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A Protective Role For Club Cell Secretory Protein-16 (CC16) In The Development of Chronic Obstructive Pulmonary Disease (COPD)

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Abstract

Rationale—Club cell secretory protein-16 (CC16) is the major secreted product of airway Club cells, but its role in the pathogenesis of COPD is unclear. We measured CC16 airway expression in humans with and without COPD and CC16 function in a cigarette smoke (CS)-induced COPD mice model.

Methods—Airway CC16 expression was measured in COPD patients, smokers without COPD, and non-smokers. We exposed wild-type (WT) and *CC16*$^{-/-}$ mice to CS or air for up to 6 months, and measured airway CC16 expression, pulmonary inflammation, alveolar septal cell apoptosis, airspace enlargement, airway MUC5AC expression, small airway remodeling, and pulmonary function.

Results—Smokers and COPD patients had reduced airway CC16 immunostaining that decreased with increasing COPD severity. Exposing mice to CS reduced airway CC16 expression. *CC16*$^{-/-}$ mice had greater CS-induced emphysema, airway remodeling, pulmonary inflammation, alveolar cell apoptosis, airway MUC5AC expression, and more compliant lungs than WT mice. These changes were associated with increased nuclear factor-κB (NFκB) activation in *CC16*$^{-/-}$ lungs. CS-induced acute pulmonary changes were reversed by adenoviral-mediated over-expression of CC16.

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Conclusions—CC16 protects lungs from CS-induced injury by reducing lung NFκB activation. CS-induced airway CC16 deficiency increases CS-induced pulmonary inflammation and injury and likely contributes to the pathogenesis of COPD.

Keywords

Chronic obstructive pulmonary disease; pulmonary inflammation; mucus; apoptosis; Club cells

Introduction

COPD is a major cause of morbidity and mortality worldwide (1). COPD results from a poorly controlled lung inflammatory response to inhaled particles primarily in cigarette smoke (CS) leading to destruction of the alveolar walls and small airway remodeling (2). The severity and progression of COPD are graded by the forced expiratory volume in one second (FEV_1) (3).

Faster rate of decline in FEV_1 in COPD is linked to current smoker status, higher baseline FEV_1, low body mass index (BMI), the degree of CT-determined emphysema, and a history of COPD exacerbations (4-6). In the ECLIPSE study, the only association observed between rate of FEV_1 decline and serum levels of various biomarkers was a protective effect associated with higher levels of Club (formerly Clara) cell secretory protein-16 (CC16) (7). This inverse relationship between serum CC16 levels and FEV_1 decline was confirmed in a cohort of mild COPD patients (8).

CC16 is also known as CC10, Club cell secretory protein, secretoglobin, family 1A, member 1 (SCGB1A1), and uteroglobin. It is a member of the secretoglobin family of disulphide-bridged dimeric proteins secreted by airway Club cells and is the most abundant protein in normal airway secretions. CC16 maintains the homeostasis of the airway epithelium (9), and has anti-inflammatory activities in lungs exposed to ozone, allergens, and viruses (10-12). Plasma CC16 levels are low in cigarette smokers, and patients with asthma and obliterative bronchiolitis (13-15). Plasma CC16 levels increase following smoking cessation (16) and increases in BAL fluid (BALF) CC16 levels correlate with regression of bronchial dysplasia in former smokers (17). Although a recent study reported that *CC16*$^{-/-}$ mice developed similar emphysema as wild-type (WT) mice when exposed to CS (8), there are knowledge gaps about the contributions of CC16 to COPD pathogenesis.

We tested the following hypotheses: 1) airway CC16 expression is reduced in smokers without COPD and COPD patients, and in COPD patients it correlates inversely with degree of airflow limitation; 2) airway CC16 levels progressively decrease in WT mice exposed to CS; and 3) when exposed to CS, CC16-deficient (*CC16*$^{-/-}$) mice have greater pulmonary inflammation, airspace enlargement, and airway pathologies than WT mice.

Methods

Airway CC16 expression in COPD patients and control subjects

Human studies were approved by institutional review boards and all subjects signed written informed consent forms. Formalin-fixed lung sections were obtained from 6 patients with

severe or very severe COPD as part of the Overholt BlueCross Emphysema Surgery Trial (OBEST) for emphysema (18), 6 patients with mild to moderate COPD, 6 healthy non-smokers (never smokers), and 7 healthy active cigarette smokers (> 20 pack/year) without COPD who had undergone lung surgery for benign nodules (see Supplemental Table 1 for demographic and clinical data). None of the subjects had lung cancer. Lung sections were double immunostained for CC16 and a marker of airway epithelial cells (pancytokeratin; see online supplement).

Animals

The Harvard Medical School Institutional Animal Care and Use Committee approved all procedures. C57BL/6 strain *CC16*$^{-/-}$ mice (19) and C57BL/6 WT control mice (from The Jackson Laboratory, Bar Harbor, USA) were studied.

CS exposures

Adult WT and *CC16*$^{-/-}$ mice (10 week old) were exposed to air or mixed mainstream and sidestream CS from 3R4F Kentucky Research cigarettes for 2 h/day on 6 days/week in Teague TE 10z chambers for 1-6 months.

Airway immunostaining for CC16, CYP2F2, and MUC5AC

Formalin-fixed lung sections from mice were immunostained for CC16, CYP2F2, and MUC5AC (see online supplement).

Airspace enlargement and airway remodeling

Respiratory mechanics were performed on mice using a mechanical ventilator (Scireq Inc., Montreal, Canada). Airspace size and was measured on Gill's-stained formalin-fixed and inflated lung sections. Small airway remodeling was measured on lung sections stained with Masson's Trichrome stain and immunostained for type-I collagen and fibronectin (see online supplement).

Lung inflammation

Leukocyte subsets were counted in BAL samples from mice. Pro-inflammatory mediators and matrix metalloproteinases (MMPs) were measured in lung samples using ELISAs or western blotting (see online supplement).

Alveolar septal cell apoptosis and lung oxidative stress levels

Alveolar septal cell death was assessed in murine lung section by TUNEL staining and immunostaining for active caspase-3. Oxidative stress levels were measured as thiobarbituric acid reactive substances (TBARS) in lung samples (see online supplement).

Cigarette smoke extract (CSE)-induced apoptosis of murine tracheal epithelial cells (MTECs)

WT and *CC16*$^{-/-}$ MTECs monolayers were exposed to 30% cigarette smoke extract (CSE) at 37°C and intracellular active caspase-3 levels quantified using a fluorogenic substrate specific for active caspase-3 (see online supplement).

Overexpression of mCC16 in murine airways

Recombinant adenoviral vectors (Vector Biolab, Philadelphia, USA) expressing the cDNA for mCC16 (Ad-CC16) or green fluorescent protein (Ad-GFP) were delivered to the lungs of WT and *CC16*$^{-/-}$ mice (5×10^7 PFU/mouse) at baseline and every 2 weeks thereafter using the oro-pharyngeal aspiration method. One week after the initial viral dose, mice were exposed to air or CS for 1 month, and BAL leukocytes enumerated or lungs removed for analyses.

Nuclear factor-κB (NFκB) activation and secretory phospholipase A_2 ($sPLA_2$) levels in murine lungs

Lung sPLA2 levels were measured using a kit and NFκB activation was measured in nuclear extracts of lungs using an electrophoretic mobility shift assay (EMSA; see online supplement).

Statistical analysis

Statistical analyses were performed using Sigma Stat™ software (SSPS Inc, San Jose, USA). Data shown are means ± SEM (unless otherwise indicated). A P-value ≤ 0.05 was considered significant.

Results

Airway CC16 expression is reduced in human smokers and COPD patients and correlates indirectly with COPD severity

Non-smokers had striking airway CC16 staining whereas staining was less intense in healthy smoker airways (Fig. 1). Airway CC16 staining was lowest in COPD airways (Fig. 1A) and decreased with increasing COPD severity as assessed by GOLD stage (Fig. 1B). Scatter plot analysis revealed modest variability in staining within the subject groups (Supplemental Fig. 1). The age and sex ratios did not differ between the groups (Supplemental Table 1). All of the COPD patients were former smokers. The GOLD stage III-IV COPD patients had greater pack-year smoking histories than the smokers without COPD. Use of inhaled corticosteroids was higher in GOLD stage III-IV than GOLD stage I-II COPD patients.

CS reduces CC16 expression in murine airways

Air-exposed WT mice had robust airway CC16 expression (Fig. 2A-2B). CS exposure caused progressive reductions in airway CC16 expression in mice over time (Fig. 2B). Air-exposed *CC16*$^{-/-}$ mice had no positive staining for CC16 or CYP2F2, another marker of Club cells (Supplemental Figs. 2-3).

CC16 deficiency increases emphysema development and airway pathologies in CS-exposed mice

The mean distal airspace size was similar in adult WT and *CC16*$^{-/-}$*mice* that were housed in a room air environment until 10 wks of age and then exposed to air for an additional 1 month (Fig.3A-3B). Thus, CC16 does not regulate lung development in mice. When 10 wk old mice were exposed to air for an additional 6 months, *CC16*$^{-/-}$ mice had a trend towards

increased airspace size compared with WT mice. However, when exposed to CS for 6 months, *CC16*$^{-/-}$ mice developed 25% increases in airspace size compared with air-exposed *CC16*$^{-/-}$ mice, while CS-exposed WT mice developed only 13% increases in airspace size compared with air-exposed WT mice (Figs. 3A-3B). Pressure-volume (P-V) flow loops were similar in *CC16*$^{-/-}$ and WT mice exposed to air for 1 month (data not shown) and 6 months (Fig. 3C). After 6 months of CS exposure, there was a left shift in the P-V flow loops of CS-exposed *CC16*$^{-/-}$ mice vs. CS-exposed WT mice (Fig. 3D). Quasi-static lung compliance did not differ in WT and *CC16*$^{-/-}$ mice exposed to air for 1 or 6 months (data not shown) but after 6 months of CS exposure was greater in *CC16*$^{-/-}$ than WT mice (0.105 ± 0.00805 vs. 0.0966 ± 0.00823 ml/cm H_2O; p = 0.036) consistent with the greater emphysema development in CS-exposed *CC16*$^{-/-}$ vs. WT mice.

There was a trend (p = 0.068) towards increased deposition of extracellular matrix (ECM) proteins around small airways in adult *CC16*$^{-/-}$ mice versus WT mice exposed to air for 1 month (Fig. 4A-4B). When mice were exposed to air for 6 months, this small airway remodeling was modestly (∼23%) greater in *CC16*$^{-/-}$ than WT mice. However, there was a much greater (∼53%) increase in small airway remodeling in *CC16*$^{-/-}$ mice vs. WT mice exposed to CS for 6 months (Fig. 4A-4B). CS-exposed *CC16*$^{-/-}$ mice also had greater staining for type-I collagen (Supplemental Fig. 4), fibronectin (Fig. 4C) around the small airways and greater airway epithelial MUC5AC immunostaining (Fig. 4D) than CS-exposed WT mice.

CC16 deficiency increases pulmonary inflammation, MMP-9 levels, and alveolar septal cell apoptosis in CS-exposed mice

Air-exposed *CC16*$^{-/-}$ mice had modestly greater BAL total leukocyte, macrophage, and PMN counts than air-exposed WT mice (Fig. 5A-5C). CS-exposed *CC16*$^{-/-}$ mice had higher BAL total leukocyte, macrophage, and PMN counts than CS-exposed WT mice at all time-points assessed (Fig. 5A-5C), but WT and *CC16*$^{-/-}$ mice did not differ in BAL lymphocyte counts (data not shown). Compared with CS-exposed WT mice, CS-exposed *CC16*$^{-/-}$ mice had higher lung levels of CCL5 and active transforming growth factor-β1 (TGF-β1), lower lung levels of interleukin-10, but similar lung levels of other pro-inflammatory mediators (Supplemental Table 2). CS induced greater increases in lung MMP-9 (but not MMP-12) levels in *CC16*$^{-/-}$ mice than WT mice (Supplemental Fig. 5).

CS exposed *CC16*$^{-/-}$ mice had increased numbers of apoptotic bronchial epithelial cells (Fig. 6A) and apoptotic alveolar septal cells (Fig. 6B-6C) as assessed by TUNEL staining and/or staining for active caspase-3. However, CSE induced similar rates of apoptosis in *CC16*$^{-/-}$ and WT MTEC cultures in vitro (Supplemental Fig. 6). Lung oxidative stress levels were similar in CS-exposed *CC16*$^{-/-}$ and WT mice measured as lung levels of TBARS (a readout of lipid peroxidation; not shown).

CC16 deficiency increases activation of NFkB but not $sPLA_2$ levels in CS-exposed lungs

CC16 inhibits two pro-inflammatory pathways in other model systems: NFκB activation (20) and $sPLA_2$ activity by binding co-factors for this enzyme (21). When we measured these pathways, air-exposed *CC16*$^{-/-}$ mice had modestly increased NFκB activation in their

lungs as assessed by EMSA. However, CS-exposed *CC16*$^{-/-}$ mice had greater NFκB activation in their lungs than CS-exposed WT mice (Figs. 7A-7B). Although air-exposed *CC16*$^{-/-}$ mice had higher lung levels of active $sPLA_2$ than air-exposed WT mice, $sPLA_2$ levels were similar in lung homogenates (Fig. 7C) and BALF (Supplemental Fig. 7) from CS-exposed WT and *CC16*$^{-/-}$ mice.

Adenoviral-mediated CC16 overexpression in murine airways reduces pulmonary pathologies induced by acute CS exposure

Delivering Ad-CC16 to the airways of *CC16*$^{-/-}$ mice induced airway immunostaining for CC16 (Supplemental Fig. 8). Delivering Ad-CC16 to both *CC16*$^{-/-}$ and WT lungs attenuated CS-induced increases in BAL macrophage (but not PMN) counts in both genotypes compared with macrophage counts in Ad-GFP-treated mice (Fig. 8A or not shown). Ad-CC16-treated *CC16*$^{-/-}$ and WT mice had lower CS-induced airway MUC5AC immunostaining, alveolar septal cell apoptosis, and lung NFκB activation (Fig. 8B-8D), but similar lung levels of $sPLA_2$ activity (data not shown) than Ad-GFP-treated mice belonging to the same genotype.

Discussion

We report several novel findings. First, airway CC16 expression was lower in COPD patients than smokers and non-smokers and indirectly correlated with airflow obstruction. Second, CS exposure progressively reduced airway CC16 expression in WT mice. Third, CS-exposed *CC16*$^{-/-}$ mice developed greater pulmonary inflammation, alveolar septal cell apoptosis, airway mucus metaplasia, emphysema, and small airway remodeling, associated with greater NFκB activation in their lungs than WT mice. Pulmonary pathologies induced by acute CS exposure were attenuated by adenoviral-mediated over-expression of CC16 in murine airways. Thus, CC16 has important roles in protecting murine lungs from the development and progression of CS-induced COPD-like pathologies in mice (at least in part) by reducing activation of NFκB in the lung which has been implicated in the pathogenesis of COPD (22).

Human studies

Only one prior study has reported decreased airway CC16 expression in severe COPD patients versus cigarette smokers (23). We now link airway CC16 deficiency to smoking in human subjects and the severity of airflow limitation in COPD. Reduced airway CC16 expression in COPD lungs may be persistent as our very severe COPD patients had stopped smoking well before the tissue was obtained. However, a prior study reported that serum CC16 levels recover somewhat when smokers without COPD quit smoking (16). The differences in these findings could be due to differences in study populations, the samples studied, or the length of time since smoking cessation.

Murine studies

CS exposure progressively reduced murine airway CC16 expression which could be due to reduced synthesis of CC16 by Club cells or loss of airway Club cells (24). Club cells have the highest levels of cytochrome P450 in the lung and are the main site of lung

detoxification of xenobiotics (25). Murine Club cells are sensitive to injury following inhalation of naphthalene [a component of CS (26)] or CS itself (24). Polymorphisms in the CC16 locus were weakly linked to COPD in the ECLIPSE cohort and associated with low plasma CC16 levels, but these findings were not replicated in other smaller COPD cohorts having different inclusion criteria (27). Additionally, the CC16 locus is hyper-methylated in COPD bronchial epithelia suggesting that epigenetic factors influence CC16 expression (28). Although CS reduced airway CC16 expression in WT mice, some CC16 was detected after 3 months of CS exposure that likely was sufficient to protect the WT lung from developing the more severe lung inflammatory response and airway and airspace disease observed in CS-exposed *CC16*$^{-/-}$ mice.

CC16 protects the murine lung from CS-induced pulmonary inflammation, emphysema development, small airway remodeling, and airway mucus metaplasia. Likely, the protective effect of CC16 in the CS-exposed murine lung is due to CC16 reducing lung macrophage and PMN counts and protecting alveolar septal cells from CS-induced apoptosis. CC16 secreted by Club cells into the epithelial lining fluid likely has paracrine crytoprotective effects on other lung epithelial cells as Club cell-free WT and *CC16*$^{-/-}$ lung epithelial cells had similar rates of CSE-induced apoptosis in vitro.

NFκB activation was increased CS-exposed *CC16*$^{-/-}$ versus WT lungs (Fig. 7) and was reduced in both CS-exposed WT and *CC16*$^{-/-}$ lung by over-expressing CC16 in their airways indicating that CC16 mediates its activities (at least in part) in the CS-exposed lung by reducing NFκB activation which promotes inflammation in COPD lungs (22). Ad-GFP-treated WT and *CC16*$^{-/-}$ mice did not differ in the extent to which CS increased NFκB activation in their lungs which may due to be virus-induced NFκB activation, but virus-mediated over-expression of CC16 attenuated NFκB activation in the lungs of CS-exposed WT and *CC16*$^{-/-}$ mice. CC16 signals through the fMLP receptor on granulocytes (29), the lipocalin-1 receptor on lung epithelial carcinoma cells (30), and cubilin in the kidney (31), and reduces macrophage toll-like receptor 4 levels (32). CC16 also inhibits $sPLA_2$ activity in other models by binding its co-factors (21) and elevated $sPLA_2$ BALF levels correlate with a proinflammatory phenotype in COPD patients (33). While *CC16*$^{-/-}$ mice had higher baseline lung $sPLA_2$ activity levels, CS-exposed WT and *CC16*$^{-/-}$ mice had similar lung levels of $sPLA_2$. The increased baseline $sPLA_2$ levels may have contributed to the increased BAL leukocyte counts in air-exposed *CC16*$^{-/-}$ mice. However, CC16 could mediate some of its anti-inflammatory effects in the CS-exposed lung by inhibiting $sPLA_2$ activity as measuring this mediator in whole lung or BALF samples may dilute $sPLA_2$ signals generated by subpopulations of pulmonary cells.

CC16 may reduce airway ECM deposition in CS-exposed lungs by reducing lung levels of active TGF-1β as levels of this mediator were higher in CS-exposed *CC16*$^{-/-}$ vs. WT lungs. CC16 could also reduce small airway remodeling and airway MUC5AC expression by restraining pulmonary inflammation as leukocyte products contribute to airway remodeling and mucin gene expression in rodents (34-36).

Our findings differ from those recently reported by Park et al. who reported that pulmonary inflammation, emphysema, and airway remodeling are similar in CS-exposed WT and

CC16$^{-/-}$ mice (8). Different *CC16*$^{-/-}$ strains vary in the severity of renal phenotypes detected (37-39) due to differences in their genetic backgrounds and/or the targeting constructs used to generate the mice. However, both studies evaluated the same *CC16*$^{-/-}$ murine stain (19) (personal communication, Don Sin, MD). Likely, the differences in the results of the two studies reflect differences in the methods used. For example, we exposed mice to whole body mixed mainstream and side-stream CS for 2 h per day for 6 days-a-week, whereas Park et al exposed the mice to mainstream CS from 3 cigarettes 5 days-a week using a nose-only technique. However, we also over-expressed CC16 in the airways of mice using adenoviral vectors which reduced CS-induced acute pulmonary changes consistent with our hypothesis that CC16 has anti-inflammatory activities in the CS-exposed lung.

We observed small increase in pulmonary inflammation and small airway remodeling in air-exposed *CC16*$^{-/-}$ mice versus WT mice. Small increases in lung leukocyte counts were detected after just 1 month of air exposure. Thus, deficiency of CC16 in the absence of CS is sufficient to cause mild pulmonary inflammation which may contribute to the modestly greater small airway remodeling in air-exposed *CC16*$^{-/-}$ vs. WT mice. Thus, CC16 has anti-inflammatory activities even in the unchallenged lung.

Limitations of our study include our relatively small sample sizes. Nevertheless, we achieved statistically significant differences between our groups and scatter-plot analysis revealed modest within-group variability in airway CC16 staining in our human subjects. The GOLD stage III-IV COPD patients had greater pack-year smoking histories than the smoker controls. All of the smokers but none of the COPD patients were current smokers which could have influenced airway CC16 staining. The use of inhaled corticosteroids by some of the COPD patients may have increased their airway CC16 expression as CC16 is steroid-responsive gene (40).

Conclusions

CS exposure reduces airway CC16 expression leading to increased pulmonary inflammation, alveolar septal cell apoptosis, mucus metaplasia, and also emphysema development and small airway remodeling which both contribute to airflow obstruction in COPD patients. Pulmonary pathologies induced by acute CS exposure are reversed by adenoviral-mediated over-expression of CC16 in both WT and *CC16*$^{-/-}$ airways. Thus, CC16 protects lungs from CS-induced injury. Future studies will determine whether CC16 can be used as a novel therapy for COPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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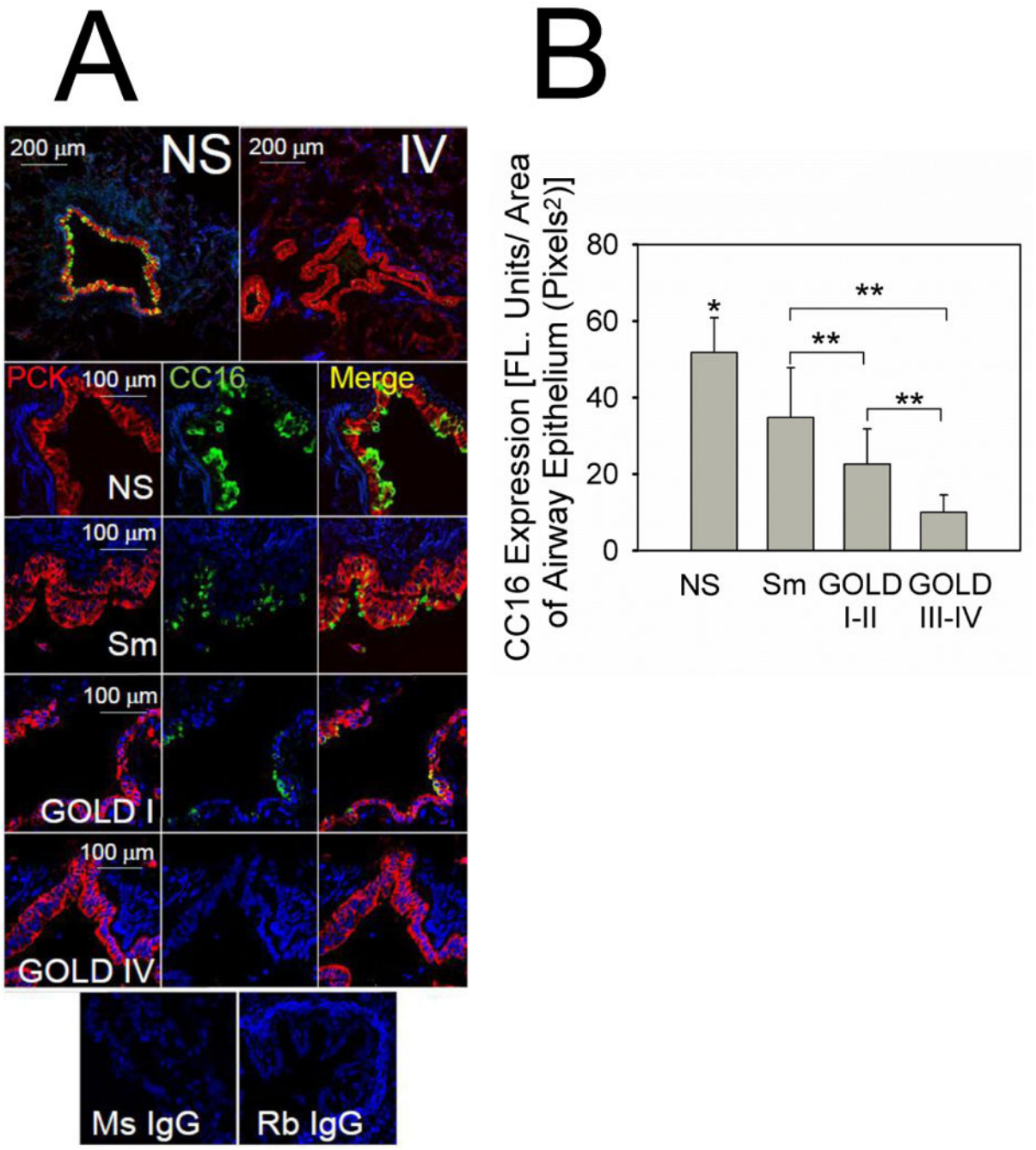

Figure 1. CC16 expression is reduced in the large airways of COPD patients and expression levels decrease with increasing airflow limitation

Double immuno-fluorescence staining for CC16 and a marker of airway epithelial cells (pancytokeratin) was performed on lung sections from 6 healthy non-smokers (never smokers, NS), 6 smokers without COPD (Sm), 7 GOLD stage I-II COPD patients (GOLD I-II), and 6 GOLD stage III-IV COPD patients (GOLD III-IV). **A** shows representative lung sections stained with a red fluorophore (left panels) and murine IgG to pancytokeratin and with a green fluorophore and rabbit IgG to CC16 (middle panels). DAPI counter-stained

lung sections were examined using a confocal microscope. Merged images are shown in the right panels. The top panel of **A** show × 100 magnification merged images of double immunostained stained lung sections from a healthy non-smoker (left) and a very-severe COPD patient (right). The panels below these in **A** show representative images (magnification × 400-600) of stained lung sections from a healthy non-smoker (NS), a cigarette smoker without COPD (Sm), and a GOLD stage II COPD patient, and a GOLD stage IV COPD patient. Lung sections of a non-smoker control stained with non-immune murine IgG (Ms IgG) or non-immune rabbit IgG (Rb IgG) showed no staining (bottom panel in **A**). In **B**, staining for CC16 in airway epithelial cells was quantified in images of the lung sections from the 4 subject groups (NS, Sm, COPD GOLD I-II, and COPD GOLD III-IV), as described in Methods. The size of the airways analyzed was similar in all groups (mean internal diameter was 763.85 ± SEM 174.4, 830.9 ± 110.2, and 808.8± 64.3 μm in the non-smokers, smokers, and COPD patients, respectively). Data are means ± SEM; n = 6-7 subjects/group. Asterisk indicates $P < 0.05$ versus all other groups; **, $p < 0.05$.

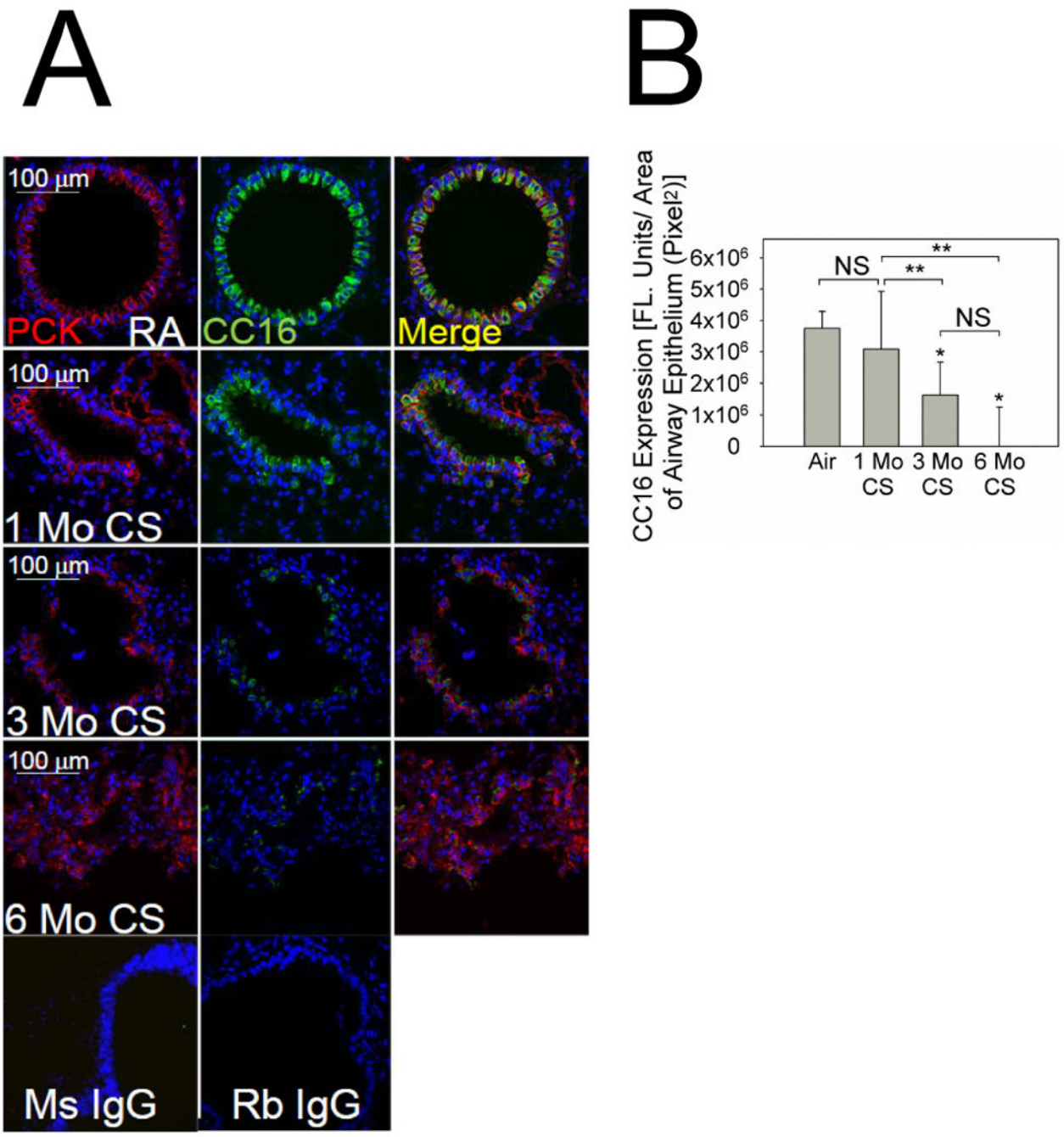

Figure 2. CS exposure reduces the expression of CC16 in murine airways

In **A**, lung sections from C57BL/6 WT mice exposed to room air (RA) or CS for 1 month, 3 months, or 6 months were stained with a red fluorophore and a murine IgG to a marker of airway epithelial cells (pancytokeratin; left panels) and a green fluorophore and a rabbit IgG to CC16 (middle panels). Merged images are shown in the right panels. Lung sections from air-exposed WT mice were stained with non-immune murine IgG (Ms IgG) or non-immune rabbit IgG (Rb IgG). **B** shows quantitative analysis of airway CC16 normalized per unit of

airway area in pixels^2. Data are means ± SEM; n = 6 air-exposed and 3-4 CS-exposed mice/group. Asterisk indicates $P < 0.001$ compared with mice exposed to air and **, $P < 0.05$.

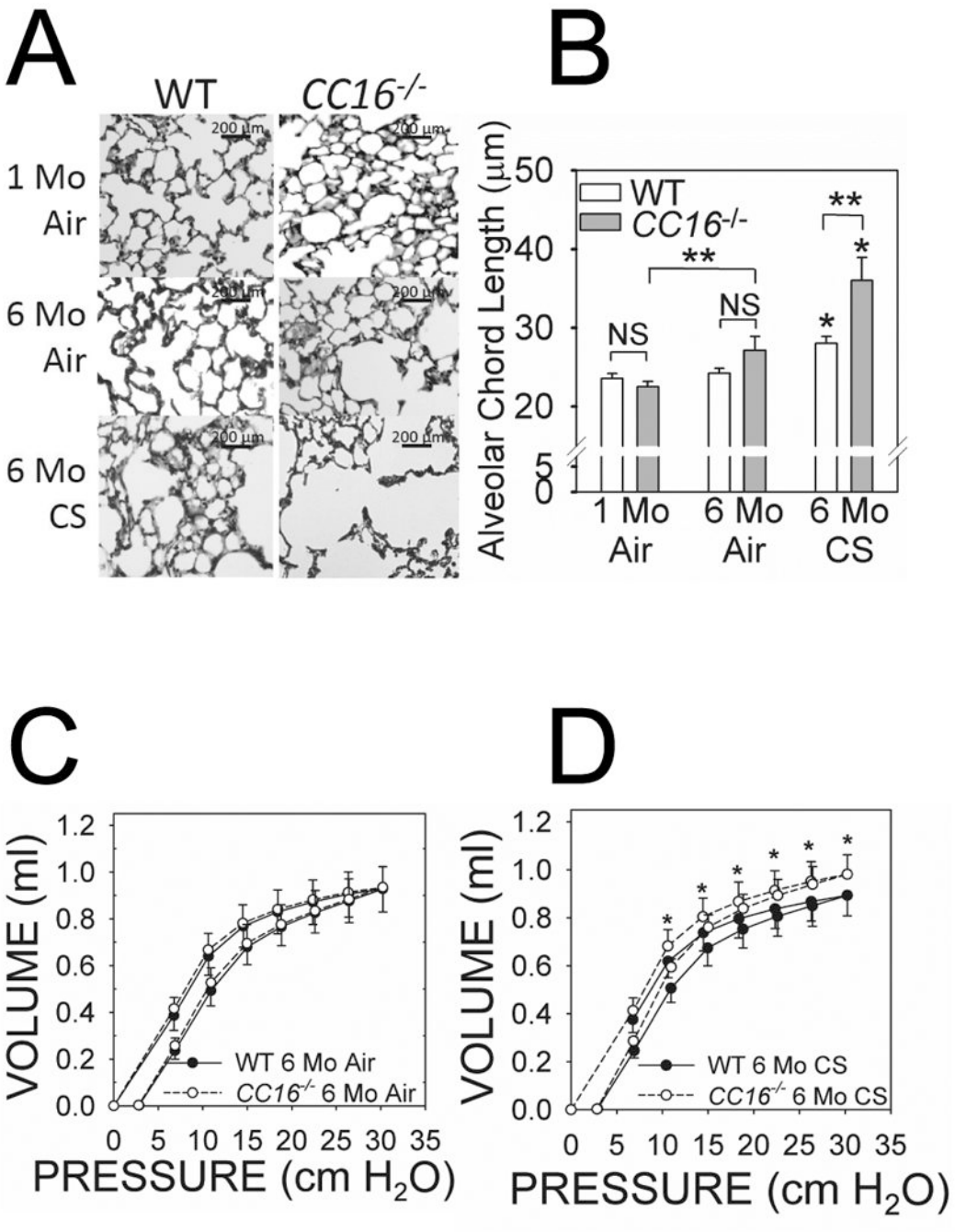

Figure 3. CC16$^{-/-}$ mice exposed chronically to CS have greater emphysema and more compliant lungs than CS-exposed WT mice

WT and *CC16*$^{-/-}$ mice were exposed to air for 1 month or 6 months, or to CS for 6 months in a Teague device. Airspace enlargement was measured in lung sections as described in Methods. **A** shows representative lung sections from each experimental group studied. **B** shows mean alveolar chord lengths (distance between the alveolar walls) in WT versus *CC16*$^{-/-}$ mice exposed to air for 1 month (8-9 mice/group) or 6 months (6-15 mice/group), or CS for 6 months (8-9 mice/group). Data are expressed as means ± SEM; asterisk indicates P

< 0.05 compared with mice exposed to air for 6 months belonging to the same genotype; **, P < 0.05. In **C-D**, a Flexivent device was used to measure pressure-volume (PV) flow loops on WT and *CC16*$^{-/-}$ mice exposed to air for 6 months (9-10 mice/group) or CS for 6 months (9-10 mice/group). In **D**, asterisk indicates P < 0.05 versus the corresponding data point on the WT PV flow loop.

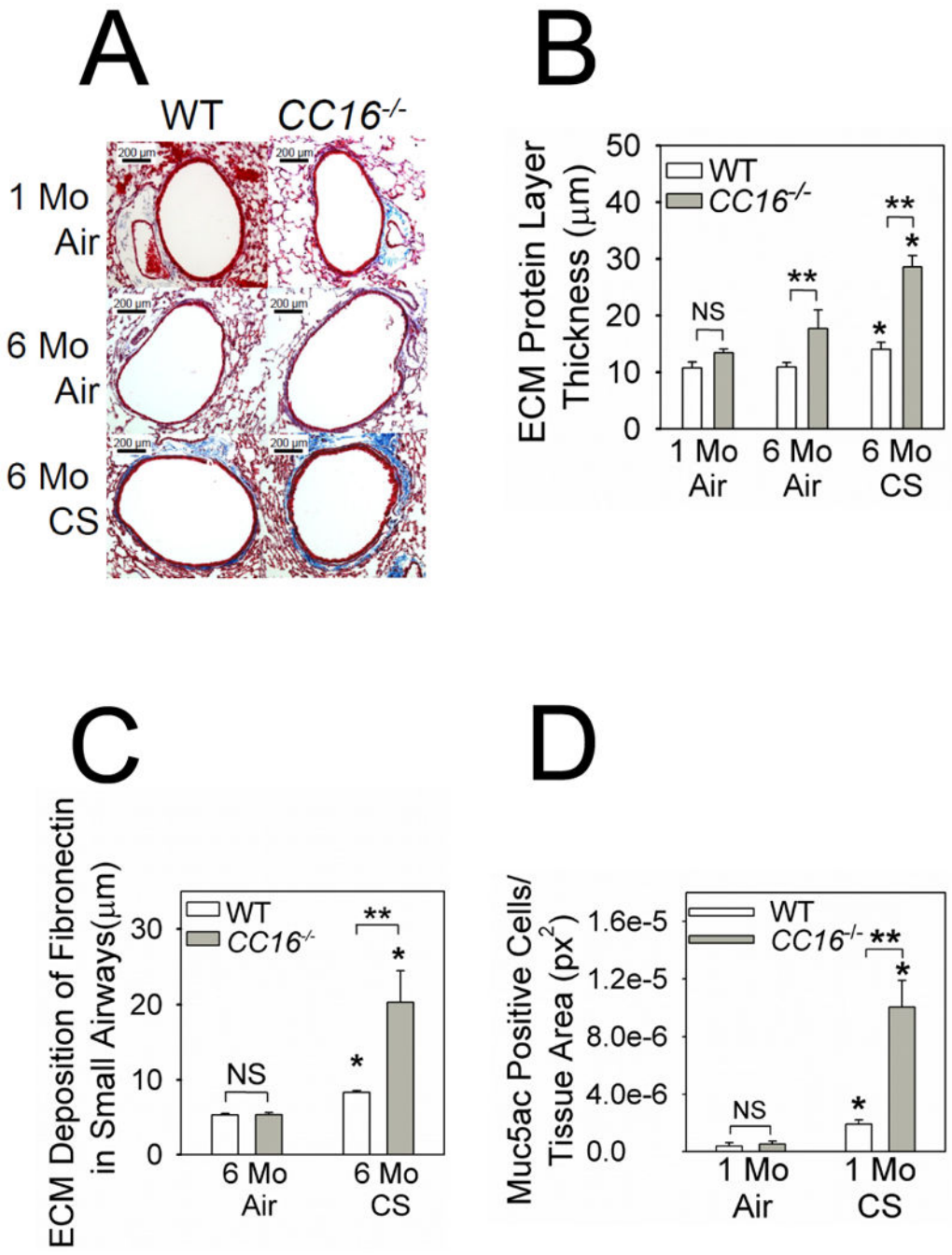

Figure 4. Small airway remodeling is greater in CC16$^{-/-}$ mice compared with WT mice exposed to CS

Starting at 10 weeks of age, WT and *CC16*$^{-/-}$ mice were exposed to air or CS for 6 months. Deposition of extracellular matrix (ECM) proteins around small airways (mean diameter between 300 and 699 μm) was quantified in Masson's Trichrome-stained lung sections. **A** shows representative stained lung sections from each experimental group. ECM proteins deposited around the small airways are stained blue. In **B**, the thickness of the ECM protein layer deposited around small airways (airways having an internal diameter from 300-699

μm) was quantified as described in Methods. Data are means ± SEM; n= 6-7 mice exposed to air for 1 month, n = 5-9 mice exposed to air for 6 months, and n = 7-8 mice exposed to CS for 6 months. Asterisk indicates $P < 0.05$ compared with mice exposed to air for 6 months belonging to the same genotype; **, $P < 0.05$. In **C**, lung sections from WT and $CC16^{-/-}$ mice exposed to air (3-4 mice/group) or CS (4 mice/group) for 6 months were stained for fibronectin as described in methods. The thickness of the layer of fibronectin deposited around small airways (internal diameter from 300-699 μm) was quantified. Asterisk indicates $p < 0.05$ compared with mice exposed to air belonging to the same genotype; **, $p < 0.05$. In **D**, lung sections from WT and *CC16*$^{-/-}$ mice exposed to air (3-5 mice/group) or CS (3-5 mice/group) for 1 month were immunostained for MUC5AC as described in Methods. The proportion of all bronchial epithelial cells that stained positively for MUC5AC was quantified. Data are means ± SEM; asterisk indicates $P < 0.05$ compared with air-exposed mice belonging to the same genotype and **, $P < 0.01$.

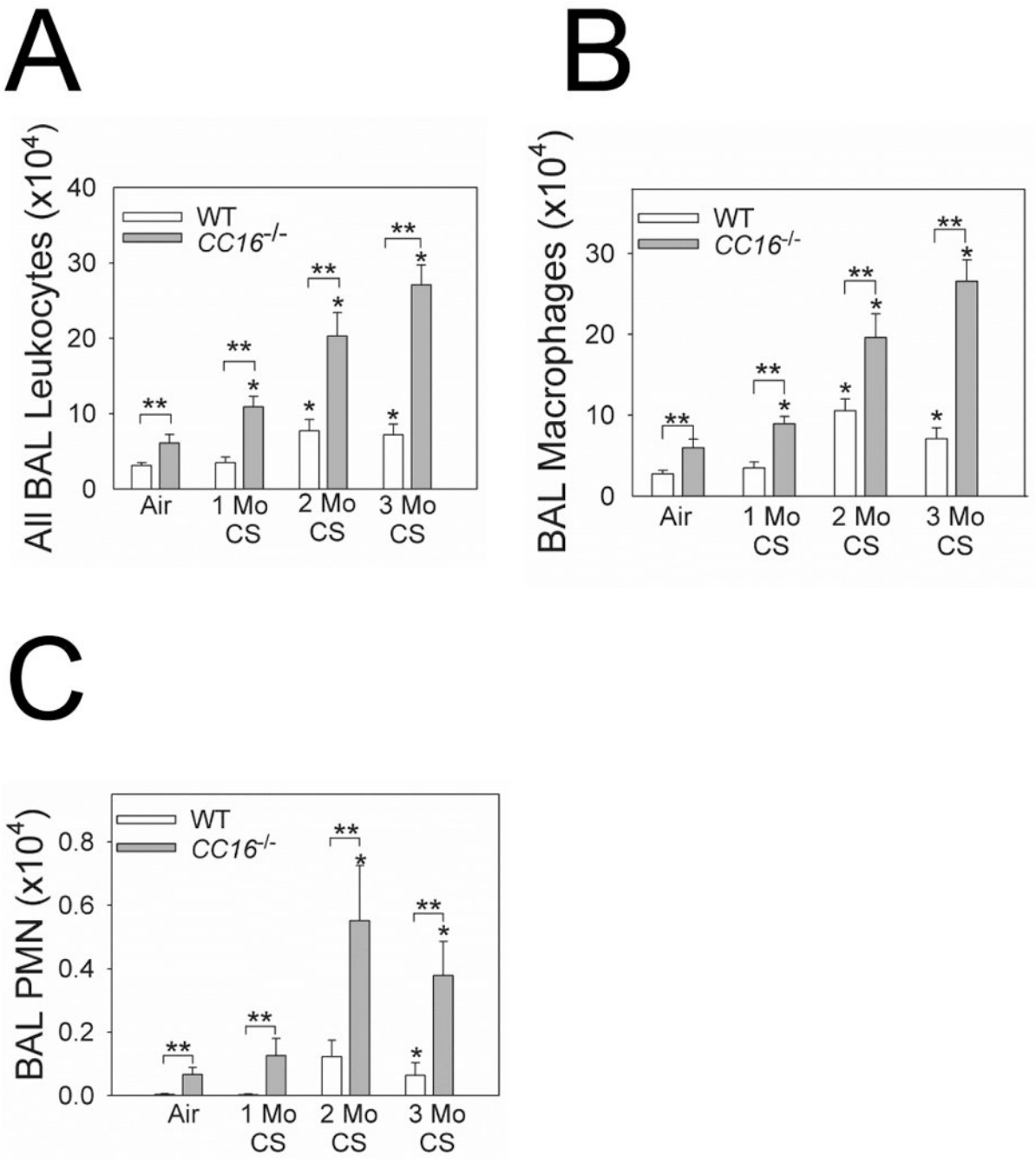

Figure 5. Lung inflammation is increased in CS-exposed CC16$^{-/-}$ mice

WT and *CC16*$^{-/-}$ mice were exposed to air or CS for 1-3 months. Absolute numbers of all leukocytes (**A**), macrophages (**B**), and PMNs (**C**) were counted in BAL samples. Data are mean ± SEM; n = 7 air-exposed mice, n = 7-10 mice exposed to CS for 1 month; n = 8 mice exposed to CS for 2 months, and n = 5-14 mice exposed to CS for 3 months. Asterisk indicates $P < 0.05$ compared with air-exposed mice belonging to the same genotype; and **, $P < 0.05$.

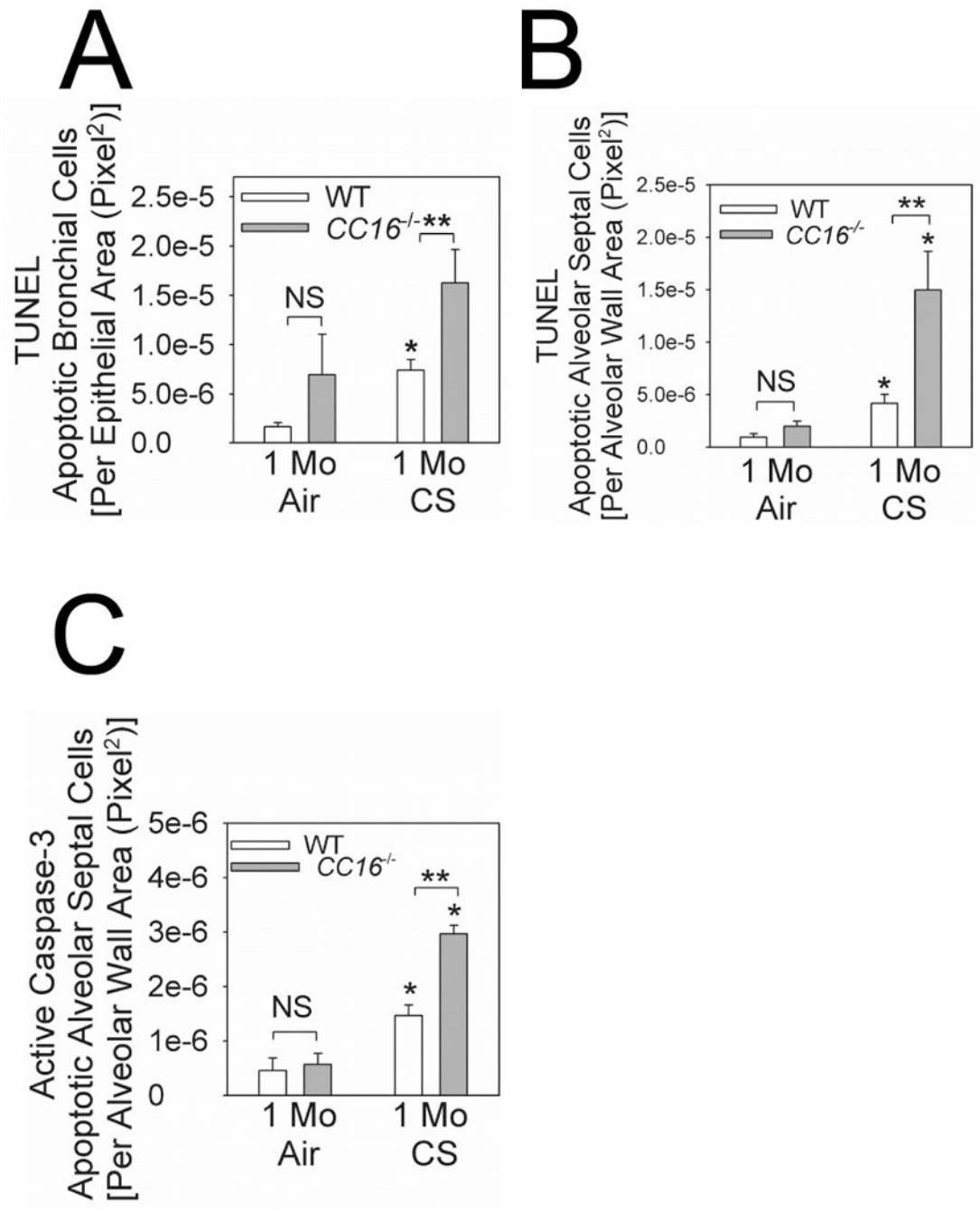

Figure 6. Apoptosis rates are increased in bronchial epithelial and alveolar septal cells in CC16$^{-/-}$ mice exposed to CS

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and immunostaining for active (cleaved) caspase-3 were performed on formalin-fixed lung sections from WT vs. *CC16*$^{-/-}$ mice exposed to air or CS for 1 month. In **A**, TUNEL-positive cells were quantified in large and medium-sized airways from 3 air-exposed WT or *CC16*$^{-/-}$ mice and 4 CS-exposed WT or *CC16*$^{-/-}$ mice. In **B**, TUNEL-positive alveolar septal cells were counted and counts were normalized to unit area of alveolar wall in 4 air-exposed WT

or *CC16*$^{-/-}$ mice and 4 CS-exposed WT or *CC16*$^{-/-}$ mice. In **C**, alveolar septal cells that stained positively for active (cleaved) caspase-3 were counted and counts were normalized per unit area of alveolar wall in 4 mice per experimental condition. In **A-C**, data are means ± SEM; asterisk indicates $P < 0.05$ compared with air-exposed mice belonging to the same genotype; ** $P < 0.05$.

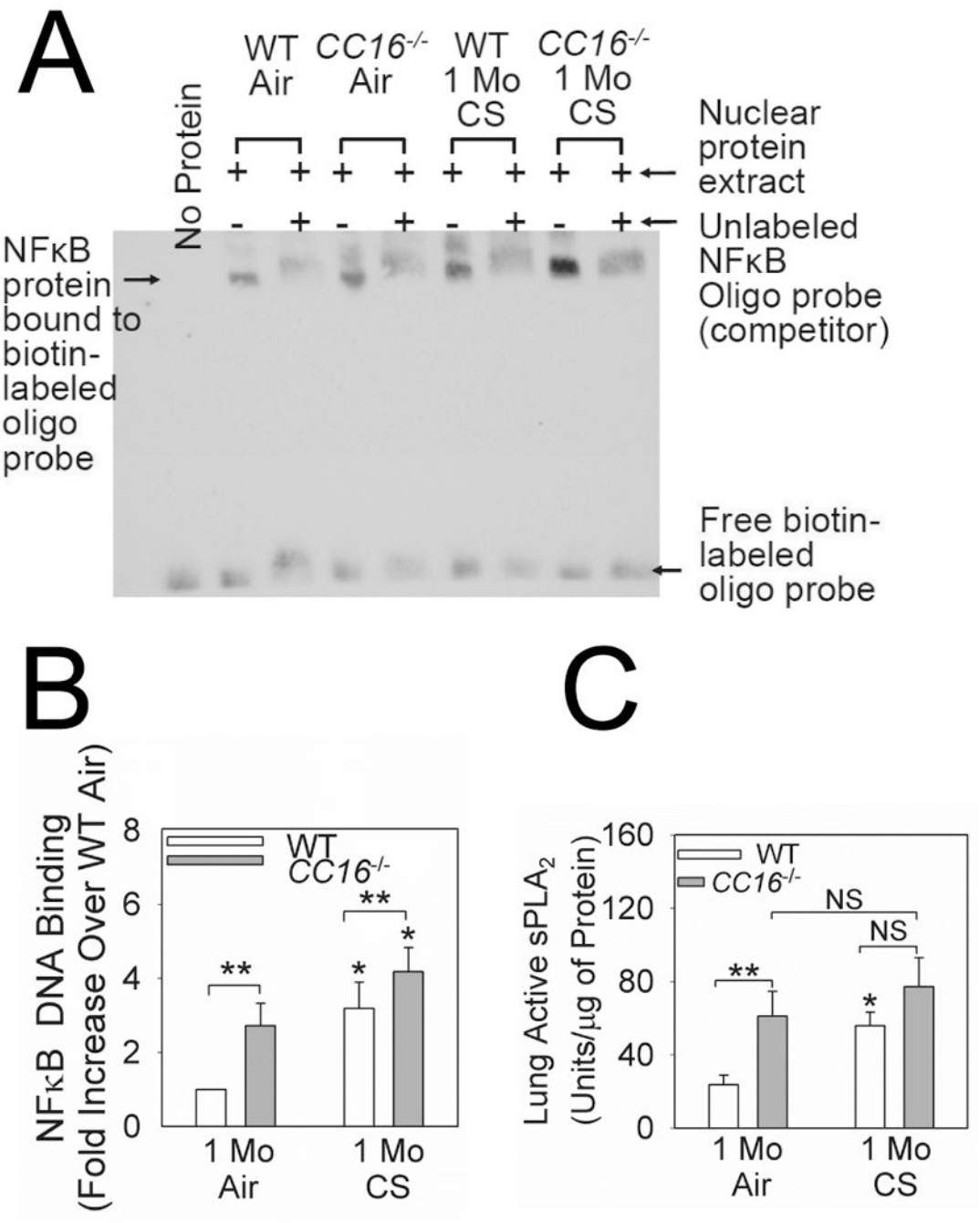

Figure 7. NFκB activation is increased in the lungs of CS-exposed CC16$^{-/-}$ mice

WT and *CC16*$^{-/-}$ mice were exposed to air or CS for 1 month. Nuclear extracts were prepared from the lungs of 5 WT or *CC16*$^{-/-}$ mice exposed to air and 6 WT or *CC16*$^{-/-}$ mice exposed to CS and equal amounts of protein (2 μg/sample) were subjected to electrophoretic mobility shifts assays (EMSAs) using a labeled oligonucleotide probe containing the NFκB consensus sequence. Assays were performed in the presence and absence of excess unlabeled probe to identify NFκB protein bound specifically to the probe. **A** shows a representative image of an EMSA analysis of nuclear extracts from all experimental groups.

Note the marked reduction in signal in the band indicated by the arrow when excess unlabeled probe is added indicating specific binding of NFκB present in nuclear extracts to the labeled oligonucleotide probe. In **B**, the intensities of the bands corresponding to NFκB-oligonucleotide complexes were quantified using densitometry, and band intensities for all groups were normalized to signals in the air-exposed WT lungs. Data are means ± SEM from six independent experiments. Asterisk indicates $P < 0.01$ compared with air-exposed mice belonging to the same genotype and **, $P < 0.05$. In **C**, lung levels of active $sPLA_2$ were measured in homogenates of lungs from mice exposed to air or CS for 1 month using a commercial kit, and levels were normalized to lung total protein levels. Data are means ± SEM; n =10 air-exposed mice and n = 11-12 CS-exposed mice. Asterisk indicates $P < 0.05$ compared with air-exposed mice belonging to the same genotype and ** $P < 0.01$.

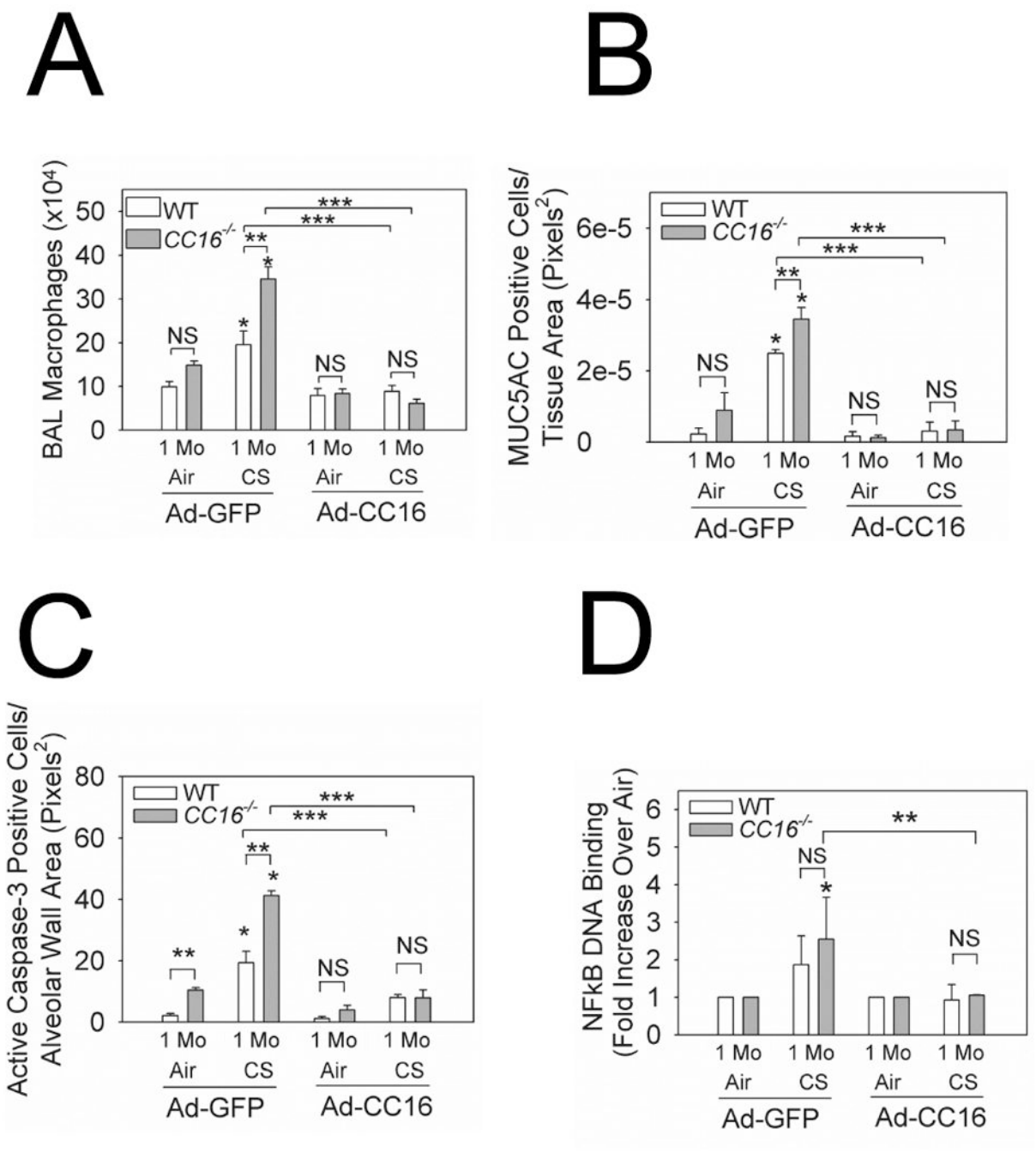

Figure 8. Adenoviral-mediated CC16 overexpression in murine airways decreases pulmonary inflammation, alveolar septal cell apoptosis, and airway mucus metaplasia

Ad-CC16 or Ad-GFP were delivered to WT and *CC16*$^{-/-}$ mice and 1 week later, the mice were exposed to air or CS for 1 month. Adenoviral vector delivery was repeated every 2 weeks for the duration of the CS exposures. In **A**, macrophage numbers were counted in BAL samples. Data are means ± SEM; n = 7-10 mice treated with Ad-GFP and exposed to air, n = 9 mice treated with Ad-GFP exposed to CS; n = 8-10 treated with Ad-CC16 and exposed to air, n = 7-9 mice treated with Ad-CC16 and exposed to CS. In **B**, the number of

alveolar septal cells staining positively for active (cleaved) caspase-3 was counted and counts were normalized per unit area of alveolar wall. Data are means ± SEM; n = 4 mice/group. In **C**, lung sections from Ad-CC16- or Ad-GFP-treated WT or *CC16*$^{-/-}$ mice exposed to air or CS for 1 month (4 mice/group) were immunostained for MUC5AC. The number of bronchial epithelial cells that stained positively for MUC5AC was quantified and normalized per unit area of airway epithelium. Data are mean ± SEM; asterisk indicates $P < 0.05$ compared with air-exposed mice belonging to the same genotype and treated with the same adenoviral vector; ** $P < 0.05$ and ***, $P < 0.05$ compared with CS-exposed mice belonging to the same genotype and treated with Ad-CC16 vs. Ad-GFP