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ABSTRACT

Chronic inflammation is hallmark of Cystic Fibrosis (CF) lung disease. Airway epithelium damage and remodelling are important components of the lung pathology progression in CF. Whether this remodelling is secondary to deleterious infectious and inflammatory mediators, or to alterations of CF human airway epithelial (HAE) cells such as their hyperinflammatory phenotype or their basic Cystic Fibrosis Transmembrane conductance Regulator (CFTR) default remains debated. We aimed in this study at evaluating the involvement of alterations of CF HAE cells in airway epithelium remodelling. HAE cells from non-CF and CF patients were cultured in an air–liquid interface with and without inflammatory stimulation along the regeneration process, and the remodelling of the reconstituted epithelium was analysed. We confirmed that CF HAE cells showed a hyperinflammatory phenotype which was lost with time. In comparison to non-CF epithelium, the CF epithelium regenerated in absence of exogenous inflammation was higher and exhibited a basal cell hyperplasia. This remodelling was mimicked by inflammatory stimulation of non-CF cells and was absent when CF HAE cells were no longer hyperinflamed. Moreover, the number of goblet cells was similar in non-CF and CF cultures and increased equally under inflammatory stimulation. Finally, whatever the inflammatory environment, the CF cultures showed a delay in ciliated cell differentiation. In conclusion, alterations of CF HAE cells partly regulated the airway epithelium remodelling following injury and regeneration. This remodelling, together with goblet cell hyperplasia only induced by exogenous inflammation, and with the alteration of ciliated cell differentiation may worsen mucociliary clearance impairment leading to injury.

Key words: Cystic Fibrosis; airway epithelium remodelling; regeneration; airway inflammation

INTRODUCTION

Cystic Fibrosis (CF) is the most common lethal autosomal recessively inherited disorder among Caucasians. Mutations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene are associated with altered ion and fluid transports leading to viscous secretions in the lungs that promote bacterial infection and chronic inflammation and resulting in a decline in lung function (1, 2). Proteases, oxidants, virulence factors and inflammatory mediators induce airway epithelial damages (3, 4, 5, 6) such as hyperplasia of surface goblet and basal cells (7-10), squamous metaplasia (8, 10, 11), increase in epithelium height (7, 12) as well as cell shedding (7-10, 12, 13). Whether this remodelling is secondary to deleterious infectious and inflammatory mediators, or to CFTR dysfunction remains debated. Indeed, in response to insults, epithelial cell migration, proliferation and differentiation are required for proper tissue repair and regeneration (14, 15) and some studies have reported a major role for CFTR in the early event of cell migration (8, 16, 17). While it is well known that some ion channels regulate cell migration (18, 19), it has been recently demonstrated that CF airway epithelial cells exhibited slower migration than non-CF cells (8, 16). As CFTR expression or activity inhibition resulted in a significant delay in wound repair, and F508del-CFTR correction significantly improved wound healing (8, 16), it is now clear that the CFTR default modulates airway epithelial cell migration. Moreover, using a humanized xenograft mouse model, we have previously shown that the CF airway epithelial regeneration is impaired even in absence of infection: the regeneration process is delayed in terms of ciliated cell differentiation and the reconstituted epithelium is remodelled, significantly higher with basal cells hyperplasia (20).

Chronic inflammation is a hallmark of CF. Inflammation begins early in the course of the disease, even in patients without clinically apparent lung disease (21). As several studies have reported the presence of increased inflammatory markers in bronchoalveolar lavages (BAL) of infants in apparent absence of CF-related pathogens (22-25), it has been postulated

that inflammation may precede infection by some direct contribution of the defective CFTR (26, 27). However, alternative explanations have been proposed, in particular the persistence of the inflammatory response after clearance of bacterial infection (3, 26). Regardless of the initial source of stimulation, inflammatory mediators, that can be produced by airway epithelial cells themselves (28-30), are elevated in sputa and BALs from CF compared to control patients and exaggeratedly produced in response to infection (22, 25, 31-35).

In the present study, using primary cultures of non-infected CF and non-CF human airway epithelial (HAE) cells from nasal origin cultured in an air–liquid interface (ALI) with and without inflammatory stimulation along the regeneration process, we demonstrated that the transient hyperinflammatory phenotype of CF cells could be partly responsible of the CF epithelium remodelling.

MATERIALS AND METHODS

Human airway tissues and cell dissociation

The use of human tissues was authorized by the bioethical law n° 2004-800 of the French Public Health Code, after written consent from the patients and the approval of our local Institutional Review Board. Non-infected human airway tissues were collected after nasal polypectomy from 9 CF non-smoker patients (median age 13; range 4-46; genotypes: F508del/F508del: 4; F508del/G85E: 2; F508del/W1282X: 1; F508del/R1158X: 1; F508del/G542A: 1) and 16 non-CF non-smoker subjects who did not suffer from any other disease (asthma, allergy, chronic obstructive pulmonary disease (COPD)) at the time of experiments were carried out (median age: 54; range 30-82). Non-CF and CF human airway epithelial (HAE) cells were obtained after dissociation by 0.05% type XIV collagenase (Pronase E; Sigma Aldrich, St Louis, MO) as described in the online supporting information.

Cell culture

Nasal HAE cells were used at passages 0 (P0) (from 9 non-CF and 9 CF donors) and at passage 1 (P1) (from 4 non-CF and 4 CF donors) (see online supporting information) in order to work with hyperinflamed (P0) and no more inflamed (P1) CF cells, as it has been previously reported (29). The ALI culture method was adapted from Fulcher *et al.* (36). Briefly, HAE cells were cultured on Transwell supports (Transwell-Clear, Corning, Acton, MA) in submerged conditions, then in an ALI for 0 to 35 days (ALI D0 to ALI D35) using, in the basal compartments, a medium consisting in 1:1 DMEM/F12 (Gibco, Paisley, UK) and BEGM (Lonza, Walkersville, MD) with the Lonza supplements for hEGF, epinephrine, BPE, hydrocortisone, insulin, triiodothyronine and transferrin, and supplemented with 200 UI/ml penicillin, 200 µg/ml streptomycin (Gibco), 0.1 nM retinoic acid (Sigma Aldrich) and 1.5 µg/ml bovin serum albumin (BSA; Sigma Aldrich), with or without pro-inflammatory cytokine (cytomix cocktail composed of TNFα (1 ng/ml; Sigma Aldrich), IL-1β (1 ng/ml; Abcys, Courtaboeuf, France) and IFNγ (1 ng/ml; Miltenyl Biotec, Auburn, CA) stimulation as described in the online supporting information.

Histological analysis

Histology of 4 non-CF and 4 CF cultures (genotypes : F508del/F508del: 3; F508del/W1282X: 1) at P0 and ~~at~~ P1 was analysed at ALI D35 on cryosections after hematoxylin-eosin staining as described in the online supporting information. Epithelium height was measured on 20 images per section and 5 different sections per culture using a Nikon Eclipse Ci microscope (Nikon, Champigny sur Marne, France) equipped with a Nikon DS-Fi2 camera, and the dedicated Nis-elements software. The results are expressed in µm.

Immunohistochemistry

To quantify the different cell types composing the non-CF and CF cultures (genotypes: F508del/F508del: 3; F508del/W1282X: 1) at P0 and P1, basal, goblet and ciliated cells were stained by immunohistochemistry on cryosections or on methanol-fixed cultures using antibodies directed against cytokeratin 13 (CK13, basal cell marker; Sigma Aldrich), MUC-5AC (goblet cell marker; Novocastra, Newcastle upon Tyne, UK), MUC-5B (provided by Dr Copin, Lille France) and β-tubulin (ciliated cell marker; Chemicon, Temecula, CA). Cycling cells were detected using antibodies anti-Ki67 (Dako, Glostrup, Denmark). Primary antibodies were revealed with goat anti-mouse Alexa Fluor 488 antibodies (Molecular Probe, Eugene, OR) and cell nuclei stained using DAPI (Sigma Aldrich). Negative controls were performed by omitting the primary antibodies. Images were acquired on an AxioImager ZI Microscope (Carl Zeiss, Göttingen, Germany) equipped with a CCD camera (CoolSnap HQ, Photometrics, Tucson, AZ) and using the dedicated Axiovision release 4.8 software (Carl Zeiss). Technical and quantification methods are described in details in the online supporting information. The results are expressed as percentage of positive cells for CK13, MUC-5AC and Ki67, and in number of positive cells per mm^2 for ciliated cells.

RT-qPCR analysis

IL-8 mRNA expression by nasal HAE cells from 7 non-CF and 5 CF donors at P0 (genotypes: F508del/F508del: 3; F508del/W1282X: 1; F508del/R1158X: 1), and from 4 non-CF and 4 CF donors at P1 (genotypes: F508del/F508del: 3; F508del/W1282X: 1) was analysed by RT-qPCR. IL-8 expression was also studied in 9 non-CF and 9 CF cultures (Passage 0; genotypes: F508del/F508del: 4; F508del/G85E: 2; F508del/W1282X: 1; F508del/R1158X: 1; F508del/G542A: 1) at ALI D0, ALI D7, ALI D15, ALI D35, and in 4 non-CF and 4 CF cultures (Passage 1; genotypes: F508del/F508del: 3; F508del/W1282X: 1) at ALI D35.

Total RNA was extracted using the High Pure RNA isolation kit (Roche Diagnostics, Indianapolis, IN), following the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) and quantitative PCR was performed using the Fast Start Universal Probe Master kit (Roche) as recommended by the manufacturer, in the LightCycler 480 Instrument (Roche). As described in the online supporting information, results were normalized to the expression levels of GAPDH and expressed as IL-8/GAPDH ratios.

Cytokine measurement

At ALI D0, ALI D7, ALI D15, ALI D25 and ALI D35, 4 non-CF and 4 CF (genotypes: F508del/F508del: 3; F508del/W1282X: 1) cultures at passage 0 were rinsed with sterile PBS, then 100 µl of fresh DMEM medium without phenol red (Gibco) were added to the basal compartment of the cultures, in contact with the porous membranes. The cultures were incubated for 4 hours at 37°C. Basal supernatants were collected and stored at -80°C until use. IL-8 was measured in these supernatants using the Human Interleukin 8 ELISA kit (Emelca Bioscience, Breda, The Netherlands) according to the manufacturer's instruction.

Statistical analysis

All experiments were performed using nasal HAE cells or ALI cultures obtained from, at least, 4 non-CF and 4 CF donors. Results are expressed as mean ± SEM. Data were analysed by the non-parametric Mann-Whitney test for statistical analysis using StatView software (SAS Institute, Inc., NC). Statistical significance was defined as $p < 0.05$.

RESULTS

In absence of exogenous infection and inflammation, the regeneration of the human CF airway epithelium leads to the reconstitution of a remodelled epithelium

Using an *in vivo* model of humanized xenograft in the mouse, we have previously reported that the CF human airway epithelial regeneration was abnormal and reconstituted a remodelled epithelium (20). In order to free oneself from any exogenous stimulation, we reproduced the experiments in the perfectly defined model of *in vitro* ALI culture. Non-CF and CF cultures were analysed at ALI D35, when they were approximately covered at 70% by ciliated cells and consequently considered as fully differentiated. As indicated by the measurement of epithelium height, the CF airway epithelium was significantly 32.87 % higher (38.03 ± 1.01 µm) than the non-CF epithelium (28.62 ± 0.75 µm) (Fig. 1C). Moreover, the CF epithelium exhibited basal cell hyperplasia (47.01 % cell number increase), with 39.37 ± 2.07 % of CK13 positive cells, while the non-CF epithelium reached 26.78 ± 0.35 % at the same time (Fig. 1F). Surprisingly, goblet cells were not more numerous in CF cultures compared to non-CF ones, with 0.32 ± 0.027 % and 0.29 ± 0.014 % of MUC-5AC positive cells, respectively (Fig. 1I), all MUC-5AC positive cells being also positive for MUC-5B (Supplementary Fig. 1). Ciliated cells were quantified during the course of their differentiation, from ALI D15 to ALI D35. At ALI D15, the number of β-tubulin positive ciliated cells was significantly 47.95 % lower in CF (235.92 ± 30.40 cells / mm^2) than in non-CF cultures (453.04 ± 46.25 cells / mm^2) (Fig. 1L), as it was also observed at ALI D25 with 349.19 ± 34.98 cells / mm^2 in CF and 642.09 ± 56.05 cells / mm^2 in non-CF cultures (decrease of 45.62 %) (Fig. 1O). In contrast, at ALI D35, both types of cultures exhibited an equivalent amount of ciliated cells with 783.85 ± 20.81 cells / mm^2 in CF and 821.81 ± 37.89 cells / mm^2 in non-CF cultures (Fig. 1R).

The quantification of cycling epithelial cells showed a decrease in the number of Ki67 positive cells during the course of regeneration in both non-CF and CF cultures. Interestingly,

the number of proliferating cells was not significantly different in non-CF and CF cultures, whatever the time point analysed (Supplementary Fig. 2).

These results, obtained in standardized cultures, showed that exogenous infection and inflammation characterizing the CF airways could not be considered as the major source of remodelling factors, and that CF epithelium remodelling was not related to an increased epithelial cell proliferation. They also suggested that alterations of CF HAE cells such as their hyperinflammatory phenotype or CFTR default could play a role in the remodelling phenomenon.

Exogenous inflammation leads to the regeneration of a remodelled epithelium in non-CF cultures

To establish the role played by inflammation in the human airway epithelium remodelling, we investigated the consequences of an inflammatory stimulation of nasal non-CF HAE cells all along the ALI culture with a mixture (cytomix) of mediators found in CF airways, i.e. IL-1β, TNFα and IFNγ (32, 37).

Cytomix-exposed non-CF cultures were remodelled at ALI D35 (Fig. 2). Inflamed non-CF cultures were significantly 29.17% higher (36.98 ± 0.40 µm) than non-stimulated cultures (28.62 ± 0.75 µm) (Fig. 2C) and showed a significant increase of 34.20 % in the number of basal cells: we quantified 35.94 ± 1.28 % of CK13 positive cells in cytomix-exposed cultures when control cultures were composed of 26.78 ± 0.35 % of basal cells (Fig. 2F). In the case of chronic stimulation with cytomix, the goblet cell differentiation was significantly 131.03 % enhanced, with 0.67 ± 0.029 % of MUC-5AC positive cells in stimulated cultures and 0.29 ± 0.014 % in control cultures (Fig. 2I). In contrast, the number of ciliated cells was not different in inflamed cultures, in comparison to non-stimulated cultures, either at ALI D15 (451.84 ± 42.22 and 453.04 ± 46.25 cells/mm2, respectively) (Fig. 2L), or at ALI D25 (616.05 ± 60.59

and 642.09 ± 56.05 cells/mm2, respectively) (Fig. 2O) or at ALI D35 (790.53 ± 42.49 and 821.81 ± 37.89 cells/mm2, respectively) (Fig. 2R).

These data demonstrated that exogenous inflammation was responsible of a remodelling of the human airway epithelium, in terms of increase in epithelium height and in basal and goblet cell number. However, the inflammatory environment did not induce modification of the kinetic of ciliated cell differentiation.

Human CF HAE cells exhibit a hyperinflammatory phenotype which is lost with time

To explore our hypothesis of the involvement of CF HAE cell transient inflammatory memory in the remodelling of the CF airway epithelium, we first verified the hyperinflammatory status of nasal CF HAE cells already reported in the literature for CF bronchial epithelial cells (28, 30). Expression of the CF inflammation marker IL-8 was assessed by RT-qPCR or ELISA. Despite huge inter-patients variability, IL-8 mRNA expression was significantly more important in CF HAE cells than in non CF HAE cells at P0 without any phase of culture, as shown in figure 3A. In contrast, after a round of amplification, CF cells at P1 did not express more IL-8 mRNA than non-CF cells at the same passage (Fig. 3B). During airway epithelial regeneration by nasal non-CF and CF HAE cells at P0, IL-8 mRNA expression was significantly higher in CF cultures than in non-CF cultures at ALI D0, decreased until ALI D7 then became similar or lower (Fig. 3C). At protein level, the IL-8 secretion by cells at P0 exhibited the same pattern of expression (Fig. 3D). Finally, at ALI D35, IL-8 mRNA expression was similar or lower in CF cultures, in comparison to non-CF cultures, either at P0 (Fig. 3C) or at P1 (Fig. 3E).

These results confirmed the hyperinflammatory status of CF HAE cells at P0 and the loss of this phenotype with time of culture when they were free of any exogenous inflammatory stimulation.

When the inflammatory memory is lost by CF HAE cells, the reconstituted epithelium is not remodelled

As the hyperinflammatory phenotype of CF cells was lost with time, we evaluated the potential remodelling of non-CF and CF epithelia regenerated from nasal HAE cells at P1. At ALI D35, epithelium height was similar in P1-non-CF (29.55 ± 1.54 µm) and P1-CF (30.61 ± 0.96 µm) cultures (Fig. 4C). Quantification of basal cells showed that P1-CF and P1-non-CF cultures exhibited an equivalent number of CK13 positive cells with 30.52 ± 1.30 % and 29.11 ± 0.70 %, respectively (Fig. 4F). Moreover, no difference in the amount of MUC-5AC expressing goblet cells was detect in P1-CF (0.35 ± 0.043 %) and P1-non-CF cultures (0.34 ± 0.038 %) (Fig. 4I). Finally, at ALI D35, both types of culture displayed a comparable number of ciliated cells, with 654.72 ± 71.37 β-tubulin positive cells / mm^2 in P1-non-CF and 574.32 ± 83.01 β-tubulin positive cells / mm^2 in P1-CF cultures (Fig. 4L).

These results obtained with P1 cultures demonstrated that when CF HAE cells lost their hyperinflammatry phenotype, they reconstituted an epithelium that was not remodelled. These data strengthen our hypothesis that the hyperinflammatory phenotype could play a role in the remodelling phenomenon.

Exogenous inflammation induces only goblet cell number increase during the CF airway epithelium regeneration.

In order to define if exogenous inflammation and inflammatory memory of CF HAE cells could play a cumulative effect on the epithelium remodelling, nasal CF HAE cells were stimulated with cytomix all along the ALI culture (Fig. 5). In terms of epithelium height at ALI D35, no significant difference was observed in cytomix-exposed (36.64 ± 1.24 µm) and control CF cultures (38.03 ± 1.01 µm) (Fig. 5C). The number of basal cells was not enhanced by

inflammatory stimulation, with 35.78 ± 2.18 % and 39.37 ± 2.07 % of CK13 positive cells in stimulated and control cultures, respectively (Fig. 5F). In contrast and as already observed above for non-CF cultures, goblet cell differentiation was significantly 96.87 % enhanced in inflamed CF cultures, with 0.63 ± 0.034 % of MUC-5AC positive cells in stimulated cultures and 0.32 ± 0.027 % in control cultures (Fig. 5I). Finally, the number of ciliated cells was not affected by exogenous inflammation in CF cultures, in comparison to non-stimulated CF cultures, either at ALI D15 (256.90 ± 18.25 and 235.92 ± 30.40 cells/mm2, respectively) (Fig. 5L), or at ALI D25 (340.35 ± 19.59 and 349.19 ± 34.98 cells/mm2, respectively) (Fig. 5O) or at ALI D35 (767.78 ± 62.26 and 783.85 ± 20.81 cells/mm2, respectively) (Fig. 5R).

These results showed that exogenous inflammation induced goblet cell hyperplasia in CF cultures but did not play any cumulative effect on the remodelling in terms of epithelium height, basal cell hyperplasia or ciliated cell differentiation delay.

DISCUSSION

We have previously reported that, in absence of exogenous infection, the CF airway epithelial regeneration was abnormal and reconstituted a remodelled epithelium (20). However, the xenograft model we used did not permit to exclude the influence of the inflammatory environment in aberrant CF regeneration. In the present study, using *in vitro* ALI cultures of primary nasal HAE cells in perfectly defined environmental conditions, we could determine the involvement of inflammation in the human CF airway epithelium regeneration. Our results demonstrate that, even in absence of exogenous inflammation, the regenerated CF epithelium is remodelled, higher than the non-CF counterpart, exhibits basal cell hyperplasia and shows a delay in ciliated cell differentiation. Surprisingly, we do not detect any goblet cell hyperplasia. All these results are consistent with our previous observations (20) and suggest for the first time

that the altered regeneration and remodelling of the CF airway epithelium could be mainly due to dysfunctions of the CF HAE cells.

In this *in vitro* study, we first show an increase in the CF epithelium height. Although detected in airway tissues, this kind of epithelial remodelling was neither described nor explained previously in *in vitro* well-differentiated cultures of primary CF HAE cells from nasal, tracheal or bronchial origin. Indeed, the increase in epithelium height has been previously reported in CF lungs, compared to lungs from subjects without chronic airway disease (7, 38) or from patients suffering from COPD (12). Airway epithelium height is also greater in these latter patients than in non-COPD subjects, at nasal and bronchial levels (39). In the same way, we highlight basal cell hyperplasia in CF ALI cultures, as it has been reported in CF (7, 9) and COPD lungs (40). Interestingly, although these two pathologies have different aetiologies, genetic in CF and mainly environmental in COPD, they are both characterized by chronic airway inflammation. This observation suggests that inflammation could be involved in the observed remodelling. Interestingly, stimulation of non-CF HAE cells with a cocktail of pro-inflammatory cytokines all along the regeneration process leads to an increase in epithelium height and in the number of basal cells at values comparable to those measured in CF cultures, while in CF cultures this stimulation does not induce more remodelling than that seen in control counterparts (Table 1). Surprisingly, CF HAE cells that lost their hyperinflammatory phenotype (P1 cells) no longer reconstitute a remodelled epithelium. Taken together, these results strongly support that the CF epithelium remodelling could be due to the hyperinflammatory phenotype of CF HAE cells that appears to be sufficient to induce such histological alterations. Our experiments with nasal HAE cells confirm this expected characteristic of hyperinflammatory phenotype of bronchial CF HAE cells and its loss with time (30, 41). In our study, the exaggerated inflammation of CF HAE cells is still detectable until 7 days of ALI culture and disappears thereafter, as attested by the transient expression of IL-8. This observation suggests

that a one week-hyperinflammation of CF HAE cells is sufficient to induce their capacity to reconstitute a remodelled epithelium. The hypothesis of pro-remodelling effect of the inflammatory memory of CF cells is consolidated by the description of airway epithelium remodelling by intrinsically-produced inflammatory mediators such as IL-1β. Indeed, in a transgenic mouse model allowing inducible expression of human IL-1β in lung epithelium, IL-1β increases thickness of the conducting airways (42). The higher airway epithelium height could be related to the increased number of basal cells. Hyperplasia has been reported in airways of CF or COPD patients as well as of smokers (43). Whereas this hyperplasia was not characterized at first (10), it was later demonstrated that cell proliferation was restricted to basal cells (7, 43, 44) shown to retain transit-amplifying cell properties and ability to restore a fully differentiated mucociliary epithelium (45). This basal cell proliferation is induced by pro-inflammatory mediators such as TNF-α, IL-1β and IL-6, and reduced by anti-TNF-α, anti-IL-1β and anti-IL-6R therapies in a model of mouse lung inflammation triggered by cigarette smoke (43). In our experiences, we postulate that a transient hyperexpression of inflammatory mediators by CF cells could be involved in basal cell hyperplasia and epithelium height increase. Although such kinds of remodelling are described in the literature, their physiopathological impact remains to be elucidated.

In another way, we confirm the interesting result obtained previously *in vivo*, i.e. absence of goblet cell hyperplasia in the CF regenerated epithelium (20). Analysis of the expression of MUC-AC that stains all mucus-secreting goblet cells of the airway surface epithelium (20) shows an increase in the number of goblet cells, only in inflamed non-CF and CF cultures. It is known that goblet cell hyperplasia is regulated by some inflammatory mediators such as Th2 cytokines (46) or neutrophil elastase (47) and by infectious endotoxin such as lipopolysaccharide from *Pseudomonas aeruginosa* (48). Our results together with the fact that MUC-5AC overexpression is induced by some inflammatory or infectious mediators

(49), lead to conclude that goblet cell hyperplasia in CF is only due to exogenous factors, and that hyperinflammatory phenotype of CF HAE cells is not sufficient to trigger mucus cell hyperplasia.

In this study, we also confirm our previous observation of a delay in ciliated cell differentiation in CF, and show that this phenomenon is independent of the inflammatory environment of the cultures (Table 1). While a lot of ultrastructural alterations of cilia in CF epithelium are consensually reported (11, 13), few data are available concerning a default in the number of airway ciliated cells. CF children exhibit less bronchial ciliated cells than children suffering from non-suppurative lung disease (50). Moreover, Danel *et al.* found less ciliated cells in CF samples recovered by bronchial brushing than in control samples, and hypothesized it was due to the high susceptibility of ciliated cells to be damage (51). As we have shown that ciliated cell differentiation delay in nasal CF cultures is not related to infection or inflammation, and together with recent data showing a down regulation of cilia genes in native nasal epithelium of CF patients (52), we propose that the decreased number of ciliated cells in CF epithelium could be due to a permanent regeneration process rather than a fragility of these cells and to a failure of differentiation related to the basic CFTR default.

In conclusion, we show that exogenous infection and inflammation characterizing the CF lung pathology are not the exclusive factors leading to airway epithelium remodelling that is partly regulated by the alterations of CF HAE cells. This could explain the CF deleterious loop: infection and inflammation are hallmarks of CF so that airway epithelium is continuously damaged and epithelial cells constantly stimulated by resident inflammation maintaining in this way their hyperinflammatory phenotype. Moreover, being in permanent regeneration, the reconstituted epithelium is remodelled, with goblet cell hyperplasia and a diminished number of ciliated cells conducting to worsen the mucociliary clearance impairment that leads to infection and inflammation.

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AUTHOR CONTRIBUTIONS

DA, JRD and CC conceived and designed the work. DA, JRD, EL, AB, JL and CC performed the experiments. DA, AB and CC analysed data. DA, JCM, MP, MA and CC were involved in drafting the manuscript for important intellectual content.

ABBREVIATIONS

CF, Cystic Fibrosis

HAE cells, human airway epithelial cells

BAL, bronchoalveolar lavages

ALI, air–liquid interface

P, passage

CK13, cytokeratin 13

MUC-5AC, mucin-5AC

D, day

COPD, chronic obstructive pulmonary disease

RT, room temperature

LIST OF SUPPORTING INFORMATION

- Supplementary materials and methods
- Supplementary Figure 1
- Supplementary Figure 2

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Table 1. Features of non-CF and CF epithelia regenerated *in vitro* from passage 0 nasal HAE cells in absence or presence of inflammatory stimulation

	Non-CF	CF	Cytomix*-stimulated Non-CF	Cytomix*-stimulated CF
Epithelium height (µm ± SEM) ALI D35	28.62 ± 0.75	38.03 ± 1.01	36.98 ± 0.40	36.64 ± 1.24
Basal cells (% ± SEM) ALI D35	26.78 ± 0.35	39.37 ± 2.07	35.94 ± 1.28	35.78 ± 2.18
Goblet cells (% ± SEM) ALI D35	0.29 ± 0.01	0.32 ± 0.03	0.67 ± 0.03	0.63 ± 0.03
Ciliated cells / mm^2 ± SEM ALI D15	453.04 ± 46.25	235.92 ± 30.40	451.84 ± 42.22	256.90 ± 18.25
Ciliated cells / mm^2 ± SEM ALI D25	642.09 ± 56.05	349.19 ± 34.98	616.05 ± 60.59	340.35 ± 19.59
Ciliated cells / mm^2 ± SEM ALI D35	821.81 ± 37.89	783.85 ± 20.81	790.53 ± 42.49	767.78 ± 62.26

Definition of abbreviations: CF = Cystic Fibrosis; ALI = air–liquid interface; D = day; HAE cells = human airway epithelial cells

* Cytomix is a mixture composed of IL-1β, TNFα and IFNγ

FIGURE LEGENDS

Figure 1: **In absence of exogenous infection and inflammation, the regeneration of CF human airway epithelium is altered and reconstitutes a remodelled epithelium.**

Non-CF (A) and CF (B) culture sections were hematoxylin-eosin stained, and then epithelium height was measured (C). Basal cells (D, E) and goblet cells (G, H) were immunostained using anti-CK13 and anti-MUC-5AC antibodies, respectively, on non-CF (D, G) and CF (E, H) culture sections at ALI D35, and the number of positive cells was determined as the percentage of all cells counterstained using DAPI (F, I). Results showed a significant increase in epithelium height (C) and in the number of basal cells (F) in CF cultures. Goblet cell number was similar in non-CF and CF cultures (I). Ciliated cells were immunostained using anti-β-tubulin antibodies (J, K, M, N, P, Q) and the number of positive cells in non-CF (J, M, P) and CF (K, N, Q) cultures at ALI D15 (J-L), D25 (M-O) and D35 (P-R) was determined and expressed as cells / mm^2. Ciliated cells were less numerous at ALI D15 (L) and D25 (O) in CF cultures than in non-CF cultures, and showed an equivalent number in non-CF and CF cultures at ALI D35 (R). Graph data are mean ± SEM, *$p<0.05$, $n = 4$. Bars = 50 μm (A-H), 100 μm (J-Q).

Figure 2: **Exogenous inflammation leads to the regeneration of a remodelled epithelium in non-CF cultures.**

Nasal non-CF HAE cells were cultured in an ALI in absence (Ctrl) or presence of a chronic stimulation with a mixture of pro-inflammatory cytokines (Cytomix) for 35 days. Non-CF control (A) and cytomix-exposed culture sections (B) were hematoxylin-eosin stained, and then epithelium height was measured (C). Basal cells (D, E) and goblet cells (G, H) were immunostained using anti-CK13 and anti-MUC-5AC antibodies, respectively, on control (D, G) and cytomix-exposed culture sections (E, H) at ALI D35, and the number of positive cells

was determined as the percentage of all cells counterstained using DAPI (F, I). Results showed that chronic inflammation led to a significant increase in epithelium height (C), and in the number of basal (F) and goblet cells (I) in non-CF cultures. Ciliated cells were immunostained using anti-β-tubulin antibodies (J, K, M, N, P, Q) and the number of positive cells in control (J, M, P) and cytomix-exposed cultures (K, N, Q) at ALI D15 (J-L), D25 (M-O) and D35 (P-R) was determined and expressed as cells / mm^2. Chronic inflammation did not modify the number of ciliated cells in non-CF cultures (L, O, R). Graph data are mean ± SEM, *$p<0.05$, n = 4. Bars = 50 µm (A-H), 100 µm (J-Q).

Figure 3: **Nasal CF HAE cells exhibit a hyperinflammatory phenotype which is lost with time.**

IL-8 mRNA expression was assessed by RT-qPCR (A, B, C, E) and IL-8 protein secretion by ELISA (D). Results showed that IL-8 mRNA expression was significantly higher in nasal CF HAE cells than in non-CF HAE cells at passage 0 (A) but was not different at passage 1 (B). During the course of regeneration by passage 0 cells, IL-8 mRNA expression was higher until ALI D7 in CF cultures, in comparison to non-CF cultures (C). At protein level, passage 0 nasal CF HAE cells secreted more IL-8 protein than non-CF cells until ALI D7, then IL-8 secretion became comparable or lower (D). Finally, IL-8 mRNA expression was not different at ALI D35 in CF and non-CF cultures at passage 1 (E). Graph data are mean ± SEM, *$p<0.05$, n = 7 non-CF and 5 CF (A), n = 4 (B, D, E), n = 9 (C).

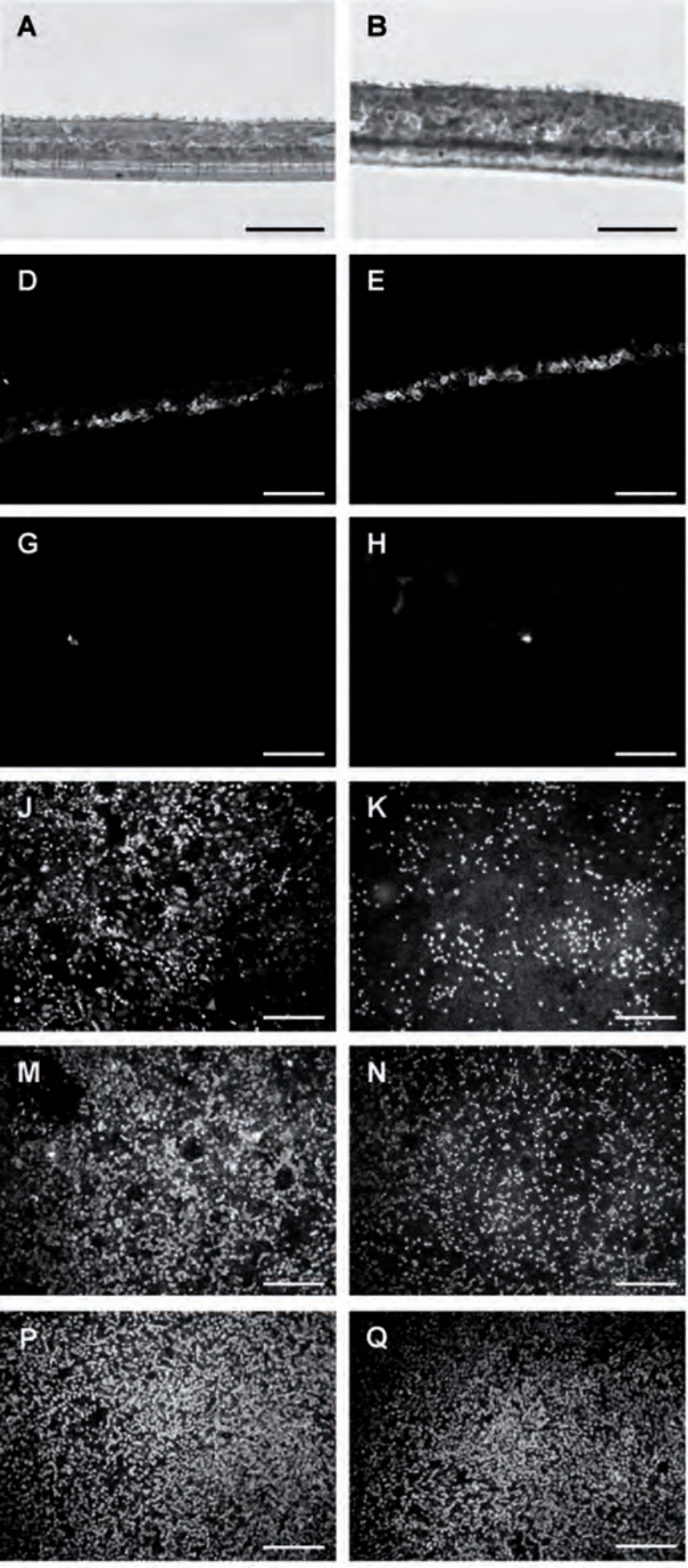
Figure 4: **When the inflammatory memory is lost by CF HAE cells, the reconstituted epithelium is no longer remodelled.**

After a round of proliferation, nasal non-CF and CF HAE cells were detached (passage 1) and cultured in an ALI for 35 days. Non-CF (A) and CF (B) culture sections were hematoxylin-

eosin stained, and then epithelium height was measured (C). Basal cells (D, E) and goblet cells (G, H) were immunostained using anti-CK13 and anti-MUC-5AC antibodies, respectively, on non-CF (D, G) and CF (E, H) culture sections at ALI D35. The number of positive cells was determined as the percentage of all cells counterstained using DAPI (F, I). Ciliated cells were immunostained using anti-β-tubulin antibodies and the number of positive cells in non-CF (J) and CF (K) cultures at ALI D35 was determined and expressed as cells / mm^2 (L). Results showed an equivalent epithelium height (C), and a similar number of basal (F), goblet (I) and ciliated cells (L) in passage 1-non-CF and passage 1-CF cultures. Graph data are mean ± SEM, n = 4. Bars = 50 μm (A-H), 100 μm (J, K).

Figure 5: **Exogenous inflammation induces only goblet cell number increase during the CF airway epithelium regeneration.**

Nasal CF HAE cells were cultured in an ALI in absence (Ctrl) or presence of a chronic stimulation with a mixture of pro-inflammatory cytokines (Cytomix) for 35 days. CF control (A) and cytomix-exposed culture (B) sections were hematoxylin-eosin stained, and then epithelium height was measured (C). Basal cells (D, E) and goblet cells (G, H) were immunostained using anti-CK13 and anti-MUC-5AC antibodies, respectively, on control (D, G) and cytomix-exposed culture (E, H) sections at ALI D35, and the number of positive cells was determined as the percentage of all cells counterstained using DAPI (F, I). D: Ciliated cells were immunostained using anti-β-tubulin antibodies (J, K, M, N, P, Q) and the number of positive cells in control (J, M, P) and cytomix-exposed cultures (K, N, Q) at ALI D15 (J-L), D25 (M-O) and D35 (P-R) was determined and expressed as cells / mm^2. Results showed that chronic inflammation led to a significant increase in the number of goblet cells (I) but did not modify either the epithelium height (C), or the number of basal (F) or ciliated cells (L, O, R) in CF cultures. Graph data are mean ± SEM, *$p<0.05$, n = 4. Bars = 50 μm (A-H), 100 μm (J-Q).


A
B
C
Epithelium height (µm)
50
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30
20
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Non-CF
CF
D
E
F
CK13 positive basal cells (%)
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*
Non-CF
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G
H
I
MUC-5AC positive goblet cells (%)
0,5
0,4
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Non-CF
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J
K
L
β-tubulin positive ciliated cells (cells/mm²) at ALI D15
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400
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100
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Non-CF
CF
M
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O
β-tubulin positive ciliated cells (cells/mm²) at ALI D25
800
600
400
200
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*
Non-CF
CF
P
Q
R
β-tubulin positive ciliated cells (cells/mm²) at ALI D35
1000
800
600
400
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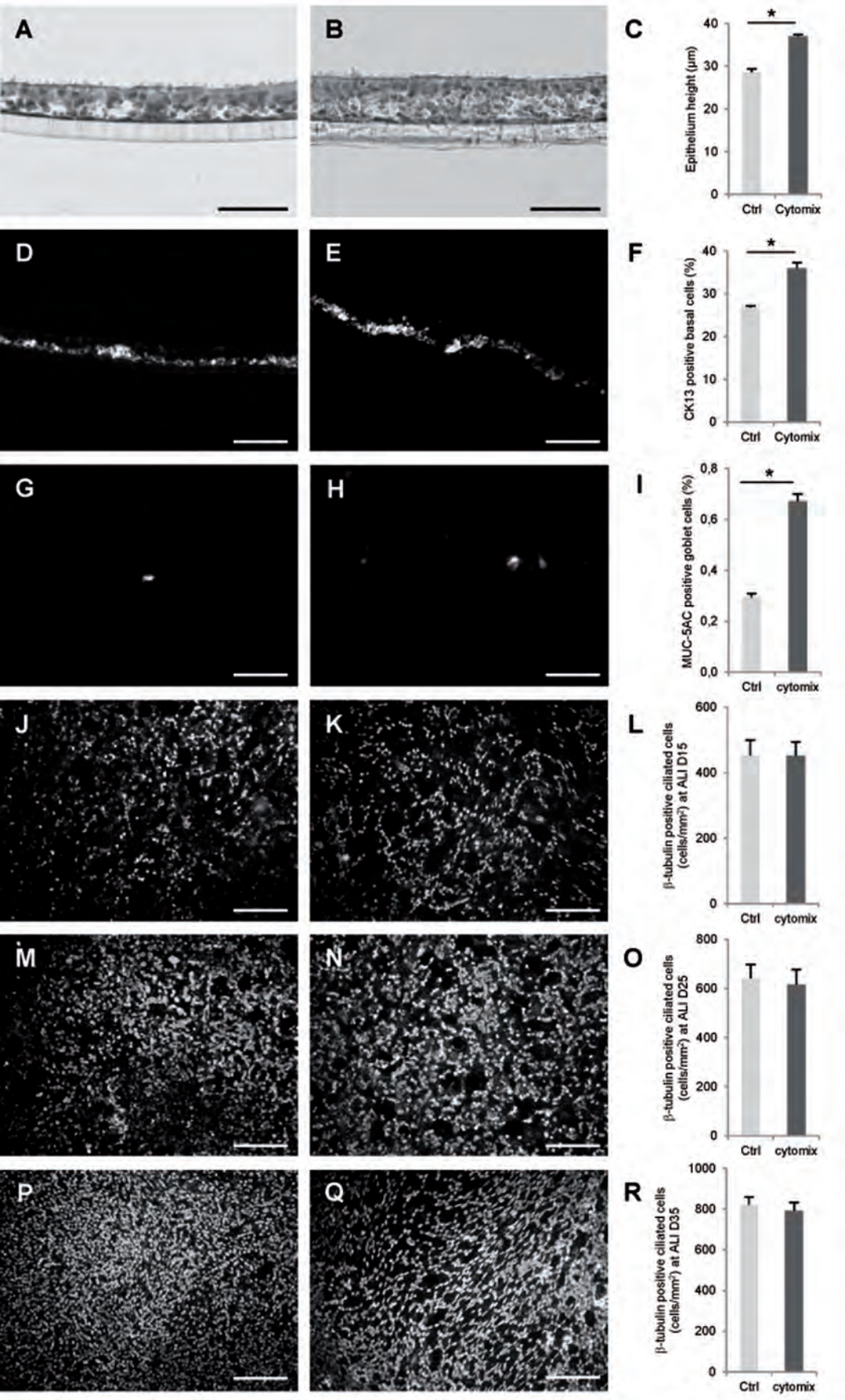
Non-CF
CF

Figure 1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Epithelium height (µm)
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CK13 positive basal cells (%)
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MUC-5AC positive goblet cells (%)
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β-tubulin positive ciliated cells (cells/mm²) at ALI D15
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β-tubulin positive ciliated cells (cells/mm²) at ALI D25
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β-tubulin positive ciliated cells (cells/mm²) at ALI D35
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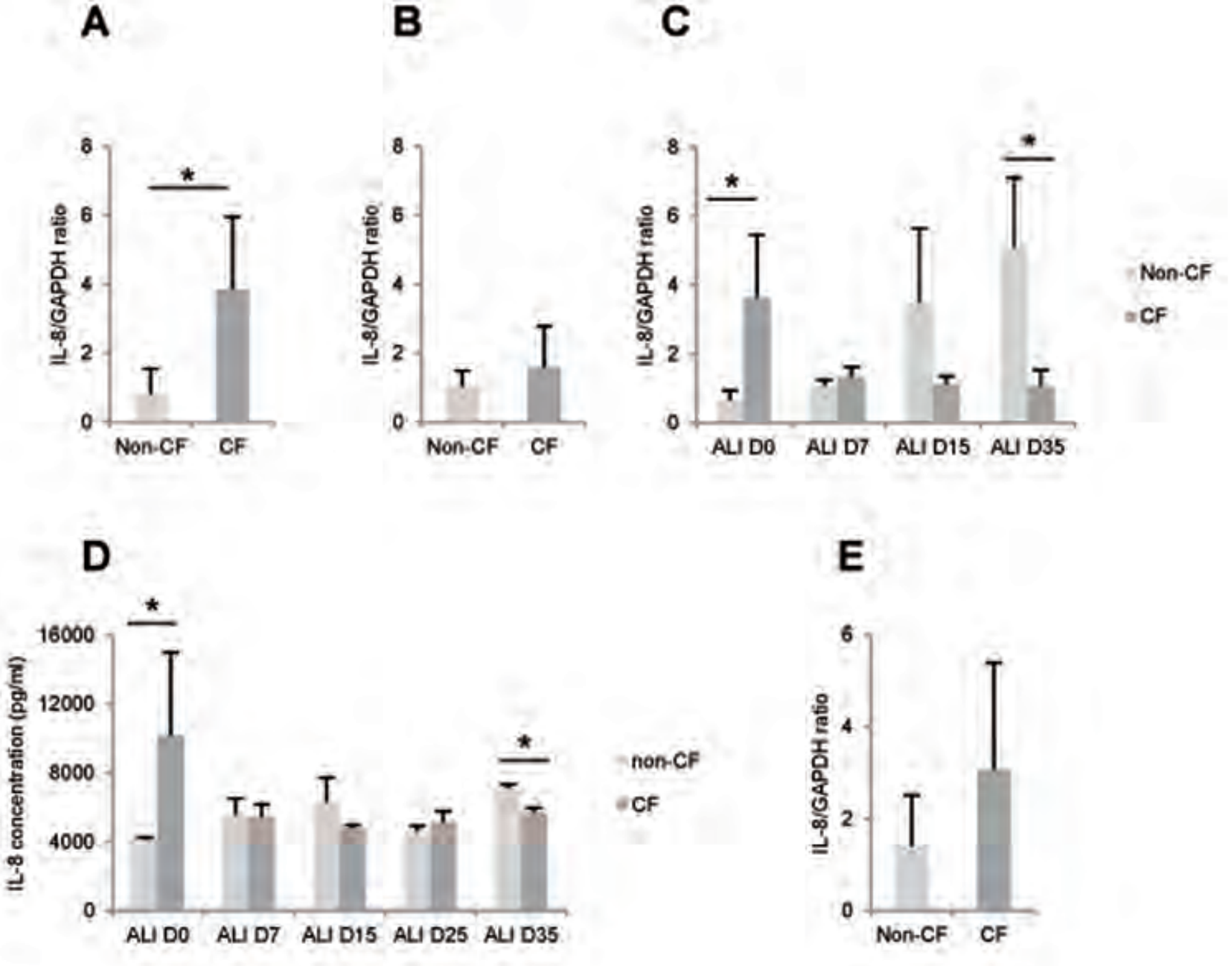
Figure 2


A
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IL-8/GAPDH ratio
IL-8 concentration (pg/ml)
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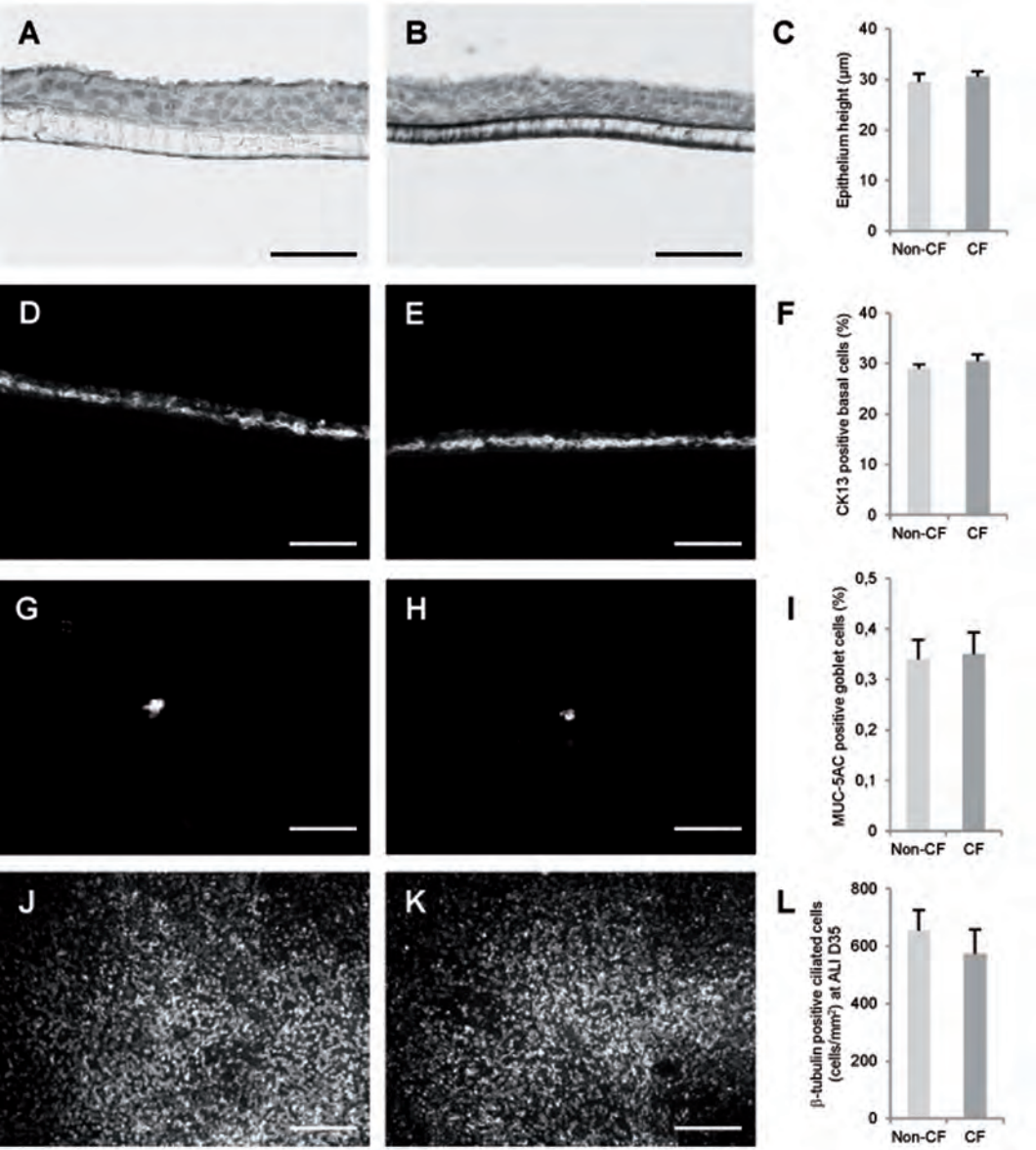
ALI D0
ALI D7
ALI D15
ALI D25
ALI D35
non-CF
*

Figure 3


A
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Epithelium height (µm)
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Non-CF
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F
CK13 positive basal cells (%)
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20
10
0
Non-CF
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G
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I
MUC-5AC positive goblet cells (%)
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Non-CF
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β-tubulin positive ciliated cells (cells/mm²) at ALI D35
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Non-CF
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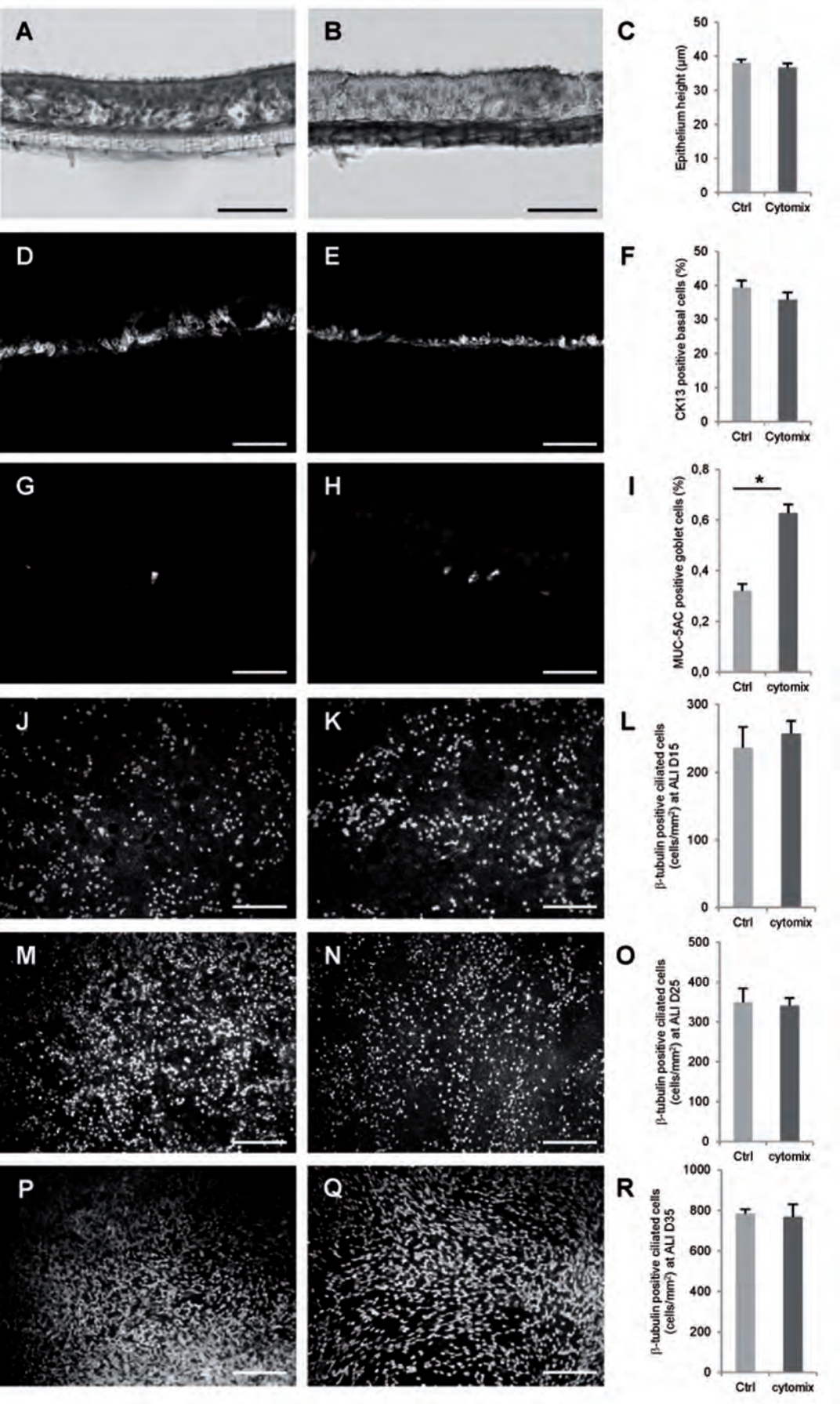
Figure 4


A
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Epithelium height (µm)
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CK13 positive basal cells (%)
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MUC-5AC positive goblet cells (%)
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β-tubulin positive ciliated cells (cells/mm²) at ALI D15
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β-tubulin positive ciliated cells (cells/mm²) at ALI D25
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β-tubulin positive ciliated cells (cells/mm²) at ALI D35
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Figure 5

SUPPORTING INFORMATION

Cystic Fibrosis airway epithelium remodelling: involvement of inflammation

MATERIALS AND METHODS

Human airway tissues and cell dissociation

Human tissues were transferred to our laboratory in RPMI 1640 medium supplemented with 20 mM HEPES (Gibco) and antibiotics (200 UI/ml penicillin, 200 µg/ml streptomycin; Gibco). Collected tissues were then dissociated by type XIV collagenase incubation in RPMI-HEPES overnight at 4°C. The dissociated nasal human airway epithelial (HAE) cells, called cells at passage 0 (P0), were counted and used for air-liquid interface (ALI) culture. They were also analysed to assess their inflammatory status by RT-qPCR after a pre-plating step in order to remove all contaminant blood cells.

Cell culture

Nasal HAE cells (P0) were suspended in CnT17 medium (CELLnTEc, Bern, Switzerland) and seeded ($5x10^4$ cells per membrane) on type IV collagen (Sigma Aldrich)-coated porous 12 mm diameter Transwell polyester membranes. Cells were grown in liquid-liquid conditions in CnT17 medium until confluent (approximately 1 week). Then the culture medium was definitively removed from the upper compartment (ALI D0) and the epithelium was allowed to differentiate at the ALI with supplemented BEGM/DMEM/F12 medium in the basal compartment of the culture chamber. All the cultures were incubated at 37°C in a 100% humidified incubator in the presence of 5% CO_2. In some experiments, the ALI medium was supplemented with a cocktail of pro-inflammatory cytokines called cytomix from ALI D0 to ALI D35. These cultures were examined at ALI D0, ALI D7, ALI D15, ALI D25 and ALI D35.

In a set of experiments, nasal non-CF and CF HAE cells were plated on type IV collagen-coated plastic flasks for cell proliferation in CnT17 medium. When confluent, the cells were detached by tryspine/EDTA (Gibco) treatment and called cells at passage P1 (P1). They were used for RT-qPCR experiments and seeded on type IV collagen-coated porous 12 mm Transwell membranes and grown as described above. These P1 cultures were examined at ALI D35.

Histological analysis

ALI cultures were embedded in optimum cutting temperature (O.C.T) compound (Tissue-Tek, Zoeterwoude, The Netherlands), frozen in liquid nitrogen and stored at -80°C until use for histology and immunohistochemistry.

Seven-micrometer frozen sections were cut with a microtome, collected on superfrost slides (Thermo Scientific, Braunschweig, Germany), and either stained with hematoxylin-eosin using Rapid-Chrome Frozen Sections Staining Kit (Thermo Shandon Inc., Pittsburgh, PA) and mounted in Eukitt (Electron Microscopy Sciences, Hartfield, PA) for histology or frozen at -20°C for immunohistochemistry.

Immunohistochemistry

Slides were air-dried, and sections were blocked with phosphate buffered saline (PBS; Gibco)-BSA 10% for 30 minutes at room temperature (RT) to prevent unspecific bindings. ALI D35 sections were incubated with mouse anti-human CK13 (dilution 1/1000), mouse anti-MUC-5AC (dilution 1/100) or mouse anti-MUC5B (dilution 1/100) antibodies, and ALI D0, D7, D15 and D35 culture sections with mouse anti-human Ki67 antibodies (dilution 1/10), diluted in PBS-BSA 3 % overnight at 4°C. After washes with PBS at RT, sections were blocked again with PBS-BSA 10 % for 30 minutes at RT, then incubated with the goat anti-mouse Alexa Fluor 488 secondary antibodies diluted in PBS/BSA 3% (dilution 1/200) for 60 minutes at RT,

washed with PBS, incubated with 4', 6'-diamino-2-phenylindole (DAPI, 200 ng/ml in PBS; Sigma Aldrich) for 15 minutes at RT, washed with PBS and finally mounted with coverslides in Aquapolymount solution (Polysciences, Warrington, PA). For quantification of basal cells, goblet cells and cycling cells, 6 different sections per culture were analysed. The number of positive cells was determined as a percentage of the total number of cells visualized with DAPI. For goblet cells double immunostaining, sections were stained with anti-MUC-5B antibodies, exposed to Alexa Fluor 488-goat anti-mouse antibodies, then incubated with goat anti-mouse Fab (H+L) fragments (30 minutes at RT; Jackson Immunoresearch, PA) to block free sites of mouse anti-MUC-5B antibodies, and finally stained with anti-MUC-5AC antibodies followed by incubation with Alexa Fluor 594-goat anti-mouse antibodies (dilution 1/200 in PBS/BSA 3%; Molecular Probe, Eugene, OR). For the quantification of ciliated cells, cultures at ALI D15, ALI D25 and ALI D35 were rinsed with PBS containing $CaCl_2$ and $MgCl_2$ (Gibco), fixed with cold methanol for 10 minutes at -20°C, washed with PBS at RT, blocked with PBS-BSA 10% for 30 minutes at RT, incubated with mouse anti-human β-tubulin (dilution 1/1000 in PBS/BSA 3%) overnight at 4°C. After washes with PBS, membranes were blocked again with PBS-BSA 10% for 30 minutes at RT, then incubated with goat anti mouse Alexa Fluor 488 secondary antibodies (dilution 1/200 in PBS/BSA 3%), washed with PBS, incubated with DAPI (200 ng/ml in PBS) for 15 minutes at RT, washed with PBS and finally mounted between slides and coverslides in Aquapolymount solution. Ten images (objective x10) per culture were analysed in order to cover approximately 80% of the total surface of cultures. Using ImageJ software (http://rsb.info.nih.gov/ij/) functionalities, the average number of ciliated cells per mm^2 was calculated after fluorescent signal treatment.

RT-qPCR analysis

Specific primers used (Eurogentec, Seraing, Belgium) were as follow for IL-8: forward, 5'-AGACAGCAGAGCACACAAGC-3'; reverse, 5'-ATGGTTCCTTCCGGTGGT-3'. Results were normalized to the expression levels of GAPDH. Primers for GAPDH were: forward, 5'-ACCAGGTGGTCTCCTCTGAC-3'; reverse, 5'-TGCTGTAGCCAAATTCGTTG-3'.Each sample was quantified using a standard curve made of serial dilutions of a batch of human placental cDNA made with the Transcriptor First Strand cDNA synthesis kit (Roche). Results were expressed as IL-8/GAPDH ratios.

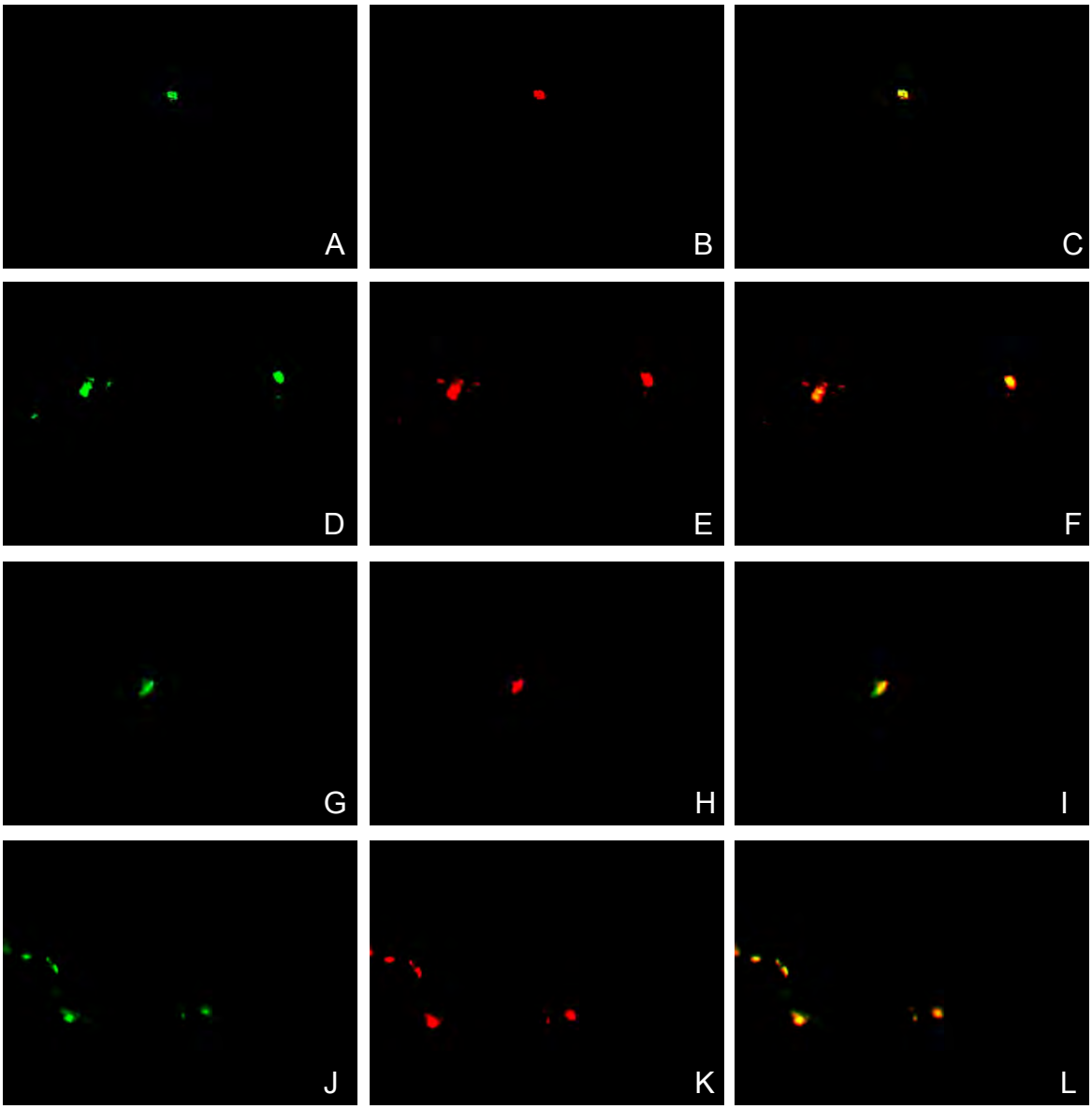
LEGEND OF SUPPLEMENTARY FIGURES

Figure 1: CF and non-CF differentiated goblet cells at ALI D35 express both MUC-5AC and MUC-5B secreted mucins, in control and cytomix-exposed cultures.

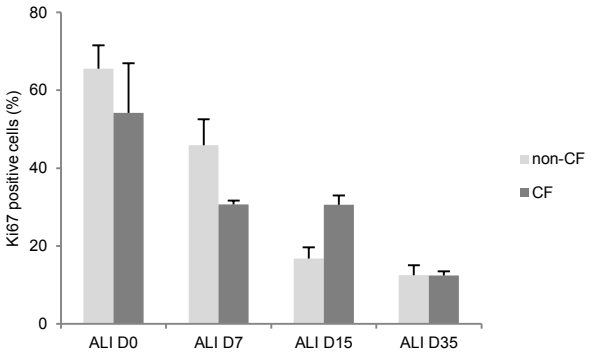
Goblet cells were immunostained using anti-MUC-5B (A, D, G, J) and anti-MUC-5AC (B, E, H, K) antibodies on sections of non-CF (A-F) and CF (G-L) cultures, exposed (D-F, J-L) or not (A-C, G-I) to cytomix. As observed in merged photographs (C, F, I, L), all goblet cells expressed both secreted mucins. n = 3. Bars = 100 μm.

Figure 2: CF HAE cells do not exhibit any hyperproliferative potential during the regeneration process in vitro.

Cycling cells were immunostained using anti-Ki67 antibodies on non-CF and CF culture sections, and the number of positive cells was determined as the percentage of all cells counterstained using DAPI. Results showed a progressive decrease in the number of proliferative nasal HAE cells in non-CF and CF cultures along the regeneration process. No significant difference between the numbers of Ki67 positive cells in non-CF and CF cultures was found, either at ALI D0, or at ALI D7, D15 or D35. Graph data are mean ± SEM, n = 4.



Supplementary Figure 1



Supplementary Figure 2