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IL-17A induces chromatin remodeling promoting IL-8 release in bronchial epithelial cells: Effect of Tiotropium

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ABSTRACT

Aims: IL-17A plays a key role in the persistence of airway inflammation, oxidative stress, and reduction of steroid-sensitivity in COPD. We studied the effect of IL-17A on chromatin remodeling and IL-8 production.
Main methods: We measured the levels of IL-8 and IL-17A in induced sputum supernatants (ISS) from healthy controls (HCs), healthy smokers (HSs), and COPD patients by enzyme-linked immunosorbent assay (ELISA). A human bronchial epithelial cell line (16HBE) was stimulated with ISS from HCs, HSs, or COPD subjects. IL-8 was evaluated in 16HBE by Western blot and real-time polymerase chain reaction (PCR). Histone deacetylase 2 (HDAC2), acetyl histone H3 (Ac-His H3) (k9) and inhibitor kappa kinase alpha (IKKα) levels were evaluated in the nuclear extract by Western blot. Finally, we evaluated the effect of IL-17A depletion in ISS, the silencing of IKKα, and the anti-inflammatory effects of Tiotropium Spiriva® (100 nM) on 16HBE.
Key findings: IL-8 and IL-17A levels were higher in ISS from COPD patients and HSs than from HCs. IL-8 protein and messenger RNA (mRNA) levels were increased in 16HBE stimulated with ISS from COPD patients compared with untreated cells. Furthermore, ISS from COPD patients reduced the nuclear levels of HDAC2 while increasing the activity of both Ac-His H3 (k9) and IKKα in stimulated 16HBE. IL-17A depletion in ISS and the IKKα silencing in 16HBE significantly increased the nuclear levels of HDAC2, reduced Ac-His H3 (k9), and promoted IL-8 synthesis in stimulated 16HBE. Tiotropium controls the proinflammatory activity generated by ISS from COPD patients in 16HBE.
Significance: IL-17A present in the airway of COPD patients, which induces chromatin remodeling, promotes the release of IL-8 in the bronchial epithelium. Tiotropium is able to control this proinflammatory activity.
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1. Introduction

The hallmarks of chronic obstructive pulmonary disease (COPD) are small-airway obstruction and emphysema which, either alone or together, are involved in the inflammation and progressive airflow limitation usually caused by tobacco smoke. However, the inflammation observed in the small and the larger airways are similar in these patients [1]. COPD subjects often show steroid resistance generated by cigarette smoke promoting the activation of macrophages, and oxidative stress (via peroxynitrite formation) specifically impairs the activity of histone deacetylase 2 (HDAC2); ultimately, the activation of nuclear factor kappa B (NFκB) is enhanced and the anti-inflammatory effects of corticosteroids are diminished [2,3].

Interleukin (IL)-17A produced by T helper cells triggers the expression of inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO). These radicals can increase the transcription of IL-8 messenger RNA (mRNA), leading to IL-8 protein production [4]. In fact, IL-17A is a potent inducer of IL-8, a chemokine with a key role in the persistence of airway inflammation and the reduction of steroid sensitivity, thereby exerting its action on human bronchial epithelial cells [5,6].

NFκB regulates the production of cytokines and chemokines associated with airway inflammation [7]. It is activated by phosphorylation, and the degradation of inhibitor kappa B (IκB) by IκB kinases (inhibitor kappa kinase alpha (IKKα) and IKKβ) leads to the nuclear translocation of NFκB and the subsequent transcription of NFκB-dependent genes [8]. In the nucleus, IKKα was found to phosphorylate histone H3 at the serine (Ser) 10 position, a step crucial for subsequent histone acetylation

Abbreviations: IL-17A, interleukin-17A; IL-8, interleukin-8; ISS, induced sputum supernatant; HDAC2, histone deacetylase 2; Ac-His H3, acetyl histone H3; IKKα, inhibitor kappa kinase alpha; COPD, chronic obstructive pulmonary disease.

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and gene transcription [9]. In this case, IKKs might be ideal molecular targets to inhibit the inflammation and the HDAC2 activity, thus regulating molecular mechanisms leading to corticosteroid resistance in COPD [1]. The production of IL-8 from airway epithelial cells is NFκB dependent, requiring IKKα and IKKβ activity [10,11]. However, it remains unclear whether IL-17A promotes the production of IL-8 by a molecular mechanism involving IKKα and IKKβ activity in bronchial epithelial cells.

The current guidelines for the treatment of COPD recommend the regular use of β2-adrenergic (AR) agonists and anticholinergic bronchodilators to maximize bronchodilation [12,13]. However, several studies suggest the potential anti-inflammatory role of these drugs. Therefore, the effect of anticholinergic drugs on the molecular mechanism involving IKKα and IKKβ activity in the IL-8 production generated by IL-17A has not yet been studied extensively in bronchial epithelial cells.

We investigated the release of IL-8 in the induced sputum supernatants (ISS) from COPD patients during the activation of bronchial epithelial cells by IL-17A present in the airways. Furthermore, we studied the effect of IL-17A on mediated chromatin remodeling mechanisms involving IKK-driven NFκB activation of IL-8 gene transcription. Finally, we tested the efficacy of the anticholinergic drug Tiotropium Spiriva®, generally used to treat COPD, on the previously mentioned activity of IL-17A in bronchial epithelial cells.

2. Materials and methods

2.1. Patients

We recruited three groups of subjects: healthy asymptomatic nonsmoking subjects with normal lung function (healthy controls, HCs) ($n = 14$), asymptomatic smokers with normal lung function (healthy smokers, HSs) ($n = 10$), and COPD subjects ($n = 16$). The diagnosis of COPD and the assessment of its severity were defined and classified based on the criteria established in the Global Initiative for Obstructive Lung Disease (GOLD) guidelines for COPD management (GOLD stage ≥I) (stage I, $n = 13$; stage II, $n = 3$) [14]. COPD subjects whose symptoms exacerbated within a month before the study were excluded. Patients with COPD had a smoking history of ≥10 pack years.

All COPD patients were in stable condition. All COPD patients with obvious emphysema on routine chest X-rays and computed tomographic scans were excluded. All patients were characterized with respect to gender, age, smoking history, COPD symptoms, comorbidity, and current history of treatment. The exclusion criteria were as follows: other systemic diseases, chronic bronchitis, chronic spontaneous sputum production, other lung diseases, upper and lower respiratory tract infections, treatment with glucocorticoids or anticholinergics within 3 months before the study, and treatment with long-acting β-AR agonists 15 days before the study.

The local ethics committee approved the study, and the participating subjects gave their informed consent.

The bronchodilator reversibility test was conducted to exclude the asthmatic component. In all COPD subjects, the increase in forced expiratory volume in the first second (FEV1) after salbutamol administration was <12% (200 mL) compared with basal values.

2.2. Sputum induction and processing

Sputum induction and processing were performed according to the plug method as described in a previous study [15]. Briefly after collecting of the sputum, the plugs were selected and processed with 4× w/v of 0.1% dithiothreitol (DDT), following which 4× w/v of phosphate-buffered saline (PBS) was added (PBS 1x; Gibco). The resulting suspension was vortexed for 30 s and then centrifuged at 1000*g* for 20 min. The ISS were then aspirated and frozen at −80 °C in separate aliquots for the subsequent biochemical analyses. The cells obtained from the induced sputum (IS) were then cytocentrifuged (Cytospin 2; Shandon, Runcorn, UK) and stained with May–Grünwald–Giemsa. For differential cell counts, two independent investigators, who counted at least 400 cells per slide, examined the slides blindly.

2.3. Measurement of IL-17A and IL-8

The levels of IL-17A in the ISS were measured using a commercial available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA). The lower limit of detection was 15 pg/mL. The levels of IL-8 in ISS and in 16HBE supernatants were determined, using commercial ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's specifications. The lower limit of detection for IL-8 was <5 pg/mL.

2.4. Bronchial epithelial cell cultures

The SV40 large T antigen-transformed 16HBE cell line (16HBE) was used in this study. 16HBE is a cell line that retains the differentiated morphology and function of normal airway epithelial cells. The cells represent a clonal diploid ($2n = 6$) cell line isolated from the human lung. 16HBE cells were cultured as adherent monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS), 1% MEM (nonessential amino acids, Euroclone), 2 mM L-glutamine, and 250 μg/mL of gentamicin at 37 °C in a humidified 5% CO_2 atmosphere. Evidence has shown that 16HBE cells are similar to primary normal human bronchial epithelial (NHBE) cells (Lonza, Brussels, Belgium), and to bronchial epithelial cells (BECs) from bronchial brushings in terms of the response to inflammatory stimuli and anti-inflammatory drugs [16].

2.5. Bronchial epithelial cells stimulation

16HBE cells (180,000 cells/well) were plated in standard six-well culture plates in MEM 10% FBS and grown to confluence (70–80%). After 1 h of incubation in 1 mL of MEM 1% FBS, the 16HBE cells were stimulated with ISS (20%) from HCs ($n = 6$), HSs ($n = 6$), and COPD subjects ($n = 6$) or with recombinant human (rh) IL-17A (20 ng/mL) ($n = 6$). ISS from COPD patients with IL-17 concentrations closest to the median of the values were selected to stimulate 16HBE. The samples selected from the COPD group belonged to GOLD stage I (five) and GOLD stage II (one). Approximately 200 μL of ISS was incubated with an anti-IL-17A antibody (Ab) for 1 h at 37 °C to neutralize the specific activity before the stimulation of 16HBE ($n = 6$). To determine the effects of anticholinergic bronchodilators on IL-17A activity, Tiotropium Spiriva® (100 nM) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) was added to 16HBE 30 min before the stimulation with ISS from COPD ($n = 6$), with rhIL-17A (20 ng/mL) ($n = 6$), or with DTT 0.1% diluted 1:2 with PBS (used as a vehicle of the ISS under experimental conditions). 16HBE cells were stimulated for 24 h to test IL-8 production by ELISA and real-time polymerase chain reaction (PCR), for 4 h to test HDAC2 and acetyl histone H3 (Ac-His H3) (k9), and for 2 h to test IKKα.

2.6. Total and cytoplasmic/nuclear protein extraction

16HBE cells were washed with cold PBS and lysed in a buffer containing 10 mmol/L Tris—HCl (pH 7.4), 50 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40. The phosphatase inhibitors consisted of 20 mmol/L β-glycerophosphate, 0.3 mmol/L Na_3VO_4, and 1 mmol/L benzamidine (ICN Biochemicals, Inc., Aurora, OH, USA), and the protease inhibitors consisted of a cOmplete protease inhibitor cocktail (Roche). Nuclear extracts were obtained using an NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific), which ensures efficient cell lysis and extraction of separate cytoplasmic and nuclear protein fractions by centrifugation. Then,

25–30 μg of the lysate was denatured under reducing conditions by boiling for 3 min in 50 mM Tris—HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, and 0.01% bromophenol blue. The total and cytoplasmic/nuclear protein extracts were analyzed by Western blot.

2.7. Western blot analysis

The total and nuclear proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred via electrophoresis onto Immobilon-P membranes (Millipore, Bedford, MA, USA). After transfer, the membranes were blocked overnight at room temperature in PBS containing 3% BSA and 0.5% Tween 20, and then incubated for 1 h at room temperature with the primary Abs. After washing, the blot was incubated for 45 min with the appropriate horseradish peroxidase-conjugated secondary Ab; the bound Ab was detected using the ECL chemiluminescence detection system (Amersham-Pharmacia, Biotech), according to the manufacturer's instructions. The membranes were stripped and reprobed with housekeeping proteins such as β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Abs to normalize the differences in protein loading. Autoradiographic films were subjected to densitometric analysis using the NIH Image/Gel Plotting analysis program (National Institutes of Health, Bethesda, MD, USA). The results were normalized and expressed as the ratio of the tested protein band intensity to β-actin.

2.8. Antibodies

Rabbit anti-human IKKα and anti-Ac-His H3 were obtained from Millipore (Temecula, CA, USA), and diluted in a ratio of 1:500. Mouse anti-human HDAC2, mouse anti-human IL-8 (B-2), and rabbit anti-human IL-17 (H-132) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and diluted 1:100. Finally, mouse monoclonal anti-β-Actin Ab was obtained from Sigma (St. Louis, MO, USA), and diluted 1:20,000.

2.9. HDAC activity

Cells were stimulated for 4 h with ISS from HCs, HSs, and COPD subjects. The protein levels were determined using a bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, IL, USA). HDAC activity in the nuclear extracts was assessed by a fluorometric HDAC activity assay (BioVision, Mountain View, CA, USA), performed according to the manufacturer's instructions, and expressed as Fluorescence Units (F.U.) normalized to micrograms of protein.

2.10. Quantitative real-time reverse transcription-PCR of IL-8

Total RNA was extracted from 16HBE cells using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. Then, it was reverse-transcribed into complementary DNA (cDNA), using M-MLV-RT and oligo$(dT)_{12-18}$ primer (Invitrogen). Quantitative real-time PCR of the IL-8 transcript was carried out in a StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for IL-8, Hs00174103m1, Assays on Demand, Applied Biosystems). IL-8 gene expression was normalized to GAPDH endogenous control gene. The relative quantitation of gene expression was performed with the comparative C_T method ($2^{-\Delta\Delta Ct}$) and plotted as fold-change compared with untreated cells as the reference sample.

2.11. Silencing

To confirm the role of IKKα in HDAC2 translocation and histone H3 acetylation, we investigated the effect of IKKα silencing in human bronchial epithelial cells using specific short interfering RNA (siRNA) transfection. 16HBE cells were plated in six-well tissue culture plates and grown in medium containing 10% FBS without antibiotics until 60–80% confluence. IKKα siRNA (10 μM; Santa Cruz Biotechnology, Inc.) was then added to 100 μL of the siRNA transfection medium, and the reaction was carried out according to the manufacturer's instructions until complete cell transfection was achieved (7 h at 37 °C). For optimal siRNA transfection efficiency, siRNA (10 μM; Santa Cruz Biotechnology, Inc.) containing a scrambled sequence, which did not lead to the specific degradation of any known cellular mRNA, was used to control the nonspecific effects. Finally, cells were stimulated with ISS from COPD (20%) patients for 24 h and the total and nuclear proteins were extracted. The silencing efficacy of IKKα RNA interference was assessed by Western blot analysis.

2.12. Co-immunoprecipitation

16HBE cells were washed with cold PBS 1x before being lysed in a mild protein lysis buffer (50 mM Tris—HCl, 150 mM NaCl, 10 mM EDTA, and 0.1% Nonidet P-40) with protease and phosphatase inhibitors. The cell lysate was precleaned with protein A agarose beads (Protein A/GPlus-Agarose, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and subsequently incubated overnight with a rabbit anti-Ac-His H3Ab (pull-down). Protein A agarose beads were added and incubated for 1 h at 4 °C. The immunoprecipitates (IPs) were washed and boiled in 2× SDS sample buffer for 5 min, and then centrifuged. The cell lysates were separated on 10% SDS/PAGE gels for Western blot using a rabbit anti-human IKKα Ab. Nonimmunized IgG was used as the pull-down control to confirm the binding specificity. The total protein without IPs was run at the same time as the control.

2.13. Statistical analysis

The Kolmogorov–Smirnov normality test was conducted to assess normal data distribution (p-value >0.05). Clinical demographics data and experimental data were not normally distributed and were analyzed using the Kruskal–Wallis test, followed by Bonferroni's test correction for multiple comparisons. In vitro experiments were normally distributed and analyzed using ANOVA, followed by Fisher's correction. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Demographic characteristic of patients and differential cell counts of IS

The patient characteristics are summarized in Table 1. According to the differential cell counts of the IS samples, the number of cells significantly increased in HSs and COPD subjects. This increase reflected an increase in the number of both macrophages and neutrophils in HSs, whereas COPD subjects showed a large increase in the number of neutrophils but significantly fewer macrophages in HSs and in HCs. A significantly greater number of eosinophils were reported in COPD subjects than in HCs, whereas lymphocytes did not differ significant among the three study groups (Table 2).

3.2. Levels of IL-8 and IL-17A in ISS

IL-8 concentrations were significantly higher in ISS from HSs and COPD patients compared with ISS from HCs ($p < 0.001$ and $p < 0.0001$, respectively) and in ISS from COPD patients compared with ISS from HSs subjects ($p < 0.05$) (Fig. 1A). Furthermore, IL-17A concentrations were significantly higher in ISS from COPD patients and from HSs than in HCs ($p < 0.0001$ and $p < 0.05$, respectively) (Fig. 1B).

3.3. Effect of ISS on IL-8 release in 16HBE cells

The protein extract from 16HBE showed a significant increase in IL-8 protein expression when stimulated with ISS from COPD patients and

Table 1
Demographic characteristics of patients.

	HCs ($n = 14$)	HSs ($n = 10$)	COPD ($n = 16$)	p-Value		
				HCs vs HSs	HCs vs COPD	HSs vs COPD
Age, year	65 ± 7	61.5 ± 8	62 ± 12	ns	ns	ns
Gender, male/female	7/7	6/4	7/9	–	–	–
FEV1, % predicted	108.6 ± 12.6	105.2 ± 10	58.7 ± 24.5	ns	<0.006	<0.03
FVC, % predicted	109.2 ± 16.7	109.1 ± 15	72.6 ± 19.2	ns	<0.04	<0.04
FEV1/FVC (%)	93.6 ± 3.4	89.3 ± 4.9	64.4 ± 9.0	ns	<0.002	<0.02
Smoking, pack/year	0	60 ± 18.0	63 ± 18.0	<0.001	<0.001	ns

Data are presented as mean ± SD. Abbreviations: HCs = healthy asymptomatic nonsmoking subjects with normal lung function; HSs = asymptomatic smokers with normal lung function; COPD = subjects with Chronic Obstructive Pulmonary Disease; FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity. Statistical analysis were performed using the Kruskal–Wallis followed by Bonferroni–Dunn correction for multiple comparison.

from HSs compared with HCs ($p < 0.019$ and $p < 0.032$, respectively), or compared with untreated 16HBE ($p < 0.022$ and $p < 0.037$, respectively (Fig. 2A). The stimulation of 16HBE with rhIL-17A significantly increased the production of IL-8 in the cell protein extract compared with untreated cells ($p < 0.035$). The preincubation of the cells with Tiotropium significantly reduced the effect of rhIL-17A on IL-8 production in 16HBE ($p < 0.05$) (Fig. 2B).

Reverse transcription (RT)-PCR analysis of IL-8 mRNA showed that 16HBE stimulated with ISS from COPD subjects showed higher levels of mRNA expression (as indicated by the fewer amplification cycles required) compared with untreated cells, 16HBE cells pretreated with Tiotropium (100 nM), or 16HBE cells stimulated with ISS from COPD subjects treated with anti-IL-17A (Fig. 2C). Furthermore, 16HBE stimulated with Tiotropium (100 nM) or with ISS from COPD patients pretreated with an anti-IL-17A Ab significantly reduced the expression of IL-8 compared with 16HBEcells stimulated with ISS from COPD patients ($p < 0.001$ and $p < 0.006$, respectively) (Fig. 2D). The treatment with anti-IL-17A Ab or Tiotropium did not affect IL-8 production in terms of protein and mRNA (data not shown) in untreated 16HBE. As Tiotropium is currently used in the pharmacological treatment of these patients, we tested its anti-inflammatory activity only in the presence of ISS from COPD subjects.

3.4. Chromatin remodeling after ISS treatment

ISS from COPD patients and HSs significantly decreased the levels of nuclear HDAC2 protein obtained by Western blot analysis ($p < 0.018$ and $p < 0.003$, respectively) (Fig. 3A) as well as the levels of nuclear HDAC activity obtained by a fluorometric assay ($p < 0.05$ and $p < 0.009$, respectively) (Fig. 3B) in 16HBE cells compared with untreated cells. Furthermore, ISS from COPD patients and HSs significantly decreased the levels of nuclear HDAC2 protein in 16HBE cells compared with the cells stimulated with ISS from HCs ($p < 0.001$ and $p < 0.007$, respectively) (Fig. 3A). Accordingly, the levels of nuclear HDAC activity ($p < 0.013$ and $p < 0.007$, respectively) (Fig. 3B) were significantly decreased in 16HBE cells stimulated with ISS from COPD patients and HSs compared with cells stimulated with ISS from HCs. We found that the nuclear levels of HDAC2 expression and activity were significantly reduced in 16HBE cells stimulated with rhIL-17A compared with untreated cells ($p < 0.023$ and $p < 0.003$, respectively). Preincubation of the cells with Tiotropium significantly reduced the effect of rhIL-17A on the nuclear levels of HDAC2 expression and activity ($p < 0.025$ and $p < 0.017$, respectively) (Fig. 3C, D). Accordingly, we showed that the pre-treatment of 16HBE cells with Tiotropium (100 nM) significantly increased HDAC2 translocation from the cytoplasm to the nucleus ($p < 0.014$) as well as HDAC activity ($p < 0.001$) in the cells stimulated with ISS from COPD patients compared with cells treated with only ISS from COPD patients. Furthermore, we showed that the stimulation of cells with ISS from COPD patients, treated with an anti-IL-17A Ab, significantly increased HDAC2 translocation from the cytoplasm to the nucleus ($p < 0.02$) and HDAC activity ($p < 0.005$) in 16HBE cells compared with cells treated with only ISS from COPD subjects (Fig. 3E, F). Treatment with Tiotropium or anti-IL-17A Ab did not affect the nuclear levels of HDAC2 and HDAC activity (data not shown) in untreated 16HBE cells.

3.5. Nuclear levels of histone H3 acetylation and IKKα

The analysis of nuclear protein lysates showed that the levels of Ac-His H3 (k9) and IKKα were significantly increased in 16HBE cells stimulated with rhIL-17A compared with untreated cells ($p < 0.006$ and $p < 0.001$, respectively). The pretreatment of 16HBE cells stimulated with Tiotropium significantly reduced this effect ($p < 0.047$ and $p < 0.003$, respectively) (Fig. 4A, B). Furthermore, we found a statistically significant increase in the Ac-His H3 (k9) levels when the cells were treated with ISS from HSs and from COPD patients compared with untreated cells ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 4C). The nuclear levels of Ac-His H3 (k9) were statistically significantly restored in 16HBE cells pretreated with Tiotropium or with ISS from COPD patients treated with an anti-IL-17A Ab, compared with cells treated with ISS from COPD patients ($p < 0.001$ and $p < 0.0001$, respectively) (Fig. 4D). These findings support an inverse correlation between Ac-His H3 (k9) and HDAC2 at the nuclear levels.

The nuclear IKKα protein level was higher in cells stimulated with ISS from COPD patients compared with untreated 16HBE cells ($p < 0.001$). Furthermore, the pretreatment of the cells with Tiotropium (100 nM) significantly reduced the translocation of nuclear IKKα

Table 2
Total and differential cell count from induced sputum.

	HCs ($n = 14$)	HSs ($n = 10$)	COPD ($n = 16$)	p-Value		
				HCs vs HSs	HCs vs COPD	HSs vs COPD
Macrophages (%)	81.5 (67.8–89.4)	51.9 (47.1–68.1)	24.6 (12.4–52.2)	<0.02	<0.003	ns
Neutrophils (%)	19.7 (10–29.1)	46.1 (30.2–48.2)	72.8 (42.2–85.5)	<0.01	<0.002	ns
Lymphocytes (%)	0.6 (0–1.2)	0.7 (0.4–1.2)	1.2 (0–1.6)	ns	ns	ns
Eosinophils (%)	0.1 (0–0.7)	0.9 (0–1.4)	0.8 (0.2–2.4)	ns	ns	ns
Epithelial cells (%)	0.7 (0.5–2.2)	0.5 (0–0.8)	0.4 (0–1.7)	ns	ns	ns
Total cells (10^6/g IS)	3.8 (2.2–7.6)	4.3 (3.6–6)	6.6 (3–15.9)	ns	ns	ns

Results are expressed as median (25th to 75th percentiles). Abbreviations: HCs = healthy asymptomatic nonsmoking subjects with normal lung function; HSs = asymptomatic smokers with normal lung function; COPD = subjects with Chronic Obstructive Pulmonary Disease; ns = not significant. Statistical analysis were performed using the Kruskal–Wallis followed by Bonferroni–Dunn correction for multiple comparison.

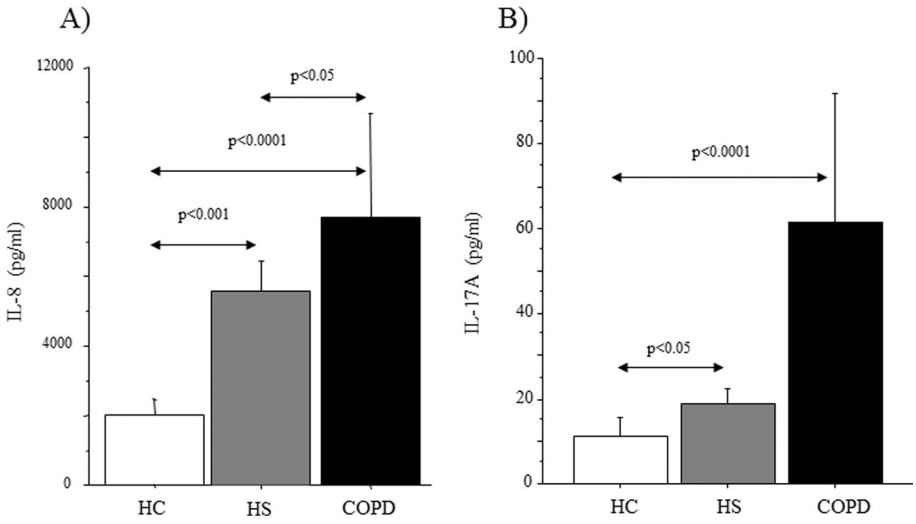

Fig. 1. IL-8 and IL-17A concentrations in ISS from HCs ($n = 14$), HSs ($n = 10$), and COPD subjects ($n = 16$). A) IL-8 and B) IL-17A levels were measured using specific commercial available kits as described in the Section "Materials and methods." Two technical replicates were performed. The bars represent the mean ± SD of values (pg/mL). Statistical analysis was performed by the Kruskal–Wallis test followed by Bonferroni–Dunn correction for multiple comparisons. A p-value <0.05 was considered statistically significant.

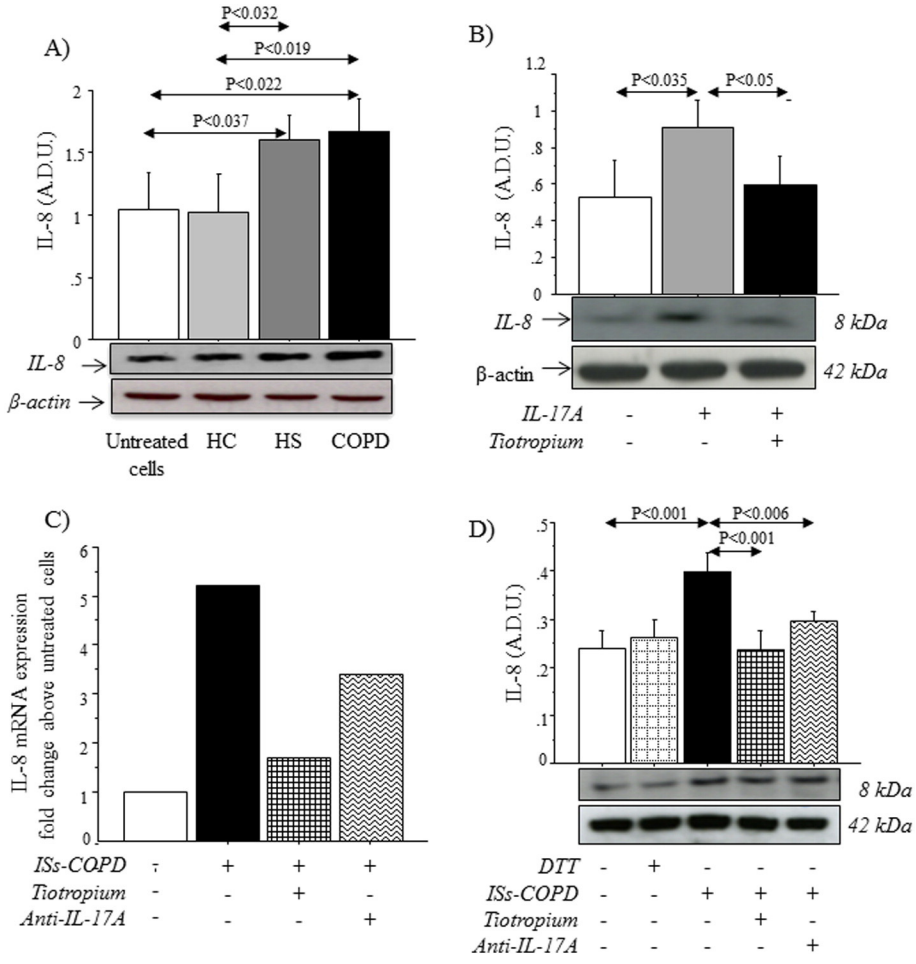

Fig. 2. IL-8 production in 16HBE cells stimulated with ISS. A) IL-8 production in protein extract from 16HBE left untreated or stimulated with ISS from HCs ($n = 6$), HSs ($n = 6$), and COPD patients ($n = 6$). B) IL-8 production in protein extract from 16HBE stimulated with rhIL-17A (20 ng/mL) in the presence or absence of Tiotropium ($n = 3$). C) IL-8 mRNA levels by RT-PCR as described in the Section "Materials and methods" ($n = 2$). Gene expression levels are expressed as fold-change compared to untreated cells chosen as the reference sample. Two technical replicates were performed for each experimental condition. D) IL-8 production in protein extract from 16HBE stimulated with ISS from COPD patients in the presence or absence of Tiotropium (100 nM), or with ISS pretreated with anti-IL-17A antibody, or with DTT ($n = 6$ for each experimental condition). IL-8 was detected by Western blot analyses as described in the "Materials and methods" Section. Bars represent mean ± SD of arbitrary densitometric units (A.D.U.), normalized to β-actin used as the loading control. Representative gel images of the experiments are shown. ANOVA with Fisher's test correction was used to analyze the data. A p-value <0.05 was statistically significant.

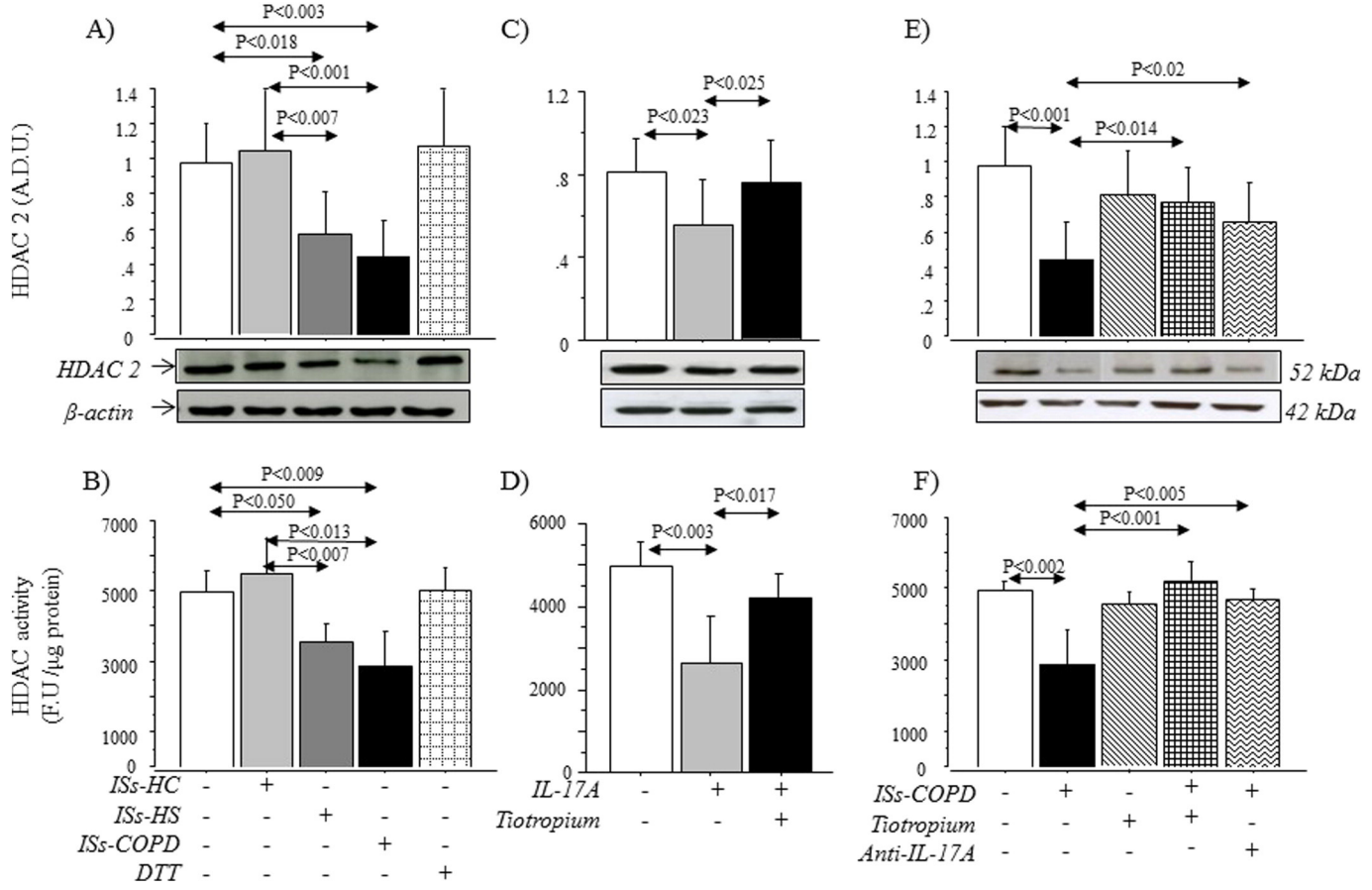

Fig. 3. Nuclear levels of HDAC2 and HDAC activity in 16HBE stimulated with ISS and rhIL-17A. A) Nuclear levels of HDAC2 expression and B) of HDAC2 activity in 16HBE cells stimulated with ISS from HCs ($n = 6$), HSs ($n = 6$), COPD patients ($n = 6$), and those treated with DTT ($n = 6$). C) HDAC2 expression and D) HDAC activity in 16HBE cells stimulated with rhIL-17A (20 ng/mL) in the presence or absence of Tiotropium (100 nM) for 4 h ($n = 6$ for each experimental condition). E) Nuclear levels of HDAC2 and F) of HDAC activity in 16HBE cells stimulated with ISS from COPD patients, with Tiotropium (100 nM) or with ISS from COPD in the presence of Tiotropium (100 nM) or with ISS treated with monoclonal anti-IL-17A Ab for 4 h ($n = 6$ for each experimental condition). HDAC2 analysis was performed by Western blot as described in the Section "Materials and methods." Bars represent mean ± SD of arbitrary densitometric units (A.D.U.), normalized to β-actin used as the loading control. Representative gel images of the experiments are shown. HDAC activity was measured using a fluorometric activity assay and expressed as Fluorescence Units (F.U.) normalized to micrograms of protein. Two technical replicates were performed for each experimental condition. ANOVA with Fisher's test correction was used to analyze the data. A p-value <0.05 was statistically significant.

protein compared with cells stimulated with ISS from COPD patients ($p < 0.001$). Furthermore, the stimulation of 16HBE cells with ISS from COPD patients treated with an anti-IL-17A Ab, significantly reduced the nuclear IKKα levels compared with 16HBE cells treated with ISS from COPD patients ($p < 0.002$) (Fig. 4E). The treatment with anti-IL-17A Ab or Tiotropium did not affect the nuclear levels of Ac-His H3 (k9) and IKKα in untreated 16HBE cells (data not shown).

3.6. Effect of IKKα silencing on HDAC 2, histone H3, and IL-8 expression

Temporary transfection of 16HBE cells with IKKα siRNA caused a statistically significant decrease in the synthesis of IKKα protein in 16HBE cells ($p < 0.001$) compared with unsilenced cells. The silencing efficacy of the RNA interference for IKKα was 40% ± 3.6% (Fig. 5A). The IKKα silencing showed a statistically significant increase in HDAC2 protein expression in 16HBE cells compared with unsilenced cells ($p < 0.002$). Furthermore, Ac-His H3 (k9) protein expression significantly decreased in IKKα-silenced cells compared to unsilenced ($p < 0.025$). Finally, the treatment of 16HBE cells with a scrambled siRNA sequence had no effect on IKKα, HDAC2, and Ac-His H3 (k9) compared to unsilenced 16HBE cells (Fig. 5B). The silencing of IKKα protein significantly increased HDAC2 protein expression ($p < 0.002$) and significantly reduced Ac-His H3 (k9) protein expression ($p < 0.025$) in 16HBE cells stimulated with 20% ISS from COPD patients compared with unsilenced stimulated cells (Fig. 5C).

IL-8 protein expression showed lower levels in cell lysates from untreated 16HBE cells silenced for IKKα protein compared with unsilenced cells ($p < 0.001$) (Fig. 6A) as well as in silenced 16HBE cells stimulated for 24 h with COPD ISS, compared with unsilenced cells stimulated with ISS ($p < 0.038$) (Fig. 6B).

3.7. Co-immunoprecipitation of His H3/IKKα

Next, we evaluated the interaction between Ac-His H3 (k9) and IKKα proteins using co-immunoprecipitation. We found that ISS from COPD patients induced a significant increase in cross-coupling between IKKα and Ac-His H3 (k9) compared with untreated 16HBE cells. The preincubation of 16HBE cells with Tiotropium or the depletion of IL-17A in ISS significantly diminished the effect of stimulation with ISS from COPD patients (Fig. 7). These findings might suggest the role of IKKα in the mechanism of histone H3 acetylation in k9 involving proinflammatory IL-8 gene expression in 16HBE cells.

4. Discussion

In this study, we described the immunological link between the IL-17A and IL-8 levels measured in the biological samples obtained from COPD patients, using an in vitro model obtained by the stimulation of bronchial epithelial cell line 16HBE with IS samples. In particular, we showed that IL-17A stimulated molecular mechanisms of chromatin

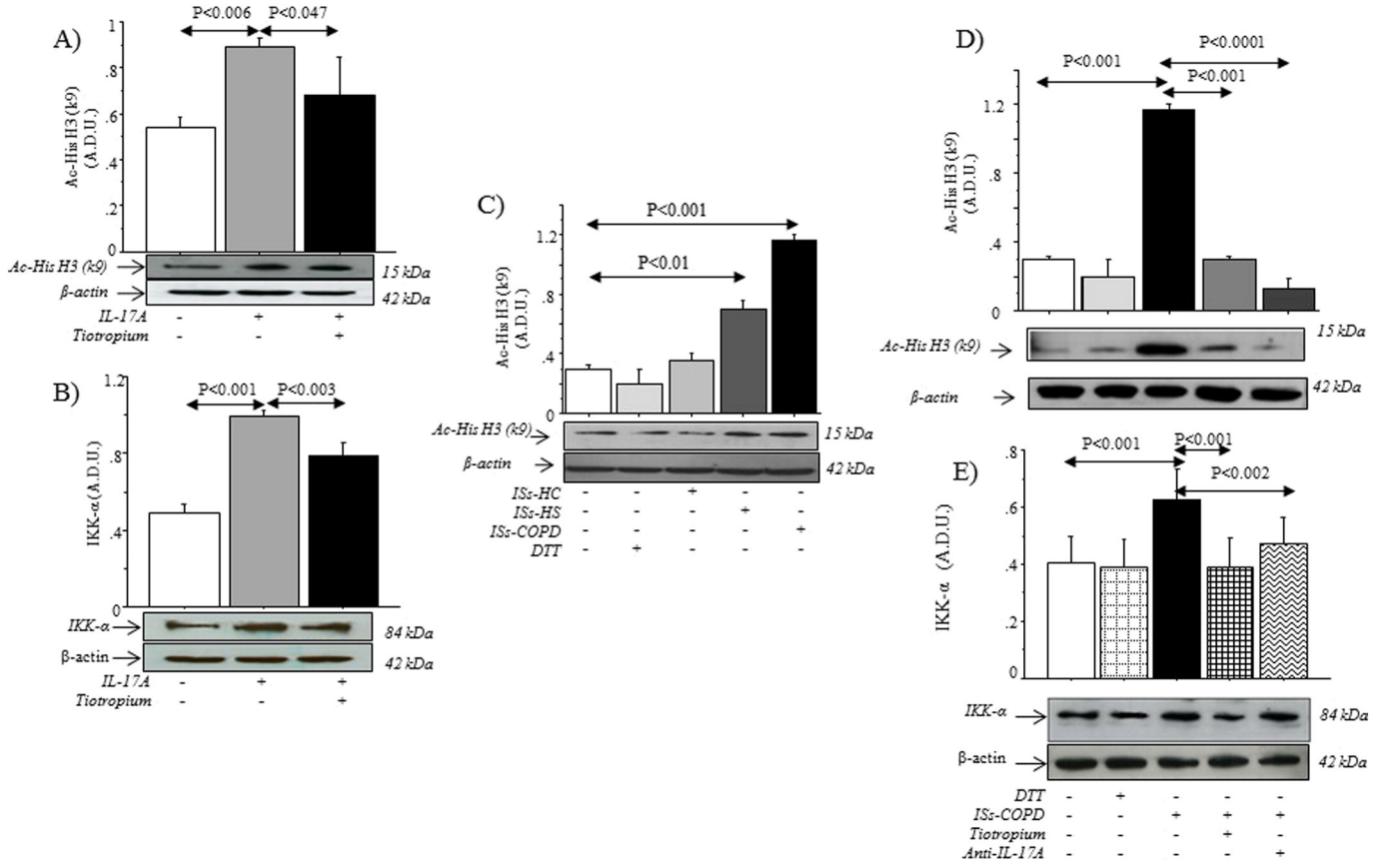

Fig. 4. A) Ac-His H3 (k9) and B) IKKα in nuclear extract of 16HBE cells treated with rhIL-17A in the presence or absence of Tiotropium (100 nM) for 4 h ($n = 6$ for each experimental condition). C) Ac-His H3 (k9) in nuclear extract of 16HBE cells stimulated with DTT ($n = 6$), ISS from HCs ($n = 6$), HSs ($n = 6$), and COPD patients ($n = 6$) for 4 h. D) Ac-His H3 (k9) and E) IKKα in nuclear extract of 16HBE cells treated with DTT, ISS from COPD patients in the presence or absence of Tiotropium (100 nM) or with ISS from COPD patients treated with anti IL-17 Ab ($n = 6$ for each experimental condition). Western blot of nuclear extract was performed as described in the "Materials and methods" Section. Bars represent mean ± SD of arbitrary densitometric units (A.D.U.), normalized to β-actin used as the loading control. Representative gel images of the experiments are shown. Two technical replicates were performed for each experimental condition. ANOVA with Fisher's test correction was used for to analyze the data. A p-value <0.05 was considered statistically significant.

remodeling, reduced HDAC2 translocation, and increased histone H3 acetylation via the promotion of IL-8 production by the activity of IKKα in bronchial epithelial cells. Finally, we provided evidence of the ability of Tiotropium (nonselective anticholinergic drug blocking muscarinic receptors) to counteract the proinflammatory activity of IL-17A.

By acting as a physical barrier, the lung epithelium regulates lung fluid balance, modulates metabolism and clearance of inhaled agents, and secretes several mediators, most of which recruit and activate inflammatory cells in response to injury [17,18]. Dysregulation of airway epithelial cell function may contribute to the pathogenesis of major chronic inflammatory diseases of the lung such as COPD. Th17 immunity and the related cytokines such as IL-17A are involved in both the innate and adaptive aspects of airway immunity, which represent a crucial cross talk between the immune system and structural cells [19]. IL-17A has been found to increase in submucosal biopsy specimens obtained from the large airways of COPD patients compared with control subjects [20]. These data support the role of IL-17A in the airway inflammation of COPD patients, which in turn promotes, alone or in combination with other cytokines, the production of IL-6, IL-8, and intercellular adhesion molecule-1 (ICAM-1) in primary epithelial cells [21]. We detected higher levels of IL-17 and IL-8 in the ISS from COPD subjects and HSs than in HCs. These findings support the cross talk between the cells of the immune system, producing IL-17A, and activated epithelial cells, releasing IL-8, during the phenomena of airway inflammation in COPD patients.

In vitro studies have shown that differentiated cell culture is an invaluable model in understanding the physiological properties of the human airway epithelium. Much of our insights into the interactions between environmental and inflammatory stimuli, and the airway epithelium have been derived extensively from in vitro cell culture models using transformed 16HBE cell line [6,16,28]. Accordingly, we studied the effect of ISS from COPD subjects, HSs, and HCs on IL-8 production in 16HBE cells. The analysis of proteins obtained from the cell extract of 16HBE, demonstrated that ISS from COPD subjects and HSs were found to increase the IL-8 production rather than ISS from HCs. Although the levels of IL-8 do not reflect the levels of IL-17A, as we did not identify a positive correlation between these cytokines in ISS from COPD patients, we observed that the depletion of IL-17A reduced the levels of IL-8 mRNA transcripts and protein production in 16HBE cells. Finally, we underline that the anti-IL-17A treatment had different levels of inhibition on IL-8 mRNA and IL-8 protein probably for a different time of mRNA stability and protein half-life. These findings might support the role of IL-17A in the activation of epithelial cells during the inflammatory process of COPD.

The inflammatory pattern observed in COPD, with increased numbers of neutrophils and levels of IL-8 and tumor-necrosis factor (TNF), increased oxidative stress and responded poorly to corticosteroids [1]. Anticholinergic drugs including Tiotropium, currently used to treat COPD [4,5], block the activation of airway secretory cells and smooth muscle; thus, in theory, these drugs may reduce vagal tone and mucus secretion in COPD, facilitating cough-induced mucus clearance [22,23]. Tiotropium reduces the exacerbation frequency in COPD, although this effect does not appear to be caused by a reduction in airway or systemic inflammation [24]. However, many studies suggest novel pharmacological strategies using Tiotropium as anti-inflammatory and anti-remodeling drugs in COPD [25–27]. In vitro, Tiotropium has been

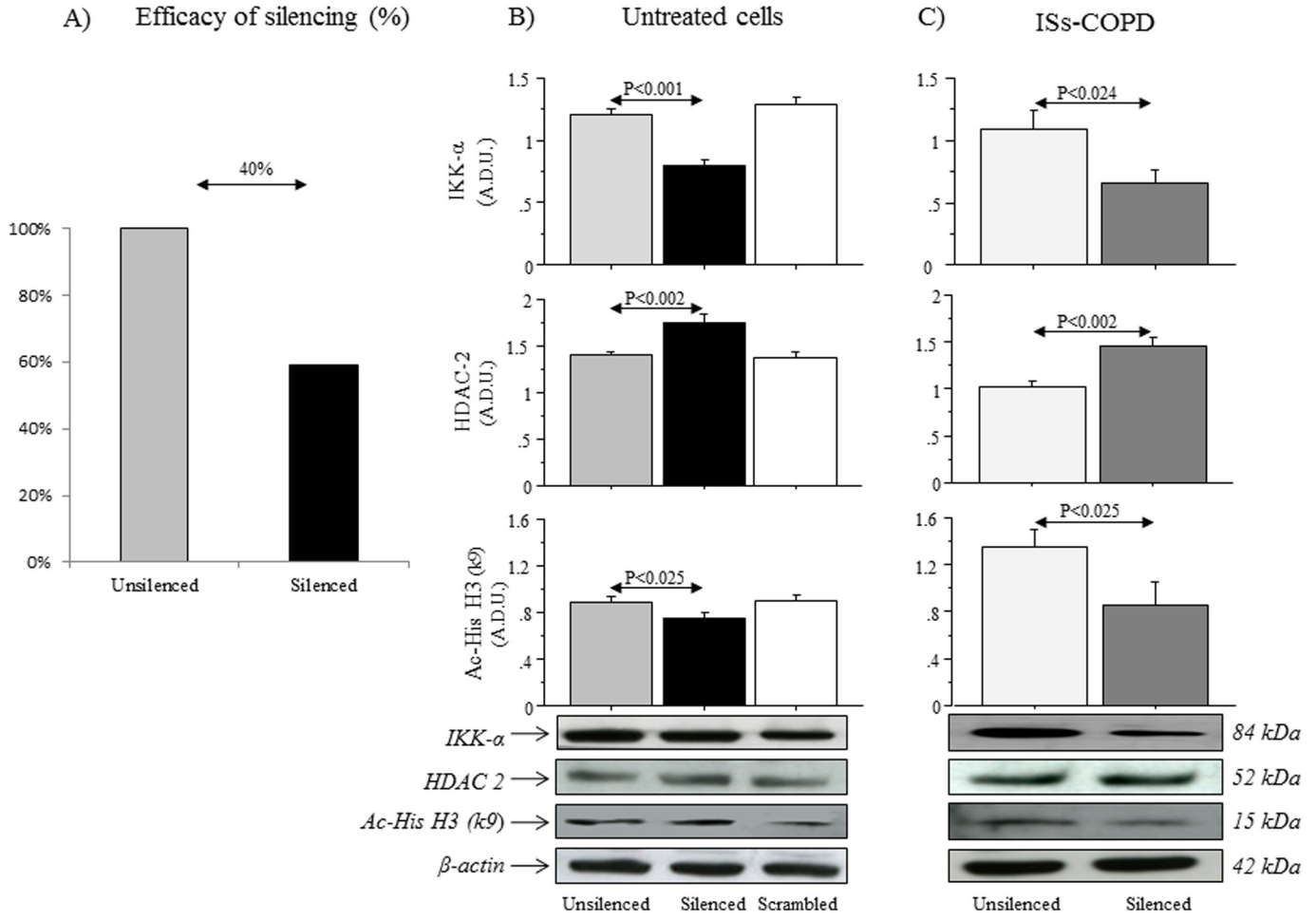

Fig. 5. Effect of IKKα silencing in 16HBE. A) A representative bar graph of the silencing efficiency. B) Expression of IKKα protein, HDAC2, and Ac-His H3 (k9) in 16HBE unsilenced, transfected with scrambled siRNA, and transfected with siRNA for IKKα ($n = 6$ for each experimental condition). C) Expression of IKKα protein, HDAC2, and Ac-His H3 (k9) in both 16HBE unsilenced and transfected with siRNA for IKKα stimulated with ISS from COPD patients for 4 h ($n = 6$ for each experimental condition). Bars represent mean ± SD of arbitrary densitometric units (A.D.U.), normalized to β-actin used as the loading control. Representative gel images of the experiments are shown. Two technical replicates were performed for each experimental condition. Statistical analysis was performed using ANOVA with Fisher's test correction. A p-value <0.05 was statistically significant.

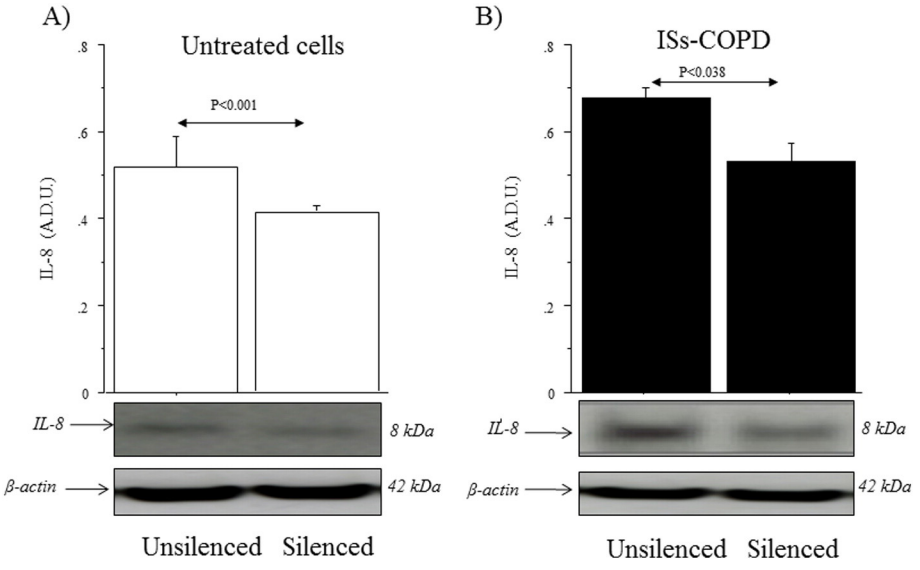

Fig. 6. Effect of IKKα silencing on IL-8 synthesis in 16HBE. A) Expression of IL-8 protein in untreated 16HBE unsilenced or transfected with siRNA for IKKα; B) Expression of IL-8 protein in both 16HBE unsilenced and transfected with siRNA for IKKα stimulated with ISS from COPD patients for 4 h. Bars represent mean ± SD of arbitrary densitometric units (A.D.U.), normalized to β-actin used as the loading control ($n = 6$). Representative gel images of the experiments are shown. Two technical replicates were performed for each experimental condition. Statistical analysis was performed using ANOVA with Fisher's test correction. A p-value <0.05 was statistically significant.

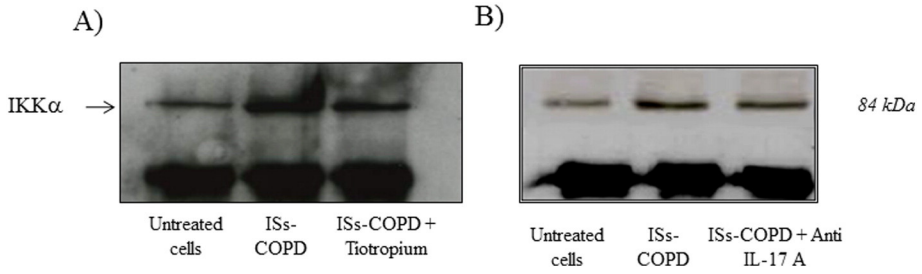

Fig. 7. IKKα binding to Ac-His H3 (k9) in 16HBE stimulated with ISS from COPD. Co-immunoprecipitation of IKKα and Ac-His H3 (k9) was performed A) in cell extract from 16HBE cells stimulated for 4 h with ISS from COPD patients in the presence or absence of Tiotropium or B) in cell extract from 16HBE cells stimulated for 4 h with ISS from COPD patients treated with anti-IL-17A Ab ($n = 3$ for each experimental condition). A representative gel image of the experiments is shown.

found to control IL-8 release from bronchial epithelial cells [28,29]. Furthermore, IL-17A induces epigenetic changes, which in turn diminishes the ability of glucocorticosteroids (GCs) to inhibit IL-8 production from human bronchial epithelial cells [8]. In this study, we showed that the pretreatment of 16HBE cells with Tiotropium may control the production of IL-8 in terms of protein and mRNA generated by IL-17A and by proinflammatory mediators present in ISS from COPD subjects. In this case, additional insight into the epithelium and its interactions with inflammatory cells obtained by our findings might open up new pharmacological perspectives to treat the epithelial dysregulation associated with Th17 immunity in inflammatory lung conditions. In light of these observations, we speculate that blockade of IL-17A downstream involving the pretreatment of epithelial cells with Tiotropium may be a novel therapeutic strategy in GC-insensitive airway inflammation of COPD patients. Furthermore, as the inflammatory components present in the airways of COPD patients can deregulate the expression and activation of muscarinic acetylcholine (ACh) receptor in bronchial epithelial cells [28,30] we suggest that this action might be exerted by IL-17A present in the ISS from COPD patients. This observation might justify the anti-inflammatory role of Tiotropium in our in vitro model of 16HBE. However, further studies might be necessary to clarify this observation.

The HDAC family of enzymes has been reported to have 17 isoforms. Each of these enzymes is differentially expressed and regulated in different cell types [31], with a role in the regulation of cell proliferation and inflammatory responses [32]. IL-17A induces GC insensitivity, probably mediated by PI3K activation and subsequent reduction of HDAC2 activity, in the airway epithelium [8]. Our findings showed that ISS from COPD patients, rather than ISS from HCs or untreated cells, led to a reduction of HDAC2 translocation from the cytoplasm to the nucleus in 16HBE cells. The depletion of IL-17A with anti-IL-17A Ab in ISS from COPD patients or the pretreatment of 16HBE cells with Tiotropium restored the nuclear levels of HDAC2 in cells stimulated with ISS from COPD patients. Our findings might suggest that anticholinergic drugs exert an anti-inflammatory effect on IL-17 activity, thus controlling chromatin remodeling responsible for the release of inflammatory cytokines such as IL-8 [33]. However, further study might be necessary to clarify the specific inhibitory effect of HDAC2 activity on IL-8 production in 16HBE cells stimulated with ISS and rhIL-17A.

The acetylation status of histones is controlled by the opposing actions of two classes of enzymes: histone acetyltransferases (HATs), which transfer acetyl groups to lysine residues within the N-terminal tails of core histones, and histone deacetylases (HDACs), which remove the acetyl groups [34]. The acetylation status of histones influences chromatin conformation and influences the accessibility of transcription factors and effector proteins to the DNA, thereby modifying gene expression [35]. Inflammation is known to induce chromatin remodeling through different molecules; the acetylation of histone H3 in lysine 9 is a modification that facilitates the access of the promoter to the transcription complex [3]. The kinase IKKα has a cytoplasmic and nuclear function. Its shuttling into the nucleus is crucial for its function as a chromatin kinase and acetylase inducing a specific modification of histones. This implies a different expression of genes in response to an inflammatory stimulus [36]. Cigarette smoke-/TNFα induced the acetylation of histone H3 and inflammation via the differential activation of IKKα in human lung epithelial cells [37]. In this study, we showed that 16HBE cells treated with ISS from COPD patients showed an increase in the nuclear levels of IKK-α and Ac-His H3 (k9) compared with untreated cells. The pretreatment of 16HBE with Tiotropium or the depletion of IL-17A with anti-IL-17A Ab in ISS from COPD patients restored the basal levels of IKKα and Ac-His H3 (k9) as well as the basal levels of IL-8 production. These findings suggest that IL-17A and inflammation present in the airways of COPD patients might stimulate IL-8 production via chromatin remodeling mechanisms that involve the IKKα-mediated acetylation of histone H3 in the gene promoter of IL-8 in bronchial epithelial cells. However, IL-17 present in the ISS from COPD patients promoted the acetylation of Ac-H3 in 16HBE by both direct and indirect action. This is further underlined by the fact that lower levels of IL-17A in ISS (8 ng/mL rather than 20 ng of rhIL-17A) eliminated this effect. Tiotropium might act as anti-inflammatory drug regulating these mechanisms. Furthermore, we found that IKKα silencing led to an increase in the nuclear levels of HADAC2, and a reduction of Ac-His H3 (k9) and IL-8 synthesis in 16HBE cells treated with ISS from COPD patients compared with unsilenced cells. Finally, the cellular extract from 16HBE (co-immunoprecipitated with anti Ac-His H3 (k9) and detected with anti-IKKα) showed higher levels of IKKα in 16HBE cells stimulated with ISS from COPD patients than in cells stimulated with ISS from COPD patients and pretreated with Tiotropium or cells stimulated with ISS depleted of IL-17A. Overall, these findings suggest that IL-17A present in the airways of COPD patients might be able to activate IKKα, which in turn can regulate the acetylation of Ac-His H3 (k9). Finally, our results suggest that the anticholinergic drug exerts its anti-inflammatory effect by regulating the molecular mechanism involving the activation of IKKα by IL-17A and the related synthesis of IL-8 in bronchial epithelial cells.

5. Conclusions

In conclusion, to the best of our knowledge, this is the first paper to demonstrate the anti-inflammatory effects of Tiotropium on IL-17A-mediated chromatin remodeling mechanisms promoting IL-8 release in bronchial epithelial cells. These findings might suggest the use of anticholinergic drugs as a useful alternative therapy to control IL-17A-induced glucocorticoid insensitivity in human bronchial epithelial cells during COPD. However, to clarify these mechanisms, further study might be necessary using primary cell cultures or differentiated cells grown on an airway–liquid interface. In addition, as the in vitro studies have a limited number of samples, we recommend that the activity of Tiotropium on the aforementioned mechanism be investigated in a larger population of subjects with COPD subjects. Adequate clinical trials must be performed to confirm the relevant contribution of anticholinergic drug therapy to corticosteroid resistance.

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Disclosure statements

The author of the manuscript MPP is an employee of Boehringer Ingelheim Pharma GmbH & Co. KG. There are no other conflicts of interest for this study.

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