



# Deleterious impact of *Pseudomonas aeruginosa* on cystic fibrosis transmembrane conductance regulator function and rescue in airway epithelial cells

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**ABSTRACT** The epithelial response to bacterial airway infection, a common feature of lung diseases such as chronic obstructive pulmonary disease and cystic fibrosis, has been extensively studied. However, its impact on cystic fibrosis transmembrane conductance regulator (CFTR) channel function is not clearly defined. Our aims were, therefore, to evaluate the effect of *Pseudomonas aeruginosa* on CFTR function and expression in non-cystic fibrosis airway epithelial cells, and to investigate its impact on  $\Delta F508$ -CFTR rescue by the VRT-325 corrector in cystic fibrosis cells.

CFTR expression/maturation was evaluated by immunoblotting and its function by short-circuit current measurements.

A 24-h exposure to *P. aeruginosa* diffusible material (PsaDM) reduced CFTR currents as well as total and membrane protein expression of the wildtype (wt) CFTR protein in CFBE-wt cells. In CFBE- $\Delta F508$  cells, PsaDM severely reduced CFTR maturation and current rescue induced by VRT-325. We also confirmed a deleterious impact of PsaDM on wt-CFTR currents in non-cystic fibrosis primary airway cells as well as on the rescue of  $\Delta F508$ -CFTR function induced by VRT-325 in primary cystic fibrosis cells.

These findings show that CFTR function could be impaired in non-cystic fibrosis patients infected by *P. aeruginosa*. Our data also suggest that CFTR corrector efficiency may be affected by infectious components, which should be taken into account in screening assays of correctors.



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**Exposure of airway epithelial cell to *P. aeruginosa* impairs CFTR function, expression and rescue by correctors** <http://ow.ly/IleTw>

This article has supplementary material available from [erj.ersjournals.com](http://erj.ersjournals.com)

Received: April 23 2014 | Accepted after revision: Dec 28 2014 | First published online: March 18 2015

Support statement: This study was funded by Cystic Fibrosis Canada (research grant E. Brochiero, studentship C. Bilodeau), CRCHUM and Université de Montréal (E. Brochiero and C. Bilodeau), the Canadian Thoracic Society, the Fondation GO and the CORAMH (Studentship N.T.N. Trinh). Funding information for this article has been deposited with FundRef.

Conflict of interest: None declared.

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## Introduction

Chloride and bicarbonate secretion through cystic fibrosis transmembrane conductance regulator (CFTR) channels is crucial in airways to maintain an adequate periciliary liquid volume and an efficient mucociliary clearance [1], as well as to control airway surface liquid pH [2], which are necessary to protect the lung against infections. Despite these mechanisms of defence, lungs are frequently exposed to infection/inflammation, which are common features of several lung pathologies including chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). The impact of infection on airway epithelia has been extensively studied and it has been established that the continuous presence of pathogens is associated with oxidative stress and chronic inflammatory responses [3–5]. However, the consequences of exposure to infections on CFTR channel expression and function have not been clearly defined. In fact, contradictory results have been reported depending on the bacterial strain, the duration of exposure to infection, as well as the cellular models tested [6–9]. However, it is crucial to understand the outcomes of microbial infection on CFTR. Indeed, a decrease of CFTR function in airways of infected patients would further reduce defence against infections, thus creating a vicious circle of infections impairing CFTR, and then dysfunctional CFTR favouring infections.

In CF, due to mutations in the *Cftr* gene, dysfunctional CFTR is associated with reduced periciliary volume and mucus accumulation, favouring bacterial colonisation and chronic inflammation [5, 10]. The prevalence of respiratory pathogens in CF patients varies as a function of age, but the most frequently found bacteria in adults are *Pseudomonas aeruginosa*, which gradually colonise CF airways [11]. Therefore, many of the current therapies in CF are directed against infection as well as towards attenuating the inflammatory response [12, 13]. In addition, many efforts have recently been put towards the development of new strategies directly targeting the basic CFTR defects rather than CF symptoms [14, 15]. New therapies, adapted to each specific class of mutations, are thus progressively emerging. Among them, small molecules called correctors are directed against class II mutations, including the most frequent mutation in CF patients, a deletion of F508 ( $\Delta$ F508-CFTR) leading to improper folding, endoplasmic reticulum trapping and protein degradation [14, 15]. Efficient CFTR maturation and delivery to the cell membrane has been reported after treatment with various corrector molecules, including corr-2b, corr-4a, VRT-325, MPB-07, KM1160, C18 and VX-809 [16–25]. However, to the best of our knowledge most high throughput screening assays to identify efficient corrector molecules are performed in the absence of infectious products. Notably, it has been previously reported that *P. aeruginosa* exposure reduced CFTR rescue induced by low temperature [6]. Because microbial infection is a key component of CF lung disease, it would thus be crucial to assess the efficiency of small molecules targeting CFTR defects in the presence of infection.

The aim of this study was first to evaluate the impact of *P. aeruginosa* diffusible material (PsaDM) on CFTR function and expression in non-CF airway epithelial cells. We then assessed the effect of PsaDM on CFTR protein degradation and synthesis as well as membrane expression. Finally, we tested if *P. aeruginosa* exoproducts could affect  $\Delta$ F508-CFTR maturation and functional rescue by the CFTR corrector VRT-325 in CF airway epithelial cells.

## Materials and methods

### Cell culture

CFBE- $\Delta$ F508 and CFBE-wt cell lines (CFBE41o– parental cells [26] stably transduced, respectively, with  $\Delta$ F508-CFTR and with wildtype (wt)-CFTR [27]) were used initially. Primary human airway cells were isolated from tissues obtained from non-CF and CF patients (with class II mutations, including  $\Delta$ F508/ $\Delta$ F508 and  $\Delta$ F508/N1303K, median age 23 years, mean forced expiratory volume in 1s of  $82 \pm 9\%$ ) undergoing nasal polypectomy at CHUM (Montreal, QC, Canada) and CHU Sainte-Justine (Montreal, QC, Canada) hospitals [28], according to approved ethical protocols. Written informed consents have been obtained from all participants. Cell isolation and culture conditions are detailed in the online supplementary material.

### Preparation of *P. aeruginosa* diffusible material

In brief, *P. aeruginosa* mucoid strain PACF508 was isolated from the sputum of a CF patient ( $\Delta$ F508/ $\Delta$ F508, CHU Sainte-Justine) and was grown in lysogeny broth (LB) medium (Life Technologies, Burlington, ON, Canada) for 72 h (corresponding to the late stationary phase) [29] at 37°C with shaking at 250 rpm. The bacterial culture was then centrifuged and the supernatant was filtered successively through 0.8, 0.45 and 0.2  $\mu$ m filters. LB was used as the control condition.

### Immunoblotting

Total proteins were extracted from CFBE cells treated or not with VRT-325 (Cystic Fibrosis Foundation Therapeutic Inc. (CFFT) Bethesda, MA, USA), LB, PsaDM, cycloheximide (CHX) (Sigma-Aldrich, St. Louis, MO, USA) and/or with MG132 (Abmole Bioscience, Kowloon, Honk Hong). For CFTR [28]

and epidermal growth factor receptor (EGFR) [30] detection, the upper section of the membrane was incubated with the polyclonal anti-CFTR 596 antibody (CFFT) or anti-EGFR antibody (anti-erbB1; Cell Signaling Technology, Danvers, MA, USA); whereas the bottom section was incubated with purified mouse anti- $\beta$ -actin monoclonal antibody (Cedarlane Laboratory, Burlington, ON, Canada) to ensure equivalent loading. The expression of CFTR was reported as percentage relative to cells with LB, after normalisation to  $\beta$ -actin signal.

#### **Biotinylation and pull-down**

CFBE-wt cells, treated with LB or PsaDM, were exposed to sulfo-NHS-SS-biotin (Thermo Fisher Scientific Inc., Waltham, MA, USA) before protein solubilisation in RIPA. The proteins were then absorbed on streptavidin beads, transferred onto a Pierce spin column (Thermo Fisher Scientific Inc.), and then eluted with a 2X sample buffer, before separation on SDS-Page and CFTR detection by immunoblotting.

#### **Immunofluorescence assay**

CFTR proteins were detected by immunofluorescence assay on CFBE cells, seeded on Lab-Tek chamber slides (Thermo Fisher Scientific Inc.) and treated with LB or PsaDM before incubation with anti-CFTR 596 antibody (CFFT) and then Alexa Fluor 488 conjugated anti-mouse antibody (Life Technologies Inc.). Slides were finally counterstained with To-Pro-3 iodide (Life Technologies Inc.) and fluorescent images were captured by a Confocal Leica TCS\_SP5 microscope (see supplementary methods for details).

#### **Electrophysiology**

Short-circuit currents ( $I_{sc}$ ) [30–32] were measured through CFBE and primary airway cells [28] that had been cultured on coated permeant filters until they reached an air-liquid interface and formed a polarised epithelium with high resistance [33, 34]. Measurements were performed (see supplementary material for details) on intact monolayers or through apical membranes after permeabilisation of the basolateral side with 7.5  $\mu$ M amphotericin B (Sigma-Aldrich), in the presence of a symmetrical physiological solution or a chloride ( $Cl^-$ ) gradient, as indicated in figure legends.

#### **CFTR mRNA expression**

Total RNA from CFBE cells treated with PsaDM or LB was purified with TRIzol reagent (Life Technologies Inc.) [28], and CFTR mRNA expression was evaluated by qPCR as described in the supplementary material. The expression of CFTR was reported as percentage relative to cells treated with LB.

#### **Statistics**

The data are presented as means $\pm$ SE. Groups were compared by paired t-test or non-parametric Wilcoxon test, with Statview (SAS Institute, Cary, NC, USA) and GraphPad (La Jolla, CA, USA) software, respectively. A probability of  $p < 0.05$  was considered to be significant.

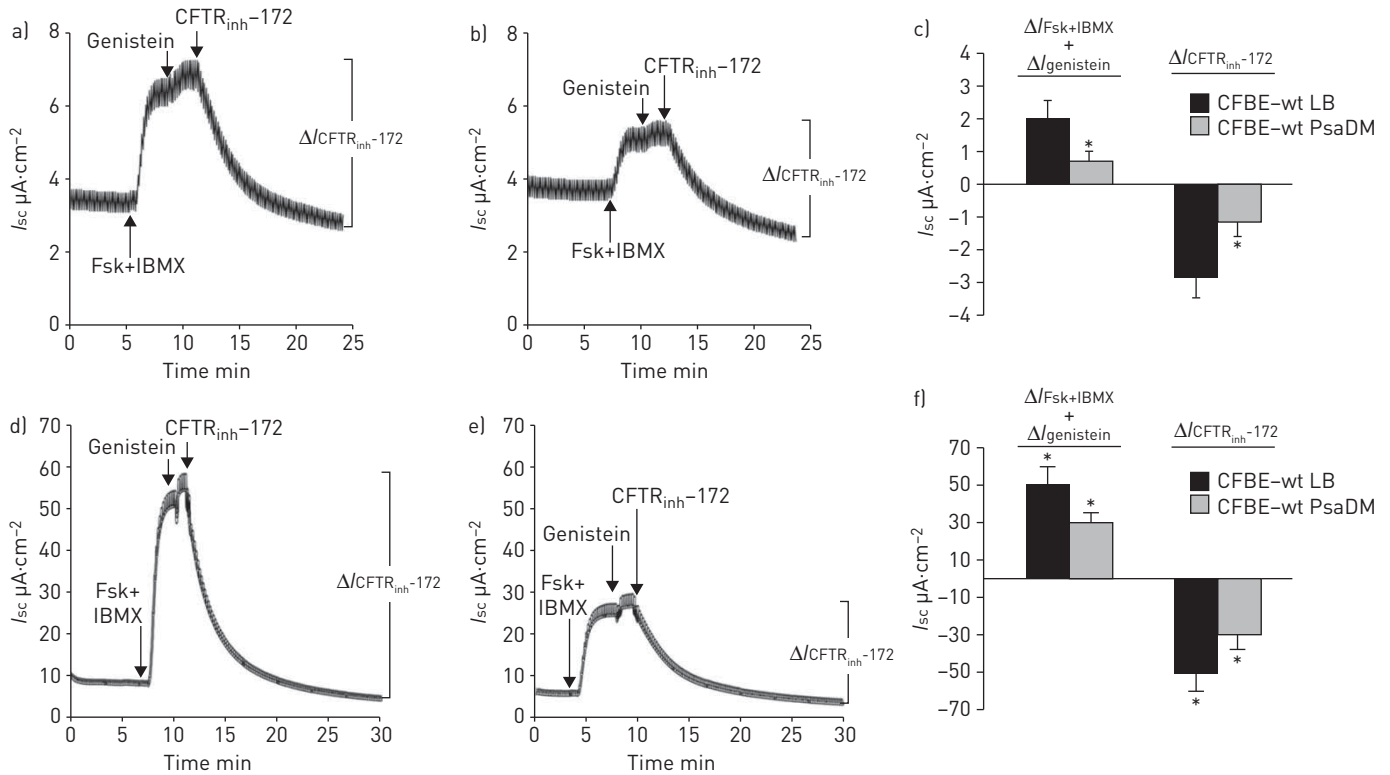
## **Results**

### **Decrease in CFTR function induced by *P. aeruginosa* exoproducts**

We first evaluated the impact of PsaDM on CFTR function in polarised airway epithelial CFBE-wt cell monolayers grown on permeant filters. Cells were treated with either LB (control) or PsaDM for a 24-h period before  $I_{sc}$  measurements. Just before mounting in an Ussing chamber, intact cell monolayers were washed and bathed with a symmetrical physiological solution supplemented with amiloride in order to inhibit sodium ( $Na^+$ ) currents through epithelial sodium channel (ENaC) channels. Forskolin (Fsk), 3-isobutyl-1-methylxanthine (IBMX) and genistein were then applied at the apical membrane to stimulate cAMP-activated  $Cl^-$  secretion through CFTR channels, which was finally inhibited by the  $CFTR_{inh-172}$  (fig. 1a). We observed that the change in  $I_{sc}$  current ( $\Delta I_{sc}$ ) after CFTR activation/potentialisation ( $\Delta I_{Fsk+IBMX+\Delta I_{genistein}}$ ) was significantly reduced in monolayers treated with PsaDM ( $0.7 \pm 0.3 \mu A \cdot cm^{-2}$ ) when compared to the LB control ( $2.0 \pm 0.5 \mu A \cdot cm^{-2}$ ,  $p < 0.02$ ) (fig. 1c). Similarly,  $CFTR_{inh-172}$ -sensitive CFTR current ( $\Delta I_{CFTR_{inh-172}}$ ) was also markedly affected by exposure to PsaDM ( $-1.2 \pm 0.4 \mu A \cdot cm^{-2}$  compared to  $-2.8 \pm 0.6 \mu A \cdot cm^{-2}$  for LB,  $p < 0.02$ ) (fig. 1c).

To assess the specific effect of 24-h treatment with PsaDM on  $Cl^-$  currents through the apical membrane, a series of experiments was performed on CFBE-wt cell monolayers after permeabilisation of the basolateral membrane and establishment of a  $Cl^-$  gradient. Although cAMP-stimulated and  $CFTR_{inh-172}$ -sensitive  $Cl^-$  currents were higher in these conditions (fig. 1d) than through intact monolayers (fig. 1a), a significant reduction was observed in PsaDM treated monolayers (fig. 1f).

We also noted a significant inhibition by PsaDM of UTP-stimulated currents, probably mediated by CaCC channels ( $11.1 \pm 1.9$  and  $5.5 \pm 0.8 \mu A \cdot cm^{-2}$ , in LB and PsaDM treated CFBE-wt cell monolayers,  $n=6$ ,  $p < 0.04$ ).



**FIGURE 1** Decrease of cystic fibrosis transmembrane conductance regulator (CFTR) function after *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment in CFBE-wt airway cell monolayers. CFBE-wt cells were cultured on permeant filters at the air-liquid interface and treated for 24 h with either 10% of lysogeny broth (LB) medium (a and d) or *P. aeruginosa* filtrate (PsaDM) (b and e) before short-circuit current ( $I_{sc}$ ) measurements in an Ussing chamber. a and b) Intact cell monolayers were bathed in a symmetrical physiological medium supplemented with 10  $\mu$ M amiloride and then sequentially exposed to a combination of 10  $\mu$ M forskolin (Fsk) and 100  $\mu$ M 3-isobutyl-1-methylxanthine (IBMX), followed by 30  $\mu$ M genistein and, finally, 20  $\mu$ M CFTR<sub>inh</sub>-172. d and e) A chloride ( $Cl^-$ ) gradient was established by bathing the cell monolayers with an asymmetrical physiological solution (low  $Cl^-$  concentration solution at the apical side and high  $Cl^-$  concentration at the basolateral side) and the basolateral membrane was permeabilised with 7.5  $\mu$ M amphotericin B. Cells were then sequentially exposed to a combination of Fsk, IBMX and genistein, followed by CFTR<sub>inh</sub>-172. Representative  $I_{sc}$  traces are reported for each condition, *i.e.* intact condition and  $Cl^-$  gradient, for LB a) and d), respectively, and for PsaDM b) and e), respectively. While quantification of the mean  $\Delta I_{sc}$  after Fsk+IBMX+genistein and  $\Delta I_{sc}$  after LB and PsaDM exposure is reported for c) intact condition and f)  $Cl^-$  gradient. For intact condition  $n=5$ ,  $p<0.02$  and for  $Cl^-$  gradient  $n=6$ ,  $p<0.006$ . \*:  $p<0.05$ .

It has to be noted that exposure to PsaDM did not elicit any decrease in transepithelial resistance ( $1018\pm39$  and  $1398\pm97 \Omega\cdot cm^2$  in LB and PsaDM conditions, respectively).

#### Decrease in CFTR expression induced by *P. aeruginosa* exoproducts

We then investigated if the observed decrease in CFTR function induced by *P. aeruginosa* exoproducts in CFBE cells was associated with reduced CFTR expression. We first tested the impact of PsaDM on CFTR mRNA expression in non-CF CFBE-wt cells as well as in CF CFBE- $\Delta F508$  cells. As shown in figure 2a, the number of CFTR mRNA copies measured by real-time PCR in CFBE-wt cells remains unchanged in the presence of PsaDM. Meanwhile, PsaDM slightly decreased the level of expression of CFTR mRNA in CFBE- $\Delta F508$  ( $p<0.006$ ) (fig. 2b).

We then assessed the impact of PsaDM on total CFTR-protein expression by immunoblotting. As expected, immature and mature CFTR bands (band B and C, respectively) were detected in CFBE-wt cells (fig. 2c) while the immature band B was visible in lysates from CFBE- $\Delta F508$  cells expressing the  $\Delta F508$ -CFTR mutant protein (fig. 2d). It was then observed that the intensity of both bands in CFBE-wt was severely reduced in the presence of PsaDM (by 73% and 40%, respectively,  $p<0.04$ ), when compared to LB control (fig. 2e). In CFBE- $\Delta F508$  cells, the immature form of CFTR was also affected by PsaDM exposure (57% decrease,  $p<0.0002$ ) (fig. 2f). It has to be noted that equal amounts of proteins were loaded in each condition and signals were normalised to  $\beta$ -actin (which was not altered by PsaDM, see  $\beta$ -actin detection from the same immunoblots, fig. 2c and d).

The effect of PsaDM on CFTR membrane expression in CFBE-wt cells was finally evaluated. Immunoblotting assays, after surface biotinylation, revealed that infectious products significantly reduced the level of CFTR plasma-membrane expression compared to the control (fig. 3a and b). Complementary confocal

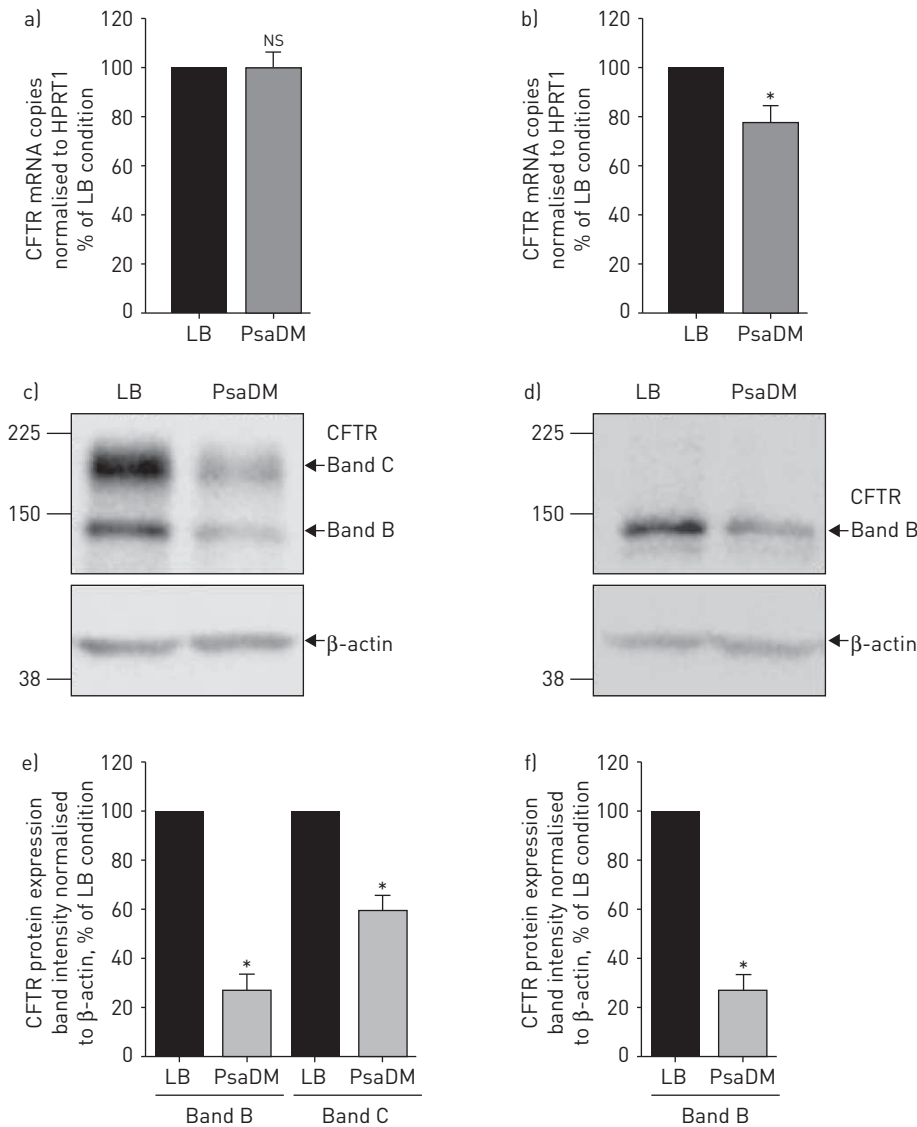
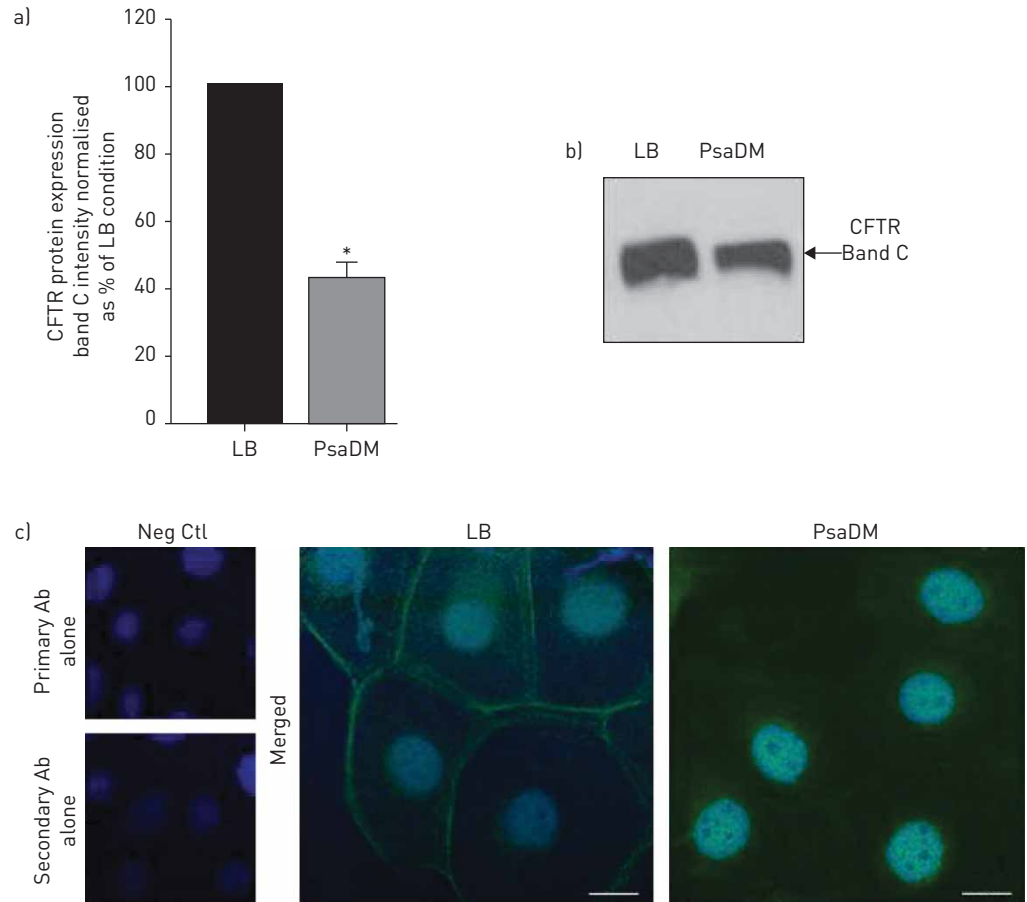


FIGURE 2 Decrease cystic fibrosis transmembrane conductance regulator (CFTR) expression after *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment. a, c and e) CFBE-wt or b, d and f) CFBE-ΔF508 cells were cultured for 8 days (until confluence was reached), then treated with either 10% of lysogeny broth (LB) medium or PsaDM for 24 h. Levels of CFTR mRNA a) CFBE-wt (n=8) and b) CFBE-ΔF508 (n=5) and protein expression for e) CFBE-wt (n=5) and f) CFBE-ΔF508 (n=5) were quantified and reported as percentage of the LB control (p<0.04). Representative immunoblots are presented in c) and d) for CFBE-wt and CFBE-ΔF508, respectively. NS: nonsignificant; \*: p<0.05.

immunofluorescence experiments also showed that CFTR membrane localisation observed in CFBE-wt cells in control conditions (*i.e.* with LB) was dramatically affected after exposure to PsaDM (fig. 3c). Taken together, these results demonstrated that bacterial exoproducts affected CFTR expression, localisation and function.

**Impact of *P. aeruginosa* exoproducts on CFTR protein degradation and synthesis**

In order to define if the observed decrease in total CFTR protein level in the presence of PsaDM could be due to enhanced protein degradation, we blocked protein translation by exposing CFBE-wt cells to CHX for 0, 2, 8, 18, 24 and 32 h, in the presence or absence of PsaDM. We then measured the remaining level of CFTR protein by immunoblotting (normalised to β-actin, and expressed as % of time 0 h in control condition). As shown in figure 4a, the decrease in CFTR protein abundance following 8, 18 and 32 h CHX treatments was slightly, but significantly, higher in the presence of PsaDM (p<0.05). However, this potential increase in CFTR degradation after exposure to PsaDM may not be sufficient to explain the large decrease in CFTR protein level shown in figure 2e. A possible impact of PsaDM on CFTR synthesis was thus assessed by exposing CFBE-wt cells to MG132, a proteasomal inhibitor, for 0, 2, 8 and 18 h in the

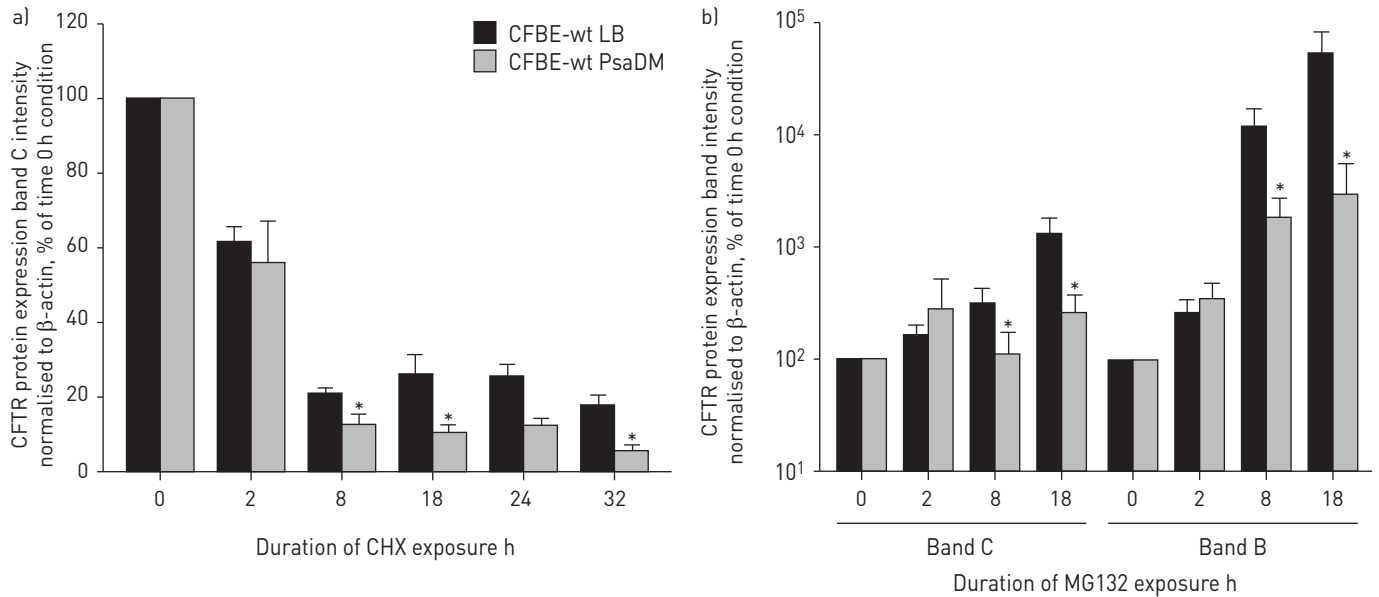


**FIGURE 3** Decrease of cystic fibrosis transmembrane conductance regulator (CFTR) expression after *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment in CFBE-wt airway cells. a and b) The level of CFTR expression at the cell surface in CFBE-wt cells treated for 24 h with lysogeny broth (LB) medium or PsaDM was measured by immunoblotting after surface biotinylation and reported as a percentage of LB control (n=6, p<0.04). b) A representative immunoblot. c) Subconfluent CFBE-wt cells were treated with either LB or PsaDM for 24 h, then fixed and stained with the anti-CFTR antibodies (Ab) coupled to Alexa Fluor 488 conjugated anti-mouse antibody (green). Nuclei were stained with To-Pro-3 in all conditions. Primary and secondary Ab were also incubated alone for negative controls. Fluorescent images were captured at  $\times 100$  magnification by a Confocal Leica TCS\_SP5 microscope. Representative images from six different experiments are reported. Neg Ctl: negative control. Scale bar=10  $\mu$ m

presence or absence of PsaDM and then measuring the accumulation of CFTR protein by immunoblotting. We observed (fig. 4b) that the accumulation of wt-CFTR protein bands B and C was higher under control conditions (LB) than in the presence of PsaDM (p<0.04), suggesting that *P. aeruginosa* exoproducts could reduce CFTR protein synthesis. To confirm that the huge accumulation of CFTR protein observed after proteasomal inhibition with MG132 (MG132+LB at 18 h) (fig. 5a and b) was secondary to newly synthesised CFTR proteins, we verified that a co-treatment with CHX (MG132+CHX+LB) totally prevented CFTR accumulation. Again, our data showed that CFTR protein accumulation was severely impacted by PsaDM (MG132+PsaDM) compared to MG132+LB condition (p<0.05). We also measured the levels of EGFR expression to define if PsaDM could also affect a non-channel membrane protein. Although EGFR accumulation after proteasomal inhibition with MG132 was lower than that of CFTR, we noted that PsaDM exposure did not significantly affected EGFR levels (fig. 5c).

#### **Impaired CFTR rescue in the presence of *P. aeruginosa* exoproducts**

In the light of this evidence pointing towards a deleterious effect of PsaDM on CFTR function and expression, we then decided to investigate if PsaDM had an impact on the ability of the CFTR corrector VRT-325 to rescue  $\Delta$ F508-CFTR maturation and function in CF cells. As previously reported [28], we first confirmed that VRT-325 partially rescued CFTR mature band C protein expression (fig. 6a and b, of note is the comparison between LB and LB+VRT-325) as well as CFTR currents through the apical membrane of CFBE- $\Delta$ F508 cell monolayers (fig. 6c and d, CFBE- $\Delta$ F508 LB versus CFBE- $\Delta$ F508 LB+VRT-325). Indeed,  $I_{sc}$  measurements revealed that CFBE- $\Delta$ F508 cell monolayers treated for 24 h with VRT-325



**FIGURE 4** Impact of *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment on the degradation and synthesis of cystic fibrosis transmembrane conductance regulator (CFTR) protein in CFBE-wt airway cell monolayers. CFBE-wt cells were treated with a) 100  $\mu\text{g}\cdot\text{mL}^{-1}$  of cycloheximide (CHX) for 2, 8, 18, 24 and 32 h, ( $n=6$ ,  $p<0.05$ ) or with b) 5  $\mu\text{M}$  of MG132 for 2, 8 and 18 h ( $n=11$ ,  $p<0.04$ ), in the presence of 10% lysogeny broth (LB) medium or PsaDM. The whole cell lysates were subjected to SDS-PAGE separation and analysed by immunoblotting with anti- $\beta$ -actin (used as loading control) and anti-CFTR antibodies to evaluate the kinetics of CFTR protein degradation in the presence of CHX (a) and the accumulation of newly synthesised CFTR proteins in the presence of the proteasomal inhibitor MG132 (b). \*:  $p<0.05$ .

elicited significantly higher cAMP-activated  $\text{Cl}^-$  currents, sensitive to the  $\text{CFTR}_{\text{inh-172}}$ , compared to the LB control condition (fig. 6c, d and f).

However, in the presence of PsaDM for 24 h (PsaDM+VRT-325) we noted a drastic decrease in band C maturation/expression, compared to the signal in the LB+VRT-325 ( $p<0.02$ ) (fig. 6a and b). The outcome of PsaDM on the rescue of  $\Delta\text{F508}$ -CFTR channel function by VRT-325 was then assessed. As shown in figure 6d–f, cAMP-activated  $\text{Cl}^-$  secretion following CFTR activation/potentialisation ( $\Delta I_{\text{Fsk+IBMX+genistein}}$ ) was notably decreased in PsaDM+VRT-325 ( $0.66\pm 0.68 \mu\text{A}\cdot\text{cm}^{-2}$ ) when compared to LB+VRT-325 ( $2.16\pm 0.32 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $p<0.03$ ). Furthermore, VRT-325-rescued  $\text{CFTR}_{\text{inh-172}}$ -sensitive CFTR currents in the presence of PsaDM (PsaDM+VRT-325,  $-1.74\pm 0.58 \mu\text{A}\cdot\text{m}^{-2}$ ) were also smaller than in LB (LB+VRT-325,  $-2.89\pm 0.47 \mu\text{A}\cdot\text{cm}^{-2}$ ,  $p<0.02$ ) (fig. 6d–f). These results indicated that the rescue of  $\Delta\text{F508}$ -CFTR channel function by the CFTR corrector VRT-325 is strongly impaired by *P. aeruginosa* exoproducts.

#### Deleterious impact of *P. aeruginosa* exoproducts on CFTR function in primary human airway epithelial cells

The effect of PsaDM on CFTR function was finally assessed in primary human airway epithelial cells isolated from non-CF and CF patients. In non-CF airway cells, we confirmed a significant reduction in cAMP-activated  $\text{Cl}^-$  secretion ( $\Delta I_{\text{Fsk+IBMX}}$  of  $13.9\pm 3.5$  versus  $8.9\pm 2.7 \mu\text{A}\cdot\text{cm}^{-2}$ , respectively under LB and PsaDM conditions,  $p<0.05$ ), as well as  $\text{CFTR}_{\text{inh-172}}$ -sensitive ( $\Delta I_{\text{CFTR}_{\text{inh-172}}}$ ,  $-18.5\pm 3.1 \mu\text{A}\cdot\text{cm}^{-2}$  in LB versus  $-13.5\pm 3.1 \mu\text{A}\cdot\text{cm}^{-2}$  in PsaDM) CFTR currents by *P. aeruginosa* exoproducts (fig. 7). Moreover, the rescue of CFTR currents in CF airway primary cells, induced by VRT-325, was also impaired in the presence of PsaDM ( $\Delta I_{\text{Fsk+IBMX}}$  of  $1.1\pm 0.4 \mu\text{A}\cdot\text{cm}^{-2}$  versus  $0.4\pm 0.4 \mu\text{A}\cdot\text{cm}^{-2}$ , respectively in the presence of LB+VRT-325 or PsaDM+VRT-325,  $p<0.03$ ) (fig. 8).  $\text{CFTR}_{\text{inh-172}}$ -sensitive CFTR currents were also slightly reduced in the presence of PsaDM.

#### Discussion

Altogether, our data demonstrated that exoproducts from *P. aeruginosa* impacted CFTR function in both CFBE-wt cells and primary human airway epithelial cells isolated from non-CF patients. A decrease in CFTR protein expression was observed, which could be due, at least in part, to enhanced protein degradation and reduced protein synthesis. *P. aeruginosa* exoproducts also affected CFTR membrane expression. Importantly, our study also revealed that the functional rescue of  $\Delta\text{F508}$ -CFTR by a CFTR corrector in CFBE- $\Delta\text{F508}$  cells and CF primary human airway epithelial cells was impaired in the presence of bacterial products.

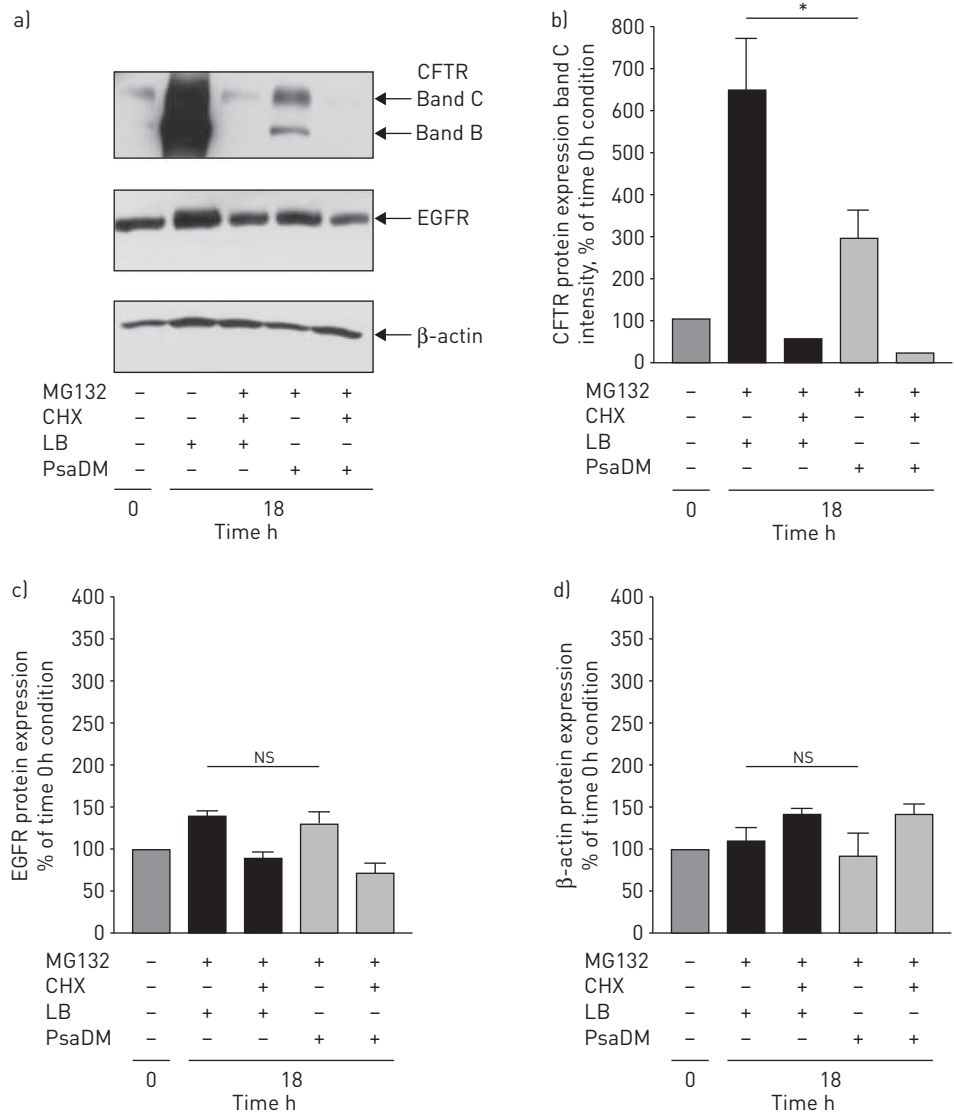
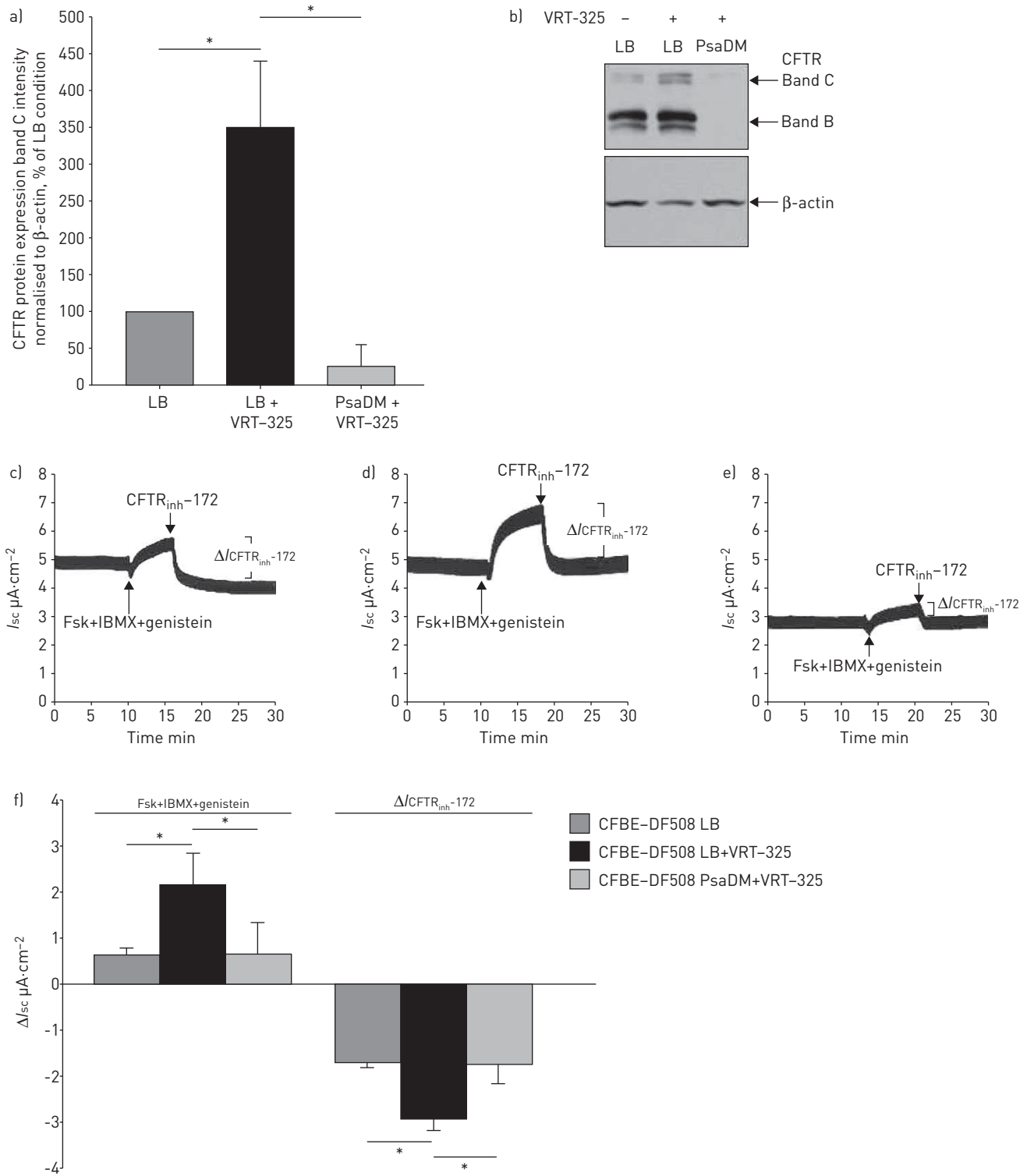


FIGURE 5 Impact of *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment on cystic fibrosis transmembrane conductance regulator (CFTR) and epidermal growth factor receptor (EGFR) proteins in CFBE-wt airway cell monolayers. CFBE-wt cells were treated with 5  $\mu$ M of MG132 (for 18 h) alone or in combination with cycloheximide (CHX) (MG132 +CHX), in the presence of lysogeny broth (LB) medium or PsaDM. a and b) CFTR, a and c), EGFR, and a and d)  $\beta$ -actin proteins were detected by immunoblotting with their respective antibodies. A representative immunoblot is presented in a) and protein levels were quantified and reported as a percentage of the LB control in b-d). NS: nonsignificant; n=6; p<0.03; \*: p<0.05.

### Impaired CFTR function by infectious products

Our Ussing chamber experiments on CFBE-wt cells presented in figure 1 demonstrated that a 24-h exposure to diffusible materials from a clinical isolate of *P. aeruginosa* (PACF508 strain) induced a significant reduction in cAMP-activated  $Cl^-$  currents through CFTR channels. We are aware that the observed decrease in cAMP-activated currents after exposure to PsaDM could be due to a secondary effect on other cAMP-dependent channels, such as the basolateral KvLQT1 potassium channel. To further define if the effect of PsaDM is specific of CFTR, another series of experiments were undertaken on CFBE-wt cells, after permeabilisation of the basolateral membrane and establishment of a  $Cl^-$  gradient, to focus on  $Cl^-$  currents through the apical membrane. As presented in figure 1d-f, a significant reduction in both cAMP-activated and CFTR<sub>inh-172</sub>-sensitive currents was observed in PsaDM treated CFBE-wt cell monolayers. This result confirmed the impact of *P. aeruginosa* exoproducts on CFTR function. It has to be noted that PsaDM also reduced UTP-activated currents through CaCC channels, thus potentially precluding a possible compensatory effect of the CaCC after inhibition of CFTR channels in infectious conditions.





**FIGURE 6** Impairment of  $\Delta F508$  cystic fibrosis transmembrane conductance regulator ( $\Delta F508$ -CFTR) functional rescue after *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment. **a)** CFTR band C protein expression in nontreated CFBE- $\Delta F508$  cells (*i.e.* lysogeny broth (LB) medium alone) and in CFBE- $\Delta F508$  cells treated with 5  $\mu M$  VRT-325 for 24 h in the presence of 10% LB (LB+VRT-325) or PsaDM (PsaDM+VRT-325) was quantified and reported as percentage of the LB control ( $n=8$ ,  $p<0.04$ ). **b)** A representative immunoblot. **c-f)** Short-circuit currents ( $I_{sc}$ ) were measured in an Ussing chamber through CFBE- $\Delta F508$  cells, cultured on permeant filters at the air-liquid interface. After mounting in an Ussing chamber, cells were bathed with an asymmetrical physiological solution (low chloride ( $Cl^-$ ) concentration solution at the apical side and high  $Cl^-$  concentration at the basolateral side) and the basolateral side was permeabilised with 7.5  $\mu M$  amphotericin B. Upon  $I_{sc}$  stabilisation, cells were exposed to a mix of forskolin (Fsk), 3-isobutyl-1-methylxanthine (IBMX) and genistein, and then with CFTR<sub>inh</sub>-172. Representative  $I_{sc}$  traces are reported for each condition: **c)** nontreated (LB), **d)** treated for 24 h before electrophysiological measurements with VRT-325 or **e)** treated with a combination of VRT-325 and PsaDM. **f)** Quantifications of the mean  $\Delta I_{Fsk+IBMX+genistein}$  and  $\Delta I_{CFTR_{inh}-172}$   $I_{sc}$  in LB, LB+VRT-325 and PsaDM+VRT-325 conditions ( $n=6$ ,  $p<0.03$ ).  $n=6$ ; \*:  $p<0.05$ .

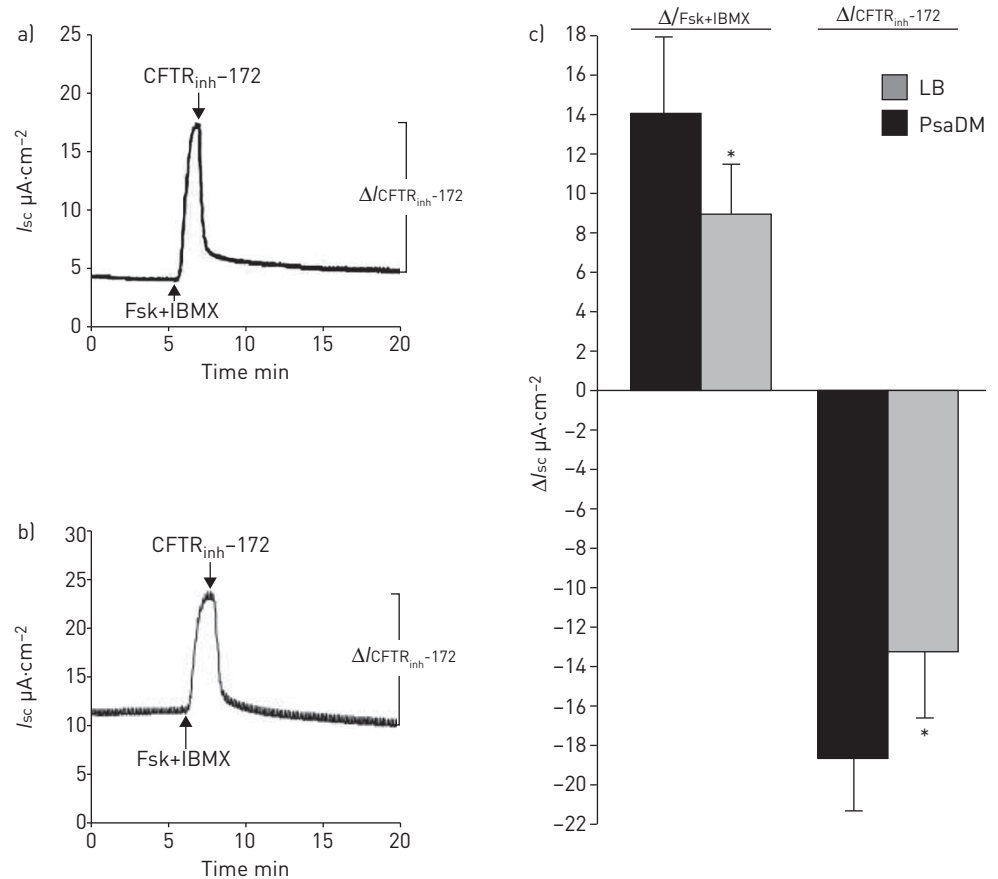


FIGURE 7 Inhibition of wildtype cystic fibrosis transmembrane conductance (wt-CFTR) function after *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment in non-cystic fibrosis (CF) human primary airway cell monolayers. Short-circuit current ( $I_{sc}$ ) measurements in an Ussing chamber were performed on non-CF human primary airway cell monolayers, cultured on permeant filters at the air-liquid interface, treated for 24 h with either 5% lysogeny broth (LB) medium or PsaDM. After mounting in an Ussing chamber, cells were bathed with an asymmetrical physiological solution (low chloride ( $Cl^-$ ) physiological solution at the apical side and high  $Cl^-$  solution at the basolateral side), supplemented with 10  $\mu M$  amiloride. Upon  $I_{sc}$  stabilisation, cells were treated with a combination of forskolin (Fsk) and 3-isobutyl-1-methylxanthine (IBMX), followed by CFTR<sub>inh</sub>-172. Representative  $I_{sc}$  traces are reported for each condition a) LB and b) PsaDM while quantifications of the mean  $\Delta I_{Fsk+IBMX}$  and  $\Delta I_{CFTR_{inh}-172}$   $I_{sc}$  are reported in c). n=7; \*; p<0.05.

Similar to our results, SWIATECKA-URBAN *et al.* [6] previously reported that a 4–6 h period of incubation with a culture of a *P. aeruginosa* laboratory isolate (PA14) reduced CFTR-mediated transepithelial  $Cl^-$  secretion through Calu-3 cells as well as CFBE41o- cells stably expressing wt-CFTR. Importantly, we now confirmed the deleterious impact of a 24-h exposure to *P. aeruginosa* exoproducts in differentiated primary cultures of non-CF human airway cells (fig. 7). In contrast with these data, it has been shown that acute (15 min) application of *P. aeruginosa* (PA14) or purified LPS increased the rate of iodide efflux, an index of  $Cl^-$  secretion, through 2WT2 epithelial cells expressing wt-CFTR [9]. Furthermore, BUYCK *et al.* [35] have shown that *P. aeruginosa* LPS-stimulated  $Cl^-$  currents through CFTR channels in 16HBE14o- cells, probably *via* a calcium signal. Similarly, N-(3-oxo-dodecanoyl)-S-homoserine lactone (3O-C12), a quorum sensing molecule secreted by *P. aeruginosa*, has been shown to rapidly enhance  $Cl^-$  and fluid secretion as well as cytosolic calcium and cAMP levels [36]. It has also been reported that *P. aeruginosa* flagellin activated CFTR-dependent anion secretion in Calu-3 (through p38 signalling) as well as in primary cultures of human bronchial epithelial cells [37]. Most of these observations were interpreted as an attempt by airway epithelia to rapidly react to bacterial infection by increasing CFTR-dependent ion and fluid flow necessary for bacterial clearance from the airways. Notably, whereas pyocyanine (N-methyl-1-hydroxyphenazine) slightly activated basal  $Cl^-$  secretion through CFBE-wt cells, this redox-active virulence factor produced by *P. aeruginosa*, severely inhibited Fsk-stimulated  $Cl^-$  secretion [38]. Our results, as well as data from the literature, thus indicated that the effects of *P. aeruginosa* infection may be time- and model-dependent. Moreover, different *P. aeruginosa* products/virulence factors present in PsaDM could be responsible for the observed effects, and further studies would be necessary to clearly define their mechanisms of actions.

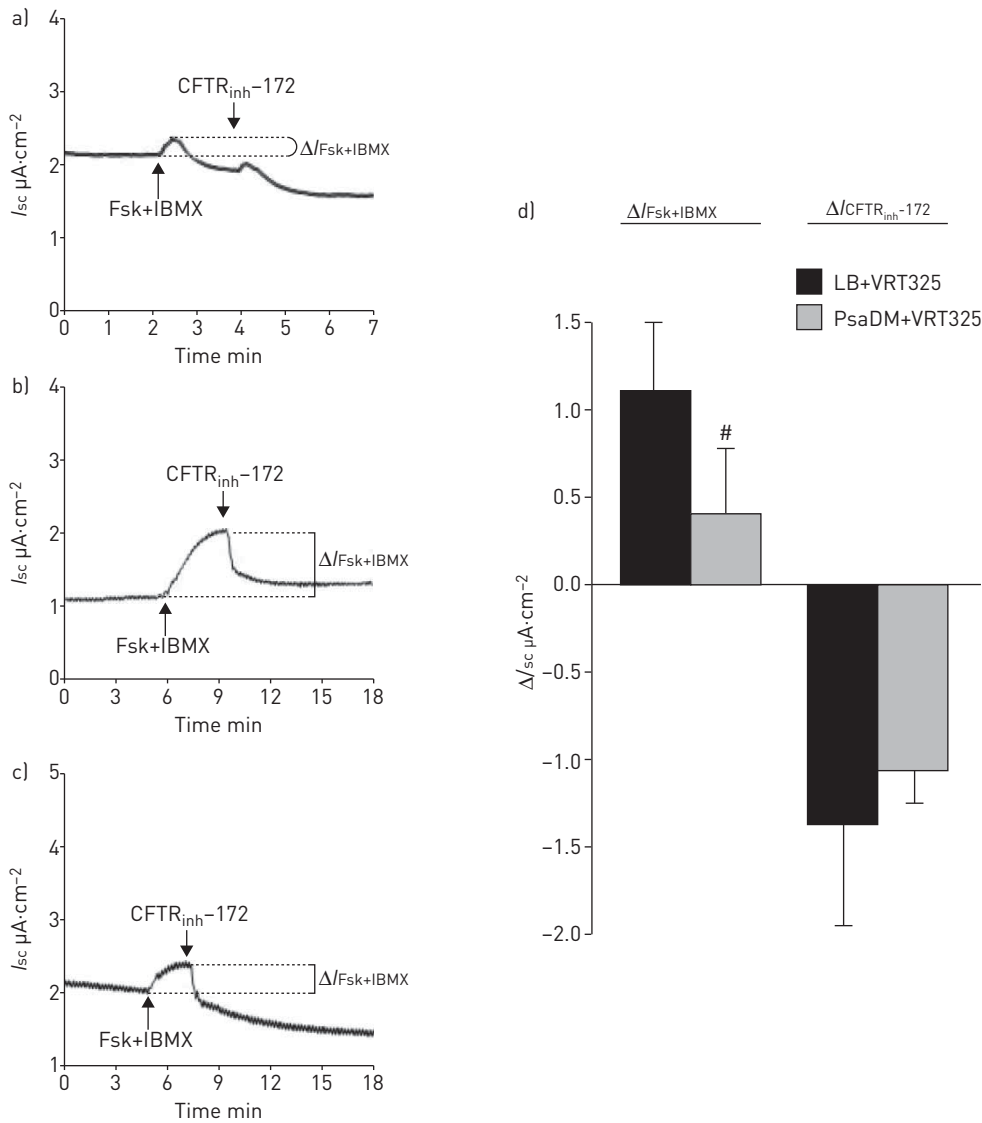


FIGURE 8 Inhibition of  $\Delta F508$  cystic fibrosis transmembrane conductance regulator ( $\Delta F508$ -CFTR) functional rescue after *Pseudomonas aeruginosa* diffusible material (PsaDM) treatment in cystic fibrosis (CF) human primary airway cell monolayers. Short-circuit current ( $I_{sc}$ ) measurements in an Ussing chamber were performed on CF human primary airway cell monolayers, cultured on permeant filters at the air-liquid interface and treated or not for 24 h with 10  $\mu M$  VRT-325 in combination with either 5% lysogeny broth (LB) medium or PsaDM. After mounting in an Ussing chamber, filters were bathed with an asymmetrical physiological solution (low chloride ion ( $Cl^-$ ) physiological solution at the apical side and high  $Cl^-$  at the basolateral side), supplemented with 10  $\mu M$  amiloride. Upon  $I_{sc}$  stabilisation, the filters were treated with a combination of forskolin (Fsk) and 3-isobutyl-1-methylxanthine (IBMX), followed by CFTR<sub>inh</sub>-172. Representative  $I_{sc}$  traces are reported for each condition a) LB, b) LB+VRT-325, and c) PsaDM+VRT-325, while d) shows quantifications of the mean  $\Delta I_{Fsk+IBMX}$  and  $\Delta I_{CFTR_{inh}-172}$   $I_{sc}$ . n=6; #: p<0.03.

### Impact of infection on CFTR expression

In order to determine if the decrease in CFTR function after chronic exposure to *P. aeruginosa* products could be due to a decrease in CFTR expression, we then undertook PCR and immunoblot measurements to assess changes in CFTR mRNA and protein expression in CFBE-wt cells. Whereas the level of wt-CFTR mRNA was not affected after a 24-h treatment with PsaDM, we observed that both immature (band B) and mature (band C) CFTR proteins were severely reduced in CFBE-wt cells. In contrast with our results, a previous study [6] indicated that live *P. aeruginosa* bacteria (PA14 strain with 4–6 h of infection) had no effect on total cellular expression of wt-CFTR. However, it was shown that PA14 decreased the expression of wt-CFTR at the apical membrane, which is in agreement with the reduced function of CFTR observed under infectious conditions in that study as well as in ours. Moreover, our immunoblot assays after surface biotinylation as well as immunofluorescence analysis with confocal microscopy (fig. 3) showed reduced CFTR membrane expression after PsaDM exposure.

It has to be noted that PACF508 used in our study is a clinical mucoid strain. However, it seems that the mucoid phenotype of *P. aeruginosa* is not responsible for the deleterious effect on CFTR since the non-mucoid laboratory strains PAO1 and PA14 have also been shown to downregulate CFTR [6, 39]. The study by RUBINO *et al.* [39] indicated that a mechanism involving bacterial pilin and flagellin as well as cellular NHERF1 could be involved in CFTR regulation by *P. aeruginosa*. In fact, various products secreted by *P. aeruginosa* have also been shown to have an impact on CFTR expression and function. Pyocyanin, for example, was found to inhibit CFTR expression at the apical membrane of airway epithelial cells [40]. The PA2394 protein (called CFTR inhibitory factor, Cif) secreted by *P. aeruginosa* is also involved in the reduction of CFTR apical membrane expression [41]. A model was then proposed where Cif secretion in *P. aeruginosa* outer membrane vesicles may reduce USP10-mediated deubiquitination of CFTR and secondarily increase the degradation of CFTR in lysosomes [42]. In agreement with CFTR degradation induced by infection, our results observed in the presence of CHX (fig. 4) indicated that the kinetics of wt-CFTR protein degradation could be slightly faster in the presence of PsaDM, compared to control conditions. However, this effect may not be sufficient to explain the huge inhibition of CFTR protein expression induced by PsaDM as shown in figure 2. In fact, our data obtained in the presence of MG132 and/or CHX (figs 4 and 5) indicated that CFTR synthesis may also be affected by PsaDM. Our study, as well as data from the literature, thus indicated that *P. aeruginosa* exoproducts may impact CFTR protein synthesis, degradation and trafficking/recycling to the cell membrane. A reduction in CFTR protein expression and function in non-CF airways exposed to chronic infection, for example in COPD patients, may have a negative impact on mucociliary clearance and consequently on the ability of airways to clear bacteria. This phenomenon could thus create a vicious circle of infection impairing CFTR, this CFTR dysfunction then favours infection.

#### **Impaired $\Delta F508$ -CFTR rescue in the presence of infection**

Our immunoblot experiments on CFBE- $\Delta F508$  cells first revealed that the immature band B of  $\Delta F508$ -CFTR was decreased by a 24-h exposure to PsaDM (fig. 2c-f). Furthermore, immunoblot and Ussing chamber measurements showed for the first time that *P. aeruginosa* exoproducts severely affected the ability of a CFTR corrector to rescue  $\Delta F508$ -CFTR maturation and function in CFBE- $\Delta F508$  cells as well as in CF primary airway epithelial cells (figs 6 and 8). These results are in agreement with a previous study [6] indicating that *P. aeruginosa* PA14 bacteria decreased CFTR currents induced by low temperature rescue in CFBE cells expressing  $\Delta F508$ -CFTR. The mechanisms whereby  $\Delta F508$ -CFTR rescue may be affected by *P. aeruginosa* remain to be elucidated.

Altogether, these data indicate that the presence of infection in CF patients may impair the ability of CFTR correctors to rescue the basic CFTR defect. These observations are critical to future screenings of CFTR correctors that should take into account the infectious component, in order to select small corrector molecules that could be efficient despite the presence of pathogens.

#### **Acknowledgements**

We acknowledge R.J. Bridges (Rosalind Franklin University, North Chicago, IL, USA) and Cystic Fibrosis Foundation Therapeutic Inc. (CFFT) (Bethesda, MA, USA) for providing VRT-325 as well as J. Riordan (University of North Carolina, Chapel Hill, NC, USA) and Cystic Fibrosis Foundation Therapeutic Inc for CFTR Ab596. Primary airway epithelial cells were from our Biobank of respiratory tissues of Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), (Montreal, QC, Canada)/ Institut de Recherches Cliniques de Montréal (IRCM) of the Respiratory Health Network of FRQS. Finally, we acknowledge the logistical assistance from the Research Support Office, CRCHUM.

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