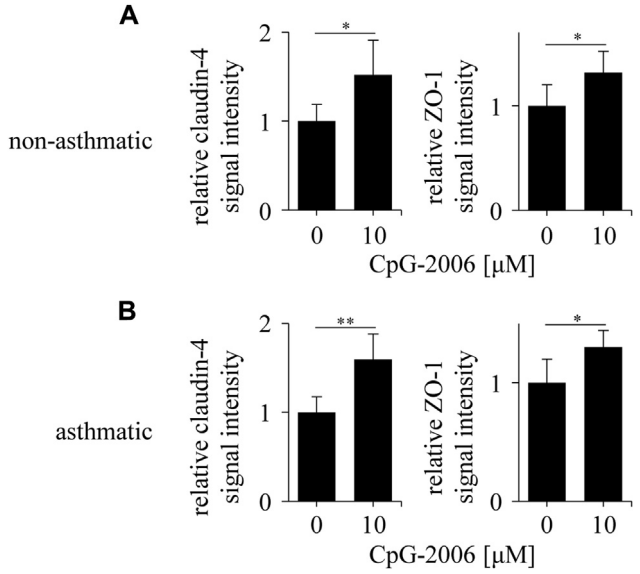

FIG E5. Relative fluorescence signal intensity of claudin-4 and ZO-1 with or without CpG-2006 stimulation in bronchial epithelial cells from nonasthmatic **(A)** and asthmatic **(B)** subjects. Each value was calculated after removing background signal by using ImageJ software. Five different view fields were compared (n = 3). $*P < .05$ and $**P < .01$.

TABLE E1. Information of donors used in this study

Donor	Asthmatic/nonasthmatic	Age (y)	Sex	Ethnic group
1	Nonasthmatic	67	Male	White
2	Nonasthmatic	24	Female	White
3	Nonasthmatic	56	Male	White
4	Nonasthmatic	56	Female	Black
5	Nonasthmatic	44	Female	Hispanic
6	Nonasthmatic	42	Male	White
7	Asthmatic	54	Male	White
8	Asthmatic	15	Female	White
9	Asthmatic	25	Male	Hispanic
10	Asthmatic	67	Male	White

TABLE E2. Primer sequences for quantitative PCR (indicated as 5′-3′)

Gene	Forward primer	Reverse primer
Claudin-1	CAG TCA ATG CCA GGT ACG AAT TT	AAG TAG GGC ACC TCC CAG AAG
Claudin-4	TGT ACC AAC TGC CTG GAG GAT	GAC ACC GGC ACT ATC ACC ATA A
Claudin-7	GGG CAT GAA GTG CAC GCG CT	CGG CAA GAC CTG CCA CGA TG
Occludin	GAT GAG CAG CCC CCC AAT	GGT GAA GGC ACG TCC TGT GT
ZO-1	ACA GTG CCT AAA GCT ATT CCT GTG A	TCG GGA ATG GCT CCT TGA G
ZO-2	CGG TTA AAT ACC GTG AGG CAA A	GGG AAC CAC TGG GTG TAA TTC A
ZO-3	TGG AGA GTC CCC GGC TTC GG	TCC CTG GGC AAC TCT GAC CGT
EF1α	CTG AAC CAT CCA GGC CAA AT	GCC GTG TGG CAA TCC AAT

EF1α, Elongation factor 1.