# Club cell 10-kDa protein attenuates airway mucus hypersecretion and inflammation

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ABSTRACT Bacterial lipopolysaccharide (LPS) and interleukin (IL)-13 increase mucus secretion and inflammatory cytokine production in normal human bronchial epithelial (NHBE) cells. We evaluated the effect of club cell 10-kDa protein (CC10), an anti-inflammatory protein produced by epithelial cells, on mucus secretion, cell morphology and inflammatory cytokine production.

NHBE cells were cultured at an air–liquid interface with CC10 or vehicle and exposed to LPS on day 14. Mucin MUC5AC, IL-8 and granulocyte-macrophage colony-stimulating factor were measured in cell supernatants. MUC5AC and IL-8 mRNA expression were measured by real-time PCR. Western blotting was used to evaluate nuclear factor (NF)-κB and extracellular signal-regulated kinase (ERK) activation. Cells were evaluated histologically. Additionally, NHBE cells were exposed to IL-13 and CC10 for 14 days, and secretion of the mucins MUC5AC and MUC5B was measured.

MUC5AC secretion stimulated either by LPS or by IL-13 was attenuated by CC10 at 20 $ng \cdot mL^{-1}$ ($p<0.05$). CC10 at 20 $ng \cdot mL^{-1}$ also attenuated IL-8 secretion ($p<0.05$). MUC5AC and IL-8 mRNA expression were also decreased by CC10 ($p<0.05$). CC10 attenuated phosphorylation of NF-κB ($p<0.05$) and ERK1/2 ($p<0.05$).

CC10 attenuates LPS-induced mucus secretion in airway cells, in part due to inhibition of NF-κB and ERK phosphorylation.

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**CC10 attenuates LPS-induced mucus secretion in airway cells and plays a role in decreasing airway inflammation** http://ow.ly/v6TDe

For editorial comments see page 831.

Received: May 10 2013 | Accepted after revision: March 24 2014 | First published online: May 15 2014

Conflict of interest: None declared.

## Introduction

Inflammatory airway diseases, such as chronic obstructive pulmonary disease and severe asthma, are associated with mucus hypersecretion and inflammatory cytokine production [1]. MUC5AC and MUC5B are the principal gel-forming mucins in the airway [2, 3]. MUC5AC production is stimulated by bacterial lipopolysaccharide (LPS) [4], primarily through the epidermal growth factor receptor, which activates nuclear factor (NF)-κB *via* the extracellular signal-regulated kinase (ERK)1/2 and phosphatidylinositol-3-kinase pathways [5, 6].

Club cell 10-kDa protein (CC10) is a small, globular, nonglycosylated, homodimetric protein member of the secretoglobin family [7]. CC10 is expressed by mucosal epithelial cells, and appears to primarily be produced by nonciliated club cells in the distal airway and nasal epithelial cells [8]. CC10 is reported to have anti-inflammatory effects [9] through the suppression of phospholipase $A_2$ activity [10], inhibition of T-helper cell (Th) type 2 T-cell differentiation [11] and inflammatory cytokine production [7].

Intratracheal LPS has been shown to decrease CC10 expression in rat lung tissue and bronchoalveolar lavage fluid [12, 13]. A lower airway concentration of CC10 has been associated with an increased severity of inflammatory airway disease [14]. CC10 knockout mice have enhanced eosinophil inflammation after allergen challenge in the lungs [7], and CC10 expression in nasal mucosa is decreased in patients with allergic rhinitis or chronic rhinosinusitis with nasal polyps [8, 15]. CC10 levels in lavage fluid from subjects with allergic rhinitis and asthma are lower than those from healthy subjects [15, 16].

The influence of CC10 on the production of mucus by bronchial epithelial cells and cell morphology are not well described. Therefore, we evaluated the effect of CC10 on mucus production and cell morphology in differentiated normal human bronchial epithelial (NHBE) cells. We also examined the effect of CC10 on cytokine secretion from these cells after LPS exposure.

## Materials and methods

### Reagents

The following reagents were purchased: recombinant human CC10 (R&D Systems, Inc., Minneapolis, MN, USA); LPS (from *Escherichia coli* serotype 0111:B4; Sigma-Aldrich Co., St Louis, MO, USA); recombinant human interleukin (IL)-13 (Biotec, Hamburg, Germany); MUC5AC monoclonal antibody (45M1; Lab Vision, Fremont, CA, USA); MUC5B rabbit polyclonal IgG (Santa Cruz Biotechnology, TX, USA); anti-mouse-IgG horseradish peroxidase (HRP)-linked whole antibody (GE Healthcare, Piscataway, NJ, USA); phospho- and nonphospho-specific ERK1/2, phospho-specific NF-κB p65 (Ser536) and nonphospho-specific NF-κB p65 and anti-rabbit-IgG HRP antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA); DMEM, Ham's F12 medium and PBS (Gibco, Grand Island, NY, USA); bronchial epithelial cell growth medium supplemented with the SingleQuotR® kit and Hanks' balanced salt solution (HBSS) (Lonza Walkersville Inc., Walkersville, MD, USA); PD98059 (Calbiochem, La Jolla, CA, USA); and PS1145 (Sigma-Aldrich Co.). Dimethylsulfoxide (DMSO) (Sigma-Aldrich Co.) was used as a solvent of PD98059 and PS1145. LPS and CC10 were dissolved in PBS at concentrations of 1 mg·mL$^{-1}$ and 100 μg·mL$^{-1}$, respectively, and stored at -80°C.

### NHBE cell culture

NHBE cells (Lonza Walkersville Inc.) were seeded at 3500 cells·cm$^{-2}$ and grown in bronchial epithelial cell growth medium supplemented with the SingleQuotR® kit at 37°C with 5% $CO_2$. The medium was changed every 48 h, and the cells were cultured until 70–80% confluence. At the second passage, the cells were seeded to polyester membrane transwell-clear inserts of 0.4 μm pore size, 6.5 mm diameter, 10 μm thickness (Corning, Lowell, MA, USA) at $2.0 \times 10^5$ cells·cm$^{-2}$ each with type I rat-tail collagen. NHBE cells were then cultured in DMEM/Ham's F12 medium with 1% insulin-transferrin-selenium A, recombinant epidermal growth factor (0.5 ng·mL$^{-1}$), triiodothyronine (10 ng·mL$^{-1}$), hydrocortisone (0.5 μg·mL$^{-1}$), all-trans retinoic acid ($1.0 \times 10^{-7}$ M), bovine serum albumin (BSA) (2.0 μg·mL$^{-1}$) and bovine pituitary extract (30 μg·mL$^{-1}$). After achieving 70–80% confluence, the apical side of the medium was removed and cells were cultured at an air–liquid interface. The medium was changed every 48 h at 37°C with 5% $CO_2$ for 14 days. After 14 days' culture at an air–liquid interface, NHBE cells were well differentiated with cilia, basal and secretory cells as described previously [17, 18].

### LPS, IL-13 and CC10 exposure

NHBE cells were grown for 14 days with CC10 (0, 1, 10 or 20 ng·mL$^{-1}$) [11, 19]. Cells were exposed to 5 μg·mL$^{-1}$ LPS from the basolateral side of the inserts for 24 h before sample harvest [20]. For IL-13 exposure, cells were grown at an air–liquid interface for 14 days with CC10 (0, 1 or 20 ng·mL$^{-1}$) and IL-13

(0, 0.5 or 1 ng·mL$^{-1}$) [21].To collect samples, HBSS was added to the apical side of the inserts, and 24-h culture supernatants and culture media from the basolateral side of inserts were harvested.

### *MUC5AC and MUC5B protein by ELISA*

MUC5AC and MUC5B protein in supernatants were harvested and prepared for ELISA as previously described [21]. The 96-well plates were coated with 50 μL sample and bicarbonate-carbonate buffer (50 μL), incubating at 37°C overnight until samples dried. After washing with 0.05% Tween 20 PBS buffer (T-PBS), 2% BSA/T-PBS was added to each well at room temperature for 1 h as blocking. After washing with T-PBS, MUC5AC monoclonal antibody (45M1) or MUC5B rabbit polyclonal IgG in T-PBS was added to a concentration of 2 μg·mL$^{-1}$ and the plate was incubated for 2 h. Anti-mouse-IgG HRP-linked whole antibody for MUC5AC or anti-rabbit-IgG HRP antibodies for MUC5B were dispensed into each well, and the plate was incubated for 1 h. After washing with T-PBS, 3,3′,5,5′-tetramethylbenzine peroxidase solution was added to the plate. The reaction was stopped with 2 N $H_2SO_4$, and absorbance was measured using an ELx808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Data are expressed as the percentage above the vehicle control.

### *IL-8, GM-CSF and CC10 secretion by ELISA*

IL-8 and CC10 protein in supernatants and culture media, and granulocyte-macrophage colony-stimulating factor (GM-CSF) protein in supernatants, were measured using human CXCL8/IL-8 ELISA DuoSet (R&D Systems), human club cell protein ELISA (BioVendor R&D, Asheville, NC, USA), and human GM-CSF ELISA DuoSet (R&D Systems). Optical density was measured at 450 nm using a microtitre plate reader (Spectra Max Plus; Molecular Devices, Sunnyvale, CA, USA) with software (Soft Max Pro version 2.0; Molecular Devices). The concentration of each sample was obtained from standard curves and calculated as the mean of the results.

### *Real-time quantitative PCR analysis*

MUC5AC and IL-8 mRNA expression was examined by real-time PCR [17, 21]. After LPS exposure, the apical side of the cells was washed three times with PBS, and total RNA was extracted using iScript RT-qPCR sample preparation reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA). The total RNA was then used to synthesise the first-strand cDNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed on the C 1000$^{TM}$ thermal cycler equipped with CFX96$^{TM}$ real-time PCR system (Bio-Rad). For the relative quantification of MUC5AC and IL-8 mRNA expression, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as an internal control. Eva Green (Biotium, Inc., Hayward, CA, USA) was used as a DNA intercalator dye to monitor amplified DNA quantification, and real-time quantitative PCR curves were analysed by CFX Manager$^{TM}$ software (Bio-Rad) in order to obtain threshold cycle values for each sample. mRNA expression level was calculated based on a standard curve. The following primers were used: MUC5AC forward 5′-TACTCCACAGACTGCACCAACTG-3′; MUC5AC reverse 5′-CGTGTATTGCTTCCCGTCAA-3′; IL-8 forward 5′-CTGCGCCAACACAGAAATTA-3′; IL-8 reverse 5′-ACTTCTCCACAACCCTCTGC-3′; GAPDH forward 5′-TGAACGGGAAGCCACGG-3′; GAPDH reverse 5′-TCCACCACCCTGTTGCTGTA-3′.

### *Western blot analysis*

Cell lysates were extracted and prepared for Western analysis [2, 17]. NHBE cells were seeded on polyester inserts and cultured with CC10 (0, 1 or 20 ng·mL$^{-1}$) for 14 days at an air–liquid interface. Culture media were changed every other day, and cells were cultured in supplement-free medium for 48 h before LPS exposure to avoid influence of growth factors on cell signalling. The cell lysates were harvested after LPS exposure. Cells were washed with cold PBS and lysed on ice in a modified ratio immunoprecipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris pH 7.5, 5 mM sodium pyrophosphate, 1 mM $NaVO_4$, 5 mM NaF, 1 μg·mL$^{-1}$ aprotinin, 1 μg·mL$^{-1}$ leupeptin and 0.1 mM phenylmethylsulfonyl fluoride) for 15 min and scraped from the plates. DNA was collected by passing the lysate through a 27-gauge needle, and insoluble material was removed by centrifugation at 20 000 × *g* for 15 min at 4°C. The protein concentration of the supernatants was quantified using the Detergent Compatible Protein Assay (Bio-Rad). Equal amounts of protein extracts were loaded on a 12% SDS-PAGE mini gel and transferred to a nitrocellulose membrane by Trans-Blot® Turbo$^{TM}$ Transfer system (Bio-Rad). Membranes were rinsed with distilled water, incubated overnight at 4°C in Tris-buffered saline (0.8% NaCl and 20 mM Tris pH 7.6) with 0.1% Tween 20 (TBS-T) with 5% nonfat dry milk to block nonspecific interactions, rinsed twice, and washed three times for 10 min with TBS-T. After washing, membranes were rinsed and incubated with the primary rabbit polyclonal IgG antibodies for 2 h in TBS-T: phospho-ERK1/2 (Thr202/Tyr204), diluted 1:2000; total ERK1/2, diluted 1:1000; phospho-NF-κB p65 (Ser536), diluted 1:1000; or total NF-κB p65, diluted 1:1000 (Cell Signaling Technology). The membranes were then


DOI: 10.1183/09031936.00080913

incubated for 2 h with the anti-rabbit-IgG HRP secondary antibody (diluted 1:2000). After washing, antibody binding was detected using LumiGLO chemiluminescent substrate peroxide (Cell Signaling Technology). Membranes were stripped with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris/HCl pH 6.7) for 20 min. Western blot images were scanned and analysed using NIH Image J software (National Institutes of Health, Bethesda, MD, USA).

### *PD98059 or PS1145 exposure*

After cells were grown for 14 days, they were exposed from the basolateral side of the inserts to vehicle (0.11% DMSO), 5 μg·mL$^{-1}$ LPS, PS1145 (an IκB kinase inhibitor) at 30 μM, or PD98059 (a mitogen-activated protein kinase (MAPK)/ERK kinase inhibitor) at 20 μM [2].

### *Histology*

Cell morphology was evaluated after haematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining [2, 21]. The transwell membrane with cells was fixed in 20% formalin neutral buffer, embedded in paraffin, then cut into 8-μm slices. We assessed the cell morphology with a light microscope (CKX41; Olympus, Tokyo, Japan) using a digital camera system (AxioCam ICc 1; Carl Zeiss, Thornwood, NY, USA).

### *Statistical analysis*

Data are expressed as mean±SD. The results are from at least three independent experiments conducted with single donor cells. Parametric testing was conducted after confirming that data were normally distributed. Statistical differences were examined by unpaired two-tailed t-test or ANOVA as appropriate. A p-value $<0.05$ was considered significant. Statistical analysis was performed using JMP Pro9 for Windows (SAS, Cary, NC, USA).

## Results

### *Effect of CC10 on LPS-stimulated MUC5AC secretion*

Although the addition of CC10 did not affect constitutive MUC5AC secretion (fig. 1a), CC10 attenuated LPS-stimulated MUC5AC production. LPS 5 μg·mL$^{-1}$ increased MUC5AC secretion in supernatants (144±36.5%) compared with PBS ($p<0.001$), and CC10 attenuated MUC5AC secretion ($p<0.05$ for CC10 at 1 and 20 ng·mL$^{-1}$, and $p<0.001$ for CC10 at 10 ng·mL$^{-1}$) (fig. 1b).

### *Effect of CC10 on LPS-stimulated MUC5AC mRNA expression*

LPS 5 μg·mL$^{-1}$ increased MUC5AC mRNA expression (3.61±2.18; $p<0.05$), and CC10 (20 ng·mL$^{-1}$) attenuated MUC5AC mRNA expression (1.79±0.87; $p<0.05$) (fig. 1c).

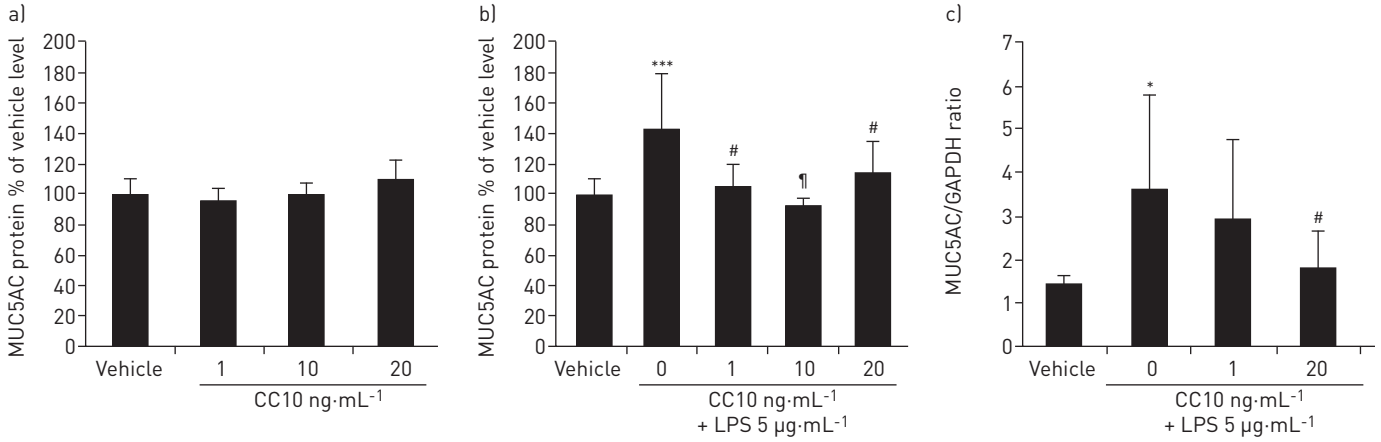


FIGURE 1 Effect of club cell 10-kDa protein (CC10) on mucin MUC5AC secretion and mRNA expression. Normal human bronchial epithelial cells were cultured for 14 days in the presence of CC10 (0, 1, 10, or 20 ng·mL$^{-1}$). Cells were then exposed to 0 or 5 μg·mL$^{-1}$ lipopolysaccharide (LPS) before extracting supernatants and total RNA. a) Constitutive MUC5AC protein secretion in the presence of CC10, shown as mean±SD percentage of value with vehicle (PBS) alone. b) MUC5AC protein secretion in the presence of LPS and CC10. c) MUC5AC mRNA expression measured by real-time PCR. Data are expressed as the ratio of MUC5AC to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and shown as mean±SD. Figures are from at least four independent experiments. *: $p<0.05$ compared to vehicle; ***: $p<0.001$ compared to vehicle; #: $p<0.05$ compared to LPS alone; ¶: $p<0.001$ compared to LPS alone.

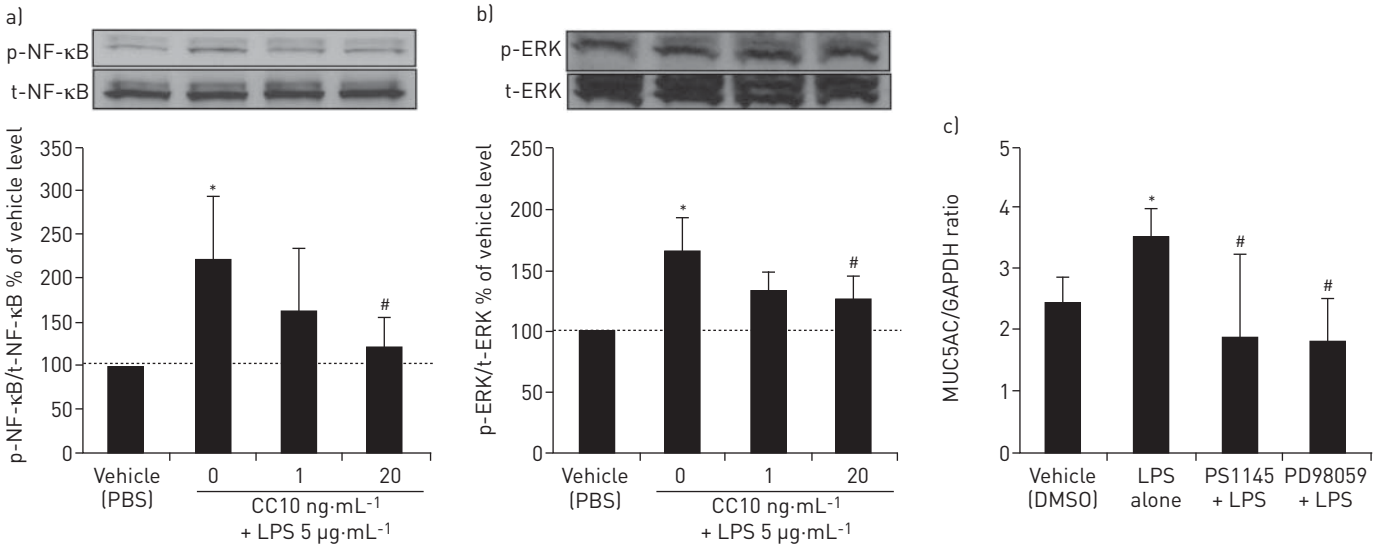


FIGURE 2 Effect of club cell 10-kDa protein (CC10) on lipopolysaccharide (LPS)-stimulated a) nuclear factor (NF)-κB and b) extracellular signal-regulated kinase (ERK)1/2 activation. Normal human bronchial epithelial (NHBE) cells were cultured for 14 days in the presence of CC10 (0, 1 or 20 ng·mL$^{-1}$). Cells were stimulated with 5 μg·mL$^{-1}$ LPS for 1 h before extracting cell lysates. a) Total (t-)NF-κB and phosphorylated (p-)NF-κB, and b) t-ERK1/2 and p-ERK1/2 were measured by Western blotting. Data are shown as mean±SD percentage of phosphorylated/total protein ratio with vehicle (PBS) alone. Figures are from at least three independent experiments. c) Regulation of mucin MUC5AC mRNA expression by PD98059 or PS1145. NHBE cells were cultured for 14 days and then exposed to vehicle (dimethylsulfoxide (DMSO)), 5 μg·mL$^{-1}$ LPS, 20 μM PD98059 or 30 μM PS1145 before extracting total RNA. MUC5AC mRNA expression was measured by real-time PCR. Data are expressed as the ratio of MUC5AC to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and shown as mean±SD. Figures are from four independent experiments. *: $p<0.05$ compared to vehicle; #: $p<0.05$ compared to LPS alone.

### *Effect of CC10 on LPS-stimulated phosphorylation of NF-κB p65 and ERK1/2*

LPS 5 μg·mL$^{-1}$ increased phospho-NF-κB expression (220±73%) compared with PBS ($p<0.05$), and this was attenuated by 20 ng·mL$^{-1}$ CC10 (122±33%; $p<0.05$) (fig. 2a). CC10 20 ng·mL$^{-1}$ also attenuated ERK1/2 phosphorylation (127±19%) induced by LPS exposure (166±27%) ($p<0.05$) (fig. 2b).

### *Regulation of MUC5AC mRNA expression by PD98059 or PS1145*

LPS 5 μg·mL$^{-1}$ increased MUC5AC mRNA expression (3.52±0.47) compared with DMSO ($p<0.05$), and this was attenuated by both the IκB kinase inhibitor, PS1145 (1.88±1.33; $p<0.05$), and by the MAPK/ERK kinase inhibitor, PD98059 (1.81±0.70; $p<0.05$) (fig. 2c).

### *Effect of CC10 on LPS-stimulated IL-8 secretion*

CC10 did not affect constitutive IL-8 secretion (fig. 3a). As expected, LPS 5 μg·mL$^{-1}$ increased IL-8 secretion compared with vehicle (44 321±8453 *versus* 21 249±4234 pg·mL$^{-1}$; $p<0.05$), and CC10 (1, 10 and 20 ng·mL$^{-1}$) attenuated LPS-induced IL-8 secretion ($p<0.05$) (fig. 3b). LPS 5 μg·mL$^{-1}$ also increased IL-8 secretion in the basal culture media compared with PBS (4583±2701 *versus* 2085±844 pg·mL$^{-1}$; $p<0.05$), and CC10 (20 ng·mL$^{-1}$) attenuated this as well (2384±814 pg·mL$^{-1}$; $p<0.05$) (fig. 3c).

### *Effect of CC10 on LPS-stimulated IL-8 mRNA expression*

LPS 5 μg·mL$^{-1}$ increased IL-8 mRNA expression (2.76±0.74; $p<0.001$), and CC10 (1 and 20 ng·mL$^{-1}$) attenuated IL-8 mRNA expression induced by LPS ($p<0.001$) (fig. 3d).

### *Effect of CC10 on LPS-stimulated GM-CSF secretion*

CC10 did not affect constitutive GM-CSF secretion (fig. 3e). LPS at 5 μg·mL$^{-1}$ increased GM-CSF secretion in supernatants compared with PBS (537±390 *versus* 163±47 pg·mL$^{-1}$; $p<0.05$), and CC10 (1 and 10 ng·mL$^{-1}$) attenuated GM-CSF secretion with LPS stimulation ($p<0.05$) (fig. 3f).

### *Effect of CC10 on NHBE cell morphology*

NHBE cells were stained with HE (fig. 4a–e) and PAS (fig. 4f–j). Cells showed differentiated morphology with metachronally beating ciliated cells at the surface of epithelial layers (fig. 4a–e) without goblet cell granules (fig. 4a). NHBE cells were weakly stained with PAS. PAS-positive nongoblet cells and the number of PAS-positive cells were moderately increased with exposure to LPS 5 μg·mL$^{-1}$ (fig. 4f and g). However, there were no differences in the presence of CC10 1 ng·mL$^{-1}$ (fig. 4c and h), 10 ng·mL$^{-1}$ (fig. 4d and i) or


DOI: 10.1183/09031936.00080913

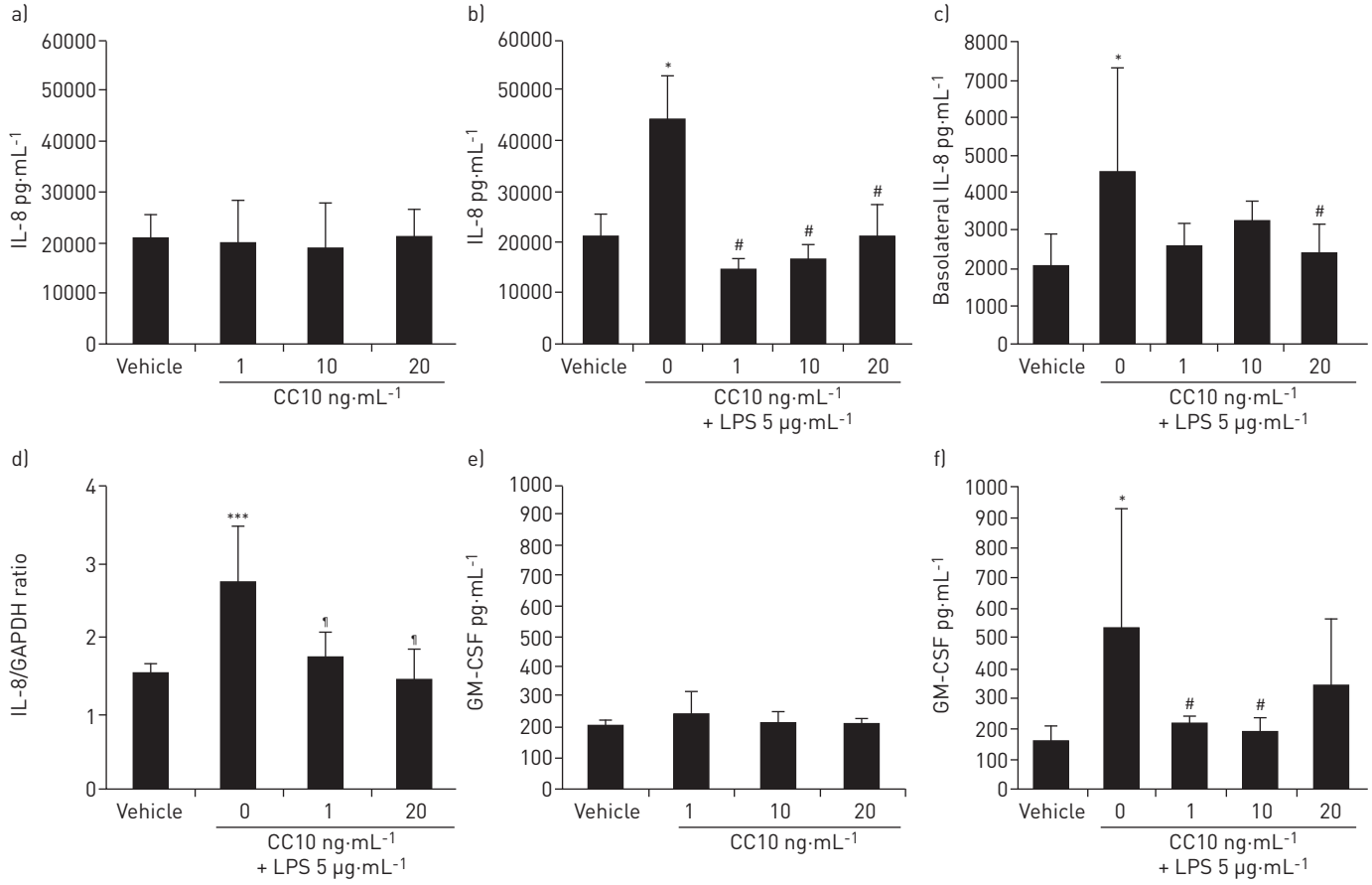


FIGURE 3 Effect of club cell 10-kDa protein (CC10) on interleukin (IL)-8 secretion, IL-8 mRNA expression and granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion. Normal human bronchial epithelial cells were cultured for 14 days in the presence of CC10 (0, 1, 10 or 20 ng·mL$^{-1}$) and then exposed to 5 μg·mL$^{-1}$ lipopolysaccharide (LPS) before extracting supernatants, culture media and total RNA. a) Constitutive IL-8 secretion in supernatants. b) LPS-stimulated IL-8 secretion in supernatants. c) LPS-stimulated basolateral IL-8 secretion. d) IL-8 mRNA expression measured by real-time PCR. Data are expressed as the ratio of IL-8 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. e) Constitutive GM-CSF secretion. f) LPS-stimulated GM-CSF secretion. Data are shown as mean±SD. Figures are from at least three independent experiments. *: $p<0.05$ compared to vehicle (PBS); ***: $p<0.001$ compared to vehicle (PBS); #: $p<0.05$ compared to LPS alone; ¶: $p<0.001$ compared to LPS alone.

20 ng·mL$^{-1}$ (fig. 4e and j), compared with PBS (fig. 4a and f). Histology suggested that 14 days of CC10 did not affect NHBE cell differentiation.

### *Effect of CC10 on IL-13-induced MUC5AC and MUC5B secretion*

We used two concentrations of IL-13 to examine the effect of CC10 on MUC5AC and MUC5B secretion. IL-13 at 0.5 and 1 ng·mL$^{-1}$ increased MUC5AC secretion more than two-fold, and CC10 (1 and 20 ng·mL$^{-1}$) significantly attenuated this IL-13-induced MUC5AC production (fig. 5a). Furthermore, IL-13 at 1 ng·mL$^{-1}$ moderately increased MUC5B secretion, and this was attenuated by CC10 at 1 ng·mL$^{-1}$ ($p<0.05$) (fig. 5b).

### *CC10 secretion exposed to LPS or IL-13*

We measured CC10 secretion in response to different concentrations of LPS (0, 1, 5 or 20 μg·mL$^{-1}$) and IL-13 (0, 0.2, 0.5 or 1 ng·mL$^{-1}$). CC10 was constitutively secreted into the apical (2.72±2.06 ng·mL$^{-1}$) and basolateral (0.68±0.30 ng·mL$^{-1}$) media ($p<0.05$) (fig. 5c), and neither IL-13 nor LPS exposure affected CC10 secretion (fig. 5d and e).

## Discussion

CC10 has been reported to suppress phospholipase $A_2$ activity [7, 10, 11], inflammatory cell migration, Th2 T-cell differentiation [11], pro-inflammatory cytokine production [7] and phagocyte chemotaxis [22]. Ovalbumin-sensitised CC10 knockout mice exhibited eosinophilic inflammation and increased mucus production in the lungs [7, 23] and CC10 in nasal lavage fluid is lower in subjects with allergic rhinitis than

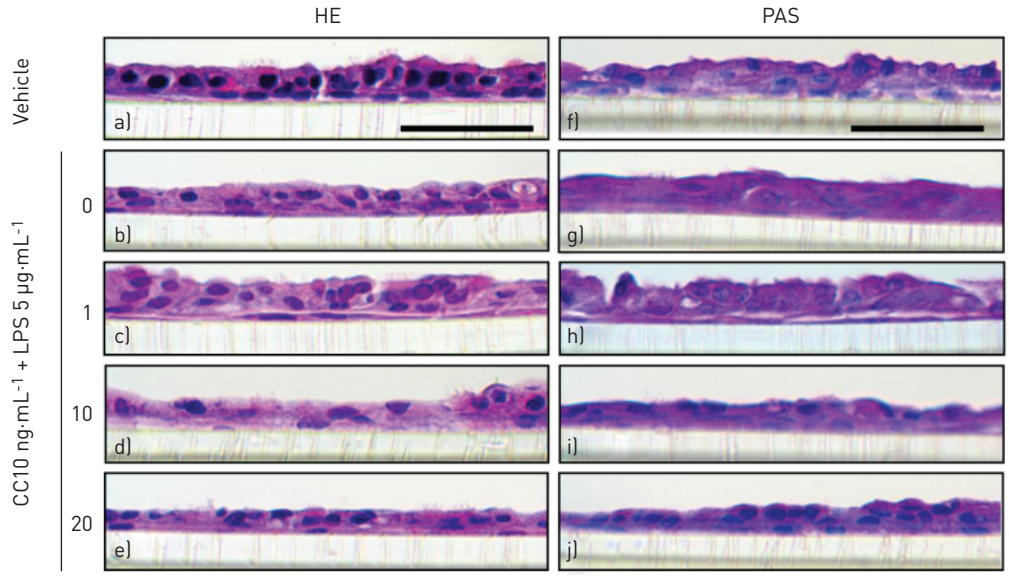


FIGURE 4 Haematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining. Normal human bronchial epithelial cells were grown for 14 days with club cell 10-kDa protein (CC10) (0, 1, 10 or 20 ng·mL$^{-1}$) and exposed to lipopolysaccharide (LPS) (5 μg·mL$^{-1}$) or LPS vehicle (PBS) on day 14. a, f) PBS alone; b, g) LPS without CC10; c, h) LPS and CC10 at 1 ng·mL$^{-1}$; d, i) LPS and CC10 at 10 ng·mL$^{-1}$; e, j) LPS and CC10 at 20 ng·mL$^{-1}$. Scale bars=50 μm.

in healthy subjects [24]. CC10 mRNA expression in sinonasal mucosa is lower in subjects with allergic rhinitis, asthma, and chronic rhinosinusitis with nasal polyps [8, 15, 16], suggesting that CC10 may modulate allergic airway inflammation. CC10 also inhibits IL-13-induced chitinase 3-like 1 (CHI3L1) gene expression in BEAS-2B cells [25]. An intravenous injection of CC10 inhibited LPS-stimulated IL-6, IL-1β and tumour necrosis factor-α in perfusates from isolated rat lungs [26]. In a pilot study, CC10 administered intratracheally to infants with respiratory distress syndrome decreased the number of neutrophils in tracheal aspirate fluid [27]. Although mucus secretion is a fundamental defence mechanism of the airway, the effect of CC10 on mucus production and secretion has not been evaluated. We have shown that CC10 attenuated MUC5AC production when cells were exposed to LPS or IL-13, but had no effect on constitutive mucin secretion. IL-8 and GM-CSF stimulated by LPS were also decreased by CC10. We also examined the effect of 24-h CC10 (1, 10 or 100 ng·mL$^{-1}$) exposure in submerged culture, and IL-8 and GM-CSF were significantly attenuated by CC10 (data not shown).

The concentration of CC10 used in this study (1–20 ng·mL$^{-1}$) was in the range of serum levels in healthy adults [19]. We used serum concentration because cells were exposed to CC10 from the basolateral (“endothelial”) side of the inserts [20]. We also measured steady-state constitutive CC10 secretion. CC10 secretion into NHBE cell epithelial lining fluid was lower than reported in bronchoalveolar lavage fluid (BALF) [19], and we suspect that BALF contains CC10 not only from bronchial cells but also from the endothelial compartment. Additionally, BALF represents fluid that has been present far longer than the dwell time of the epithelial lining fluid washing from cells grown on transwell inserts [28]. With exposure to CC10 over 14 days, it is likely that the intracellular concentration of CC10 was at steady state [29]. There were no side-effects reported with tracheal or intranasal administration of CC10 in humans and piglets [27, 29]. Consistent with this, we saw no changes in cell histology over 14 days with any concentration of CC10.

Airway epithelial cells are covered by a mucus fluid layer, containing the gel-forming mucins MUC5AC and MUC5B [4, 30]. Diverse stimuli increase mucin production in airway epithelial cells [4, 31]. LPS enhances mucus hypersecretion in human airways, signalling through ERK and NF-κB downstream [5]. MUC5AC is increased in NHBE cells after LPS exposure *via* NF-κB activation [32], and the intratracheal instillation of LPS increases MUC5AC secretion *via* ERK activation [33]. These reports are consistent with our results that MUC5AC mRNA expression was attenuated by IκB kinase inhibitor and MAPK/ERK kinase (MEK, an upstream kinase of ERK1/2) inhibitor. NF-κB phosphorylation precedes NF-κB nuclear translocation [34, 35].

To further examine the effect of CC10 on mucus secretion, NHBE cells were exposed for 14 days to IL-13, a Th2 mediator of allergic inflammation, and we measured MUC5AC and MUC5B secretion [21]. Like LPS, IL-13 also induced MUC5AC secretion [2, 36] and this was attenuated by CC10. IL-13 secretion is attenuated by CC10 in splenocytes of ovalbumin-sensitised rats and CC10 knockout mice [7, 37].


DOI: 10.1183/09031936.00080913

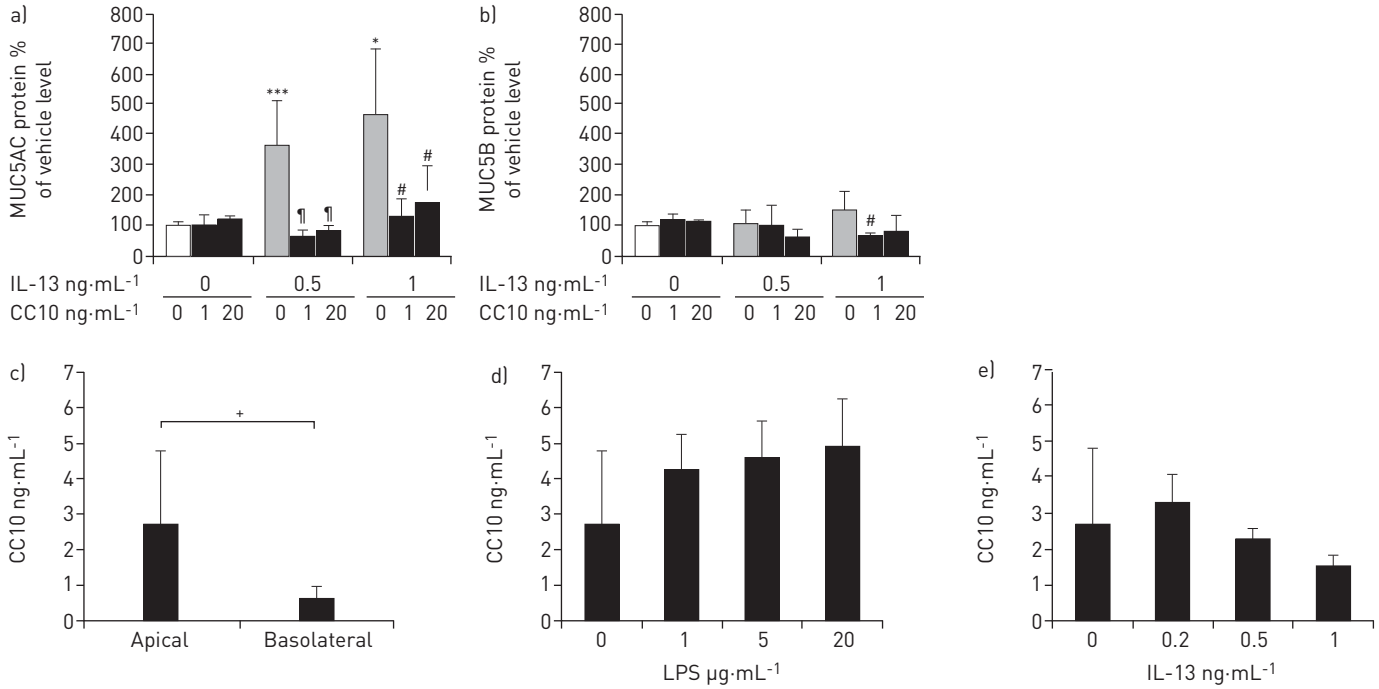


FIGURE 5 Effect of club cell 10-kDa protein (CC10) on secretion of mucins a) MUC5AC and b) MUC5B. Normal human bronchial epithelial (NHBE) cells were cultured for 14 days in the presence of CC10 (0, 1 or 20 ng·mL$^{-1}$) and interleukin (IL)-13 (0, 0.5 or 1 ng·mL$^{-1}$). Data are shown as mean ± SD percentage of value with vehicle (PBS) alone. c–e) Effect of lipopolysaccharide (LPS) or IL-13 on CC10 secretion. NHBE cells were cultured for 14 days and exposed to LPS (0, 1, 5 or 20 µg·mL$^{-1}$), or cultured for 14 days in the presence of IL-13 (0, 0.2, 0.5 or 1 ng·mL$^{-1}$). c) CC10 secretion from apical and basolateral sides of inserts; d) CC10 secretion with LPS exposure; e) CC10 secretion with IL-13 exposure. Data are shown as mean ± SD. Figures are from three independent experiments. *: $p<0.05$ compared to vehicle; ***: $p<0.001$ compared to vehicle; #: $p<0.05$ compared to IL-13 alone; ¶: $p<0.001$ compared to IL-13 alone; +: $p<0.05$.

Furthermore, ovalbumin-sensitised CC10 knockout mice had eosinophilic inflammation and increased mucus production in the lungs [23].

Our data show that neither IL-13 nor LPS had a direct effect on CC10 secretion. These data are consistent with a report that, although intratracheal IL-13 exposure increases CC10 mRNA expression *in vivo*, IL-13 did not affect CC10 expression in cultured NCI-H292 cells [38]. It was reported that CC10 secretion in the nose is lower after intranasal LPS exposure [13, 39], and is also decreased in subjects with asthma, allergic rhinitis, acute lung injury or chronic obstructive pulmonary disease [16, 24, 40, 41]. IL-8 and GM-CSF are thought to be biomarkers of LPS-induced injury [42]. They activate neutrophils, dendritic cells and macrophages, and increase cytokine production *via* NF-κB and ERK [6, 42]. We showed that CC10 blocked LPS-induced NF-κB and ERK phosphorylation, and this inhibitory effect of CC10 on cell signalling is consistent with decreased MUC5AC and IL-8 mRNA expression.

LPS can induce airway inflammation, including cytokine production and mucus hypersecretion, but our data show that LPS can also modestly increase CC10, which will attenuate this inflammatory response. IL-13, a Th2 cytokine, greatly increases airway mucin production, and this too is attenuated by CC10 in physiological concentrations.

## Acknowledgements

We thank Jorge A. Almenara, histotechnologist, for technical assistance in the tissue processing, sectioning and staining, and Melissa A. Yopp for technical assistance (both at Virginia Commonwealth University, Richmond, VA, USA).

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 DOI: 10.1183/09031936.00080913