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Pseudomonas aeruginosa reduces the expression of CFTR via post-translational modification of NHERF1

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Abstract *Pseudomonas aeruginosa* infections of the airway cells decrease apical expression of both wild-type (wt) and F508del CFTR through the inhibition of apical endocytic recycling. CFTR endocytic recycling is known to be regulated by its interaction with PDZ domain containing proteins. Recent work has shown that the PDZ domain scaffolding protein NHERF1 finely regulates both wt and F508delCFTR membrane recycling. Here, we investigated the effect of *P. aeruginosa* infection on NHERF1 post-translational modifications and how this affects CFTR expression in bronchial epithelial cells and in murine lung. Both in vitro in bronchial cells, and in vivo in mice, infection reduced CFTR expression and increased NHERF1 molecular weight through its hyperphosphorylation and ubquitination as a consequence of both bacterial pilin- and flagellin-mediated host–cell interaction. The ability of *P. aeruginosa* to down-regulate mature CFTR expression was reduced both in vivo in NHERF1 knockout mice and in vitro after silencing NHERF1 expression or mutations blocking its phosphorylation at serines 279 and 301. These studies provide the first evidence that NHERF1 phosphorylation may negatively regulate its action and, therefore, the assembly and function of multiprotein NHERF1 complexes in response to infection. The identification of molecular mechanisms responsible for these effects could identify novel targets to block potential *P. aeruginosa* interference with the efficacy of potentiator and/or corrector compounds.

Keywords Lung infection · NHERF1 · CFTR · Phosphorylation · Ubquitination

Abbreviations

CF	Cystic fibrosis
NHERF1	Na^+/H^+ exchanger regulatory factor 1
CFTR	CF transmembrane conductance regulator
siRNA	Small interfering RNA

Introduction

Although intensive and early anti-infectious and nutritional treatment has prolonged the life span of individuals suffering from cystic fibrosis (CF), it is still a life-threatening disease. Mutated or absent CFTR alters muco-ciliary clearance, which ultimately increases infection of the airways by a few opportunistic bacterial species. This infection represents a key

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pathogenetic event of lung damage and respiratory insufficiency. Indeed, CF patients become colonized with inhaled bacteria soon after birth and by their late teens, 80 % of CF patients develop chronic endobronchial infections, mainly due to *Pseudomonas aeruginosa*. This results in progressive obstructive pulmonary disease, respiratory failure and ultimately death.

The focus of novel drug development strategies in CF disease is presently on discovering compounds that restore chloride transport by either increasing the export of F508delCFTR from the ER (correctors), or by activating defective protein (potentiators), thereby increasing CFTR apical membrane function. The hope is that this will enable bronchial epithelial cells to reinstate mucociliary clearance and eradicate *P. aeruginosa* from the CF airway.

Recently, a novel pathogenic mechanism has been observed in which *P. aeruginosa* infection directly reduces the apical membrane expression and the chloride secretion of both wt and rescued F508delCFTR in respiratory epithelia by inhibiting apical endocytic recycling [32], possibly through the secretion of the soluble CFTR inhibitory factor, Cif [25]. More recently, it was demonstrated that Cif reduces the USP10-mediated deubiquitination of CFTR, thereby increasing the degradation of CFTR in lysosomes and reducing the cell and plasma membrane CFTR protein levels [4]. In this way, *P. aeruginosa* further creates an environment conducive for its colonization in CF airways by eliminating any residual chloride secretion and airway surface liquid volume, which further reduces mucociliary clearance, establishing chronicity of the bacterial infection and, eventually, biofilm formation. Thus, the presence of *P. aeruginosa* infection could per se render the recent CF therapies less effective, as any increase in CFTR function would be reversed due to the activity of the infecting *P. aeruginosa*.

NHERF1 plays a key role in the turnover of CFTR as suggested by works demonstrating that: (1) deletion of the PDZ interacting domain of CFTR reduces the half-life of CFTR at the apical membrane [31], (2) over expression of the PDZ domain containing protein, NHERF1, increases wt CFTR apical expression and rescues F508delCFTR surface expression [14]; (3) NHERF1 knockdown reduces surface expression of wt-CFTR and enhances the degradation of rescued F508delCFTR [10, 21]; and (4) the NHERF1-dependent increases of apical membrane wt and F508del CFTR expression occur via the re-organization of the actin cytoskeleton induced via the formation of a NHERF1–RhoA–ROCK–ezrin–actin multiprotein complex [11, 27]. Since CFTR endosomal recycling requires tight coordination between polarized trafficking, signaling events and cytoskeleton/membrane remodeling, NHERF1 represents the archetypal molecular structure where this tight integration is achieved through the highly coordinated interaction of the two PDZ and ERM domains.

Altogether these data suggest a possible additional mechanism for how *P. aeruginosa* could reduce CFTR expression, likely involving NHERF1. Here, we examined, both in vitro and in vivo, the effect of the infection with *P. aeruginosa* on the expression of CFTR and NHERF1 and determined the role NHERF1 in the reduction of CFTR expression by *P. aeruginosa* infection. Lastly, we also observed that the alteration induced by these bacteria in NHERF1 expression is due to its post-translational modifications.

Materials and methods

Cell cultures

Experiments were performed with two normal human bronchial epithelial cell lines: NuLi-1 cells (from A. Klingelhutz, P. Karp and J. Zabner, University of Iowa, Iowa City, IA, USA) and 16HBE14o- cells (from D. Gruenert, University of California, San Francisco, CA, USA) grown as described [27, 38].

Silencing *NHERF1* gene

16HBE14o- cells at 70–80 % confluence were transiently transfected with small interfering RNA (siRNA) targeting NHERF1 (siGENOME Smart pool reagent; Dharmacon RNA Technologies) or with scrambled siRNA (Dharmacon RNA Technologies). Transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen) and the experiments were conducted 72 h later.

Transfection of 16HBE14o- with mutated NHERF1

Cells at 70–80 % confluence were transiently transfected with pcDNA vector containing wt NHERF1 cDNA or with NHERF1 cDNA mutated in both serine 271 and serine 301 to alanine (S271/S301A) which was the kind gift of Dr. Massimo Tommasino of the Infections and Cancer Biology Group, International Agency for Research on Cancer, World Health Organization, Lyon, France.

Plasmid constructs were mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and the PAO1 infections (for 4 h at 37 °C) were conducted 72 h later.

P. aeruginosa strains

P. aeruginosa laboratory strains, PAO1; PAK a wild-type (wt), non-mucoid, piliated, motile strain; PAK/NP, non mucoid, non piliated, motile strain; PAK/*fliC* a non motile derivative of PAK [35] have been kindly donated by Alice Prince (Columbia University). Bacteria were grown on trypticase soy broth (TSB) or agar (TSA) (Difco).

Cell infection

Preliminary experiments were performed to gain insights on the effect of different doses of PAO1 on CFTR expression (see Fig. S3). On the basis of these results, we chose to use 100–200 CFU/cell PAO1 dose, at higher doses we observed cellular damage.

Before the experiment, bacteria from overnight cultures in TSA plates were grown in 20 ml TSB broth at 37 °C with shaking until there was an OD (at 660 nm) of about 1×10^9 colony-forming units (CFU)/ml, determined by dilution plating. Bacteria were washed twice with PBS at 4 °C to eliminate products secreted into the extracellular environment, and resuspended to a final dilution in cell culture medium. The doses of bacteria were determined by plating aliquots of dilutions on TSA plates. Cells were infected with different strains (100–200 CFU/cell) at 37 °C, 5 % CO_2 for 4 h in the absence of antibiotics as previously described [8].

Animal models and infection protocol

NHERF1-knockout mice, originally generated in the laboratory of E. Weinman Jr. at the University of Maryland, were bred onto a FVB/N background for >10 generations at the Hannover Medical School from heterozygote parents. Mice were bred at the animal care facility under standard temperature and light conditions, and allowed free access to food and water. Care was taken to match the mice not only as sex-matched littermates but also in terms of an equal number of weight-matched male and female pairs of WT and KO mice in each group of experiments. WT refers to the +/+ littermates of the respective strain. Paired KO and WT littermates were housed under identical conditions mostly in the same cages to ensure identical bacterial flora. All experiments involving animals were approved by the Hannover Medical School and the Infections and Cystic Fibrosis Unit, San Raffaele Scientific Institute Committees on investigations involving animals and independent committees assembled by the local authorities.

PAO1 (1×10^5 CFU) or sterile PBS was administrated in both wt and NHERF1 null mice by intratracheal (i.t.) injection. Mice were sacrificed 4 h later, lungs were perfused with PBS, excised, frozen in liquid nitrogen and stored at −80 °C for protein expression study. In these experiments, two mice per group were utilized for airway histology, the lungs of these animals were perfused, excised, fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E).

Western blot analysis

Cells or tissues proteins were extracted and separated on 7 % or 10 % SDS-PAGE for CFTR and NHERF1, respectively, followed by transfer to Immobilon P filters (Millipore) for immunoblotting. The primary antibodies used were anti-human CFTR monoclonal antibody against C terminus (R&D Systems, Minneapolis, MN, USA; MAB25031, dilution 1:500), purified mouse anti-NHERF1 monoclonal antibody (BD Transduction Laboratories™; 611161, diluted 1:500), Ubiquitin (P4D1) monoclonal antibody (Cell Signaling; 3936, diluted 1:1,000), monoclonal anti-β-Actin clone AC-15 (Sigma-Aldrich; A5441, diluted 1:5,000). The secondary antibody was horseradish peroxidase-coupled anti-mouse IgG (Sigma). Immunocomplexes were detected with ECL Plus Western Blotting detection system (Amersham Biosciences). Image processing and densitometric quantification were carried out using Image J.

Statistical analysis

Data are presented as means ± SEM. One-way ANOVA Kruskal/Wallis test was used to compare more than two groups, and Student's *t* test (two-tailed) was used to compare two groups. A value of $p<0.05$ was considered significant, assuming equal variances on all experimental data sets. All analyses were performed with InStat (GraphPad Software, La Jolla, CA, USA).

Results

P. aeruginosa infection decreased the CFTR membrane expression and increased NHERF1 molecular weight

To determine the effect of the *P. aeruginosa* on CFTR and NHERF1 expression, we infected both NuLi-1 and 16HBE14o- (HBE) bronchial epithelial cell lines expressing wt CFTR with the PAO1 strain for 4 h as described in Materials and methods. As expected, *P. aeruginosa* infection induced a reduction of CFTR expression in both cell lines as shown by western blot analysis (Fig. 1a, NuLi-1; Fig. 1b, 16HBE14o-). The reduction in intensities of the CFTR bands were approximately 70 % and 60 % in the NuLi-1 and 16HBE14o- cell lines, respectively, as shown in the histograms (Fig. 1c and d, respectively). Interestingly, this reduction of CFTR expression was accompanied by an increase in NHERF1 expression and a shift of its molecular weight from 50 to 65 kDa in both cell lines.

To assess whether the increase in NHERF1 molecular weight was, at least in part, due to its hyper-phosphorylation, an extract from PAO1 infected NuLi-1 cells was exposed to increasing levels of the de-phosphorylating enzyme, alkaline phosphatase (AP) (Fig. 2a). This resulted in a reduction of the molecular weight of NHERF1, demonstrating that the increase in molecular weight after exposure to *P. aeruginosa* was in part due to its hyper-phosphorylation, but a certain amount was resistant to this enzyme. This could be due to phosphate residues resistant to AP or, possibly, due to other PAO1 infection–

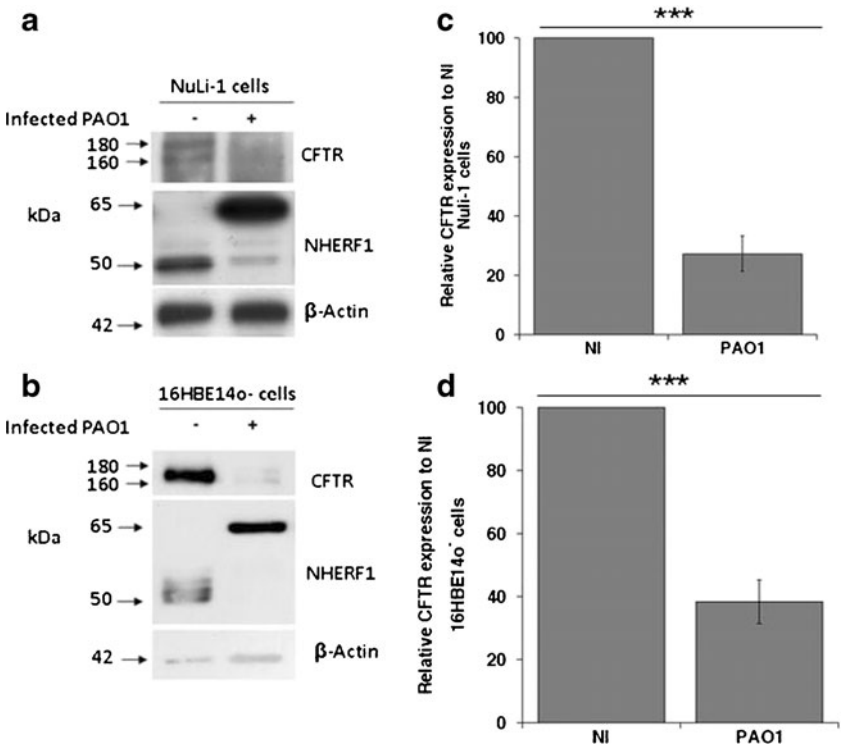

Fig. 1 *P. aeruginosa* decreased the CFTR expression and changed the NHERF1 mobility shift. NuLi-1 (**a** and **c**) and 16HBE14o- (**b** and **d**) cells were incubated with 100 CFU/cells of *P. aeruginosa* for 4 h. Cells were then lysed and Western Blots were performed using an antibodies directed against NHERF1 or CFTR. The intensities of CFTR bands, in NuLi-1 (n=4 independent experiments) and in 16HBE14o- (n=5 independent experiments) were quantified and are represented in the histograms (**c** and **d**, respectively). Significance in Student's t test between not infected and PAO1 infected cells: ***$p<0.001$

dependent post-translational modifications and we looked at ubiquitination. In order to determine the presence of ubiquitin molecules bound to NHERF1, we performed a Western Blot analysis by using a specific antibody against ubiquitin in the extract from infected NuLi-1. Infection with PAO1 greatly increased the total level of protein ubquitination by approximately 7-fold, especially in the higher molecular weights (see Supporting Information). As can be seen in Fig. 2b, the observed shift of NHERF1 from 50 to 65 kDa upon infection of the cells was mirrored by the appearance of a ubiquitined protein at the same molecular weight of shifted NHERF1. Altogether, these data demonstrate that *P. aeruginosa* infection induced both hyperphosphorylation, as well as, increased ubiquitination.

Pilin and flagellin of *P. aeruginosa* were responsible for NHERF1 mobility shift

Since *P. aeruginosa* engages multiple receptors and activates several parallel transmembrane pathways, we investigated the role of interaction of flagellum or pili with the pattern recognition receptors (PRRs) expressed in respiratory cells on NHERF1 phosphorylation. For this study, we infected the NuLi-1 cells with (1) PAK, a wt, non-mucoid, piliated, motile strain, (2) PAK/NP, a non mucoid, non piliated, motile strain, or (3) PAK/*fliC* a non motile derivative of PAK in which the *fliC* gene encoding flagellin was replaced by homologous recombination with a mutant gene interrupted by a gentamicin resistance cassette. As shown in Fig. 3, the infection of the cells with PAK induced a reduction of CFTR expression, an increase in NHERF1 expression and a shift of its molecular weight from 50 to 65 kDa, as observed when the cells were infected with PAO1 (see Fig. 1a and b). This mobility shift of

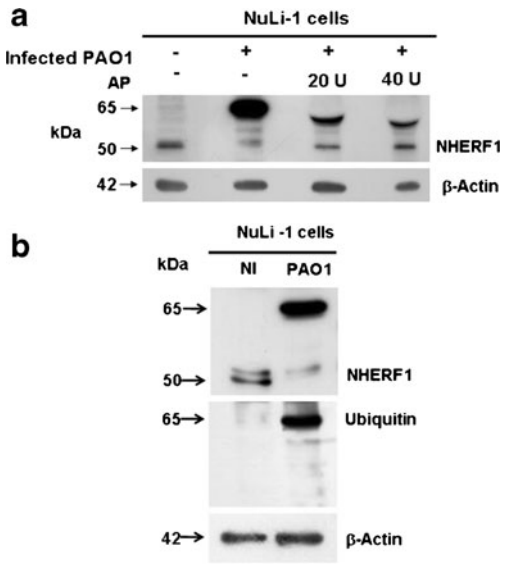

Fig. 2 The increase in NHERF1 molecular weight is due to its hyperphosphorylation. The extract from Nuli-1 infected cells was exposed to 0, 20 or 40U of AP, as indicated. NHERF1 expression was analyzed by Western Blot as described in Materials and methods (**a**). In another experiment, the lysates from NuLi-1 infected cells were analyzed by Western blot to investigate the presence of ubiquitin molecules (**b**). Results are representative of three independent experiments

NHERF1 induced by whole bacteria cells was completely abolished in cells infected with PAK/NP and partially abolished in cells infected with PAK/*fliC*. This suggested that, the mobility shift of NHERF1 was dependent on the interaction of pilin and flagellin with their cell receptors.

Transient knockdown of NHERF1 or NHERF1 S271A/S301A mutation reduced the *P. aeruginosa*-mediated decrease in CFTR surface expression

NHERF1 knockdown has been shown to induce a reduction in expression of CFTR [10, 21]. To investigate the role of NHERF1 on the expression of CFTR in our cell model, HBE respiratory cells were transiently transfected with siRNA against NHERF1 and the expression of CFTR and NHERF1 were assayed by Western Blot. As shown in Fig. 4, transient transfection of siRNA against NHERF1 in HBE cells resulted in an 87 % inhibition of NHERF1 expression and a reduction of about 49 % of CFTR expression. In order to ascertain the involvement of NHERF1 in the events driven by *P. aeruginosa* infection both cells transfected with siRNA against NHERF1 and their relative scrambled controls were infected with *P. aeruginosa* for 4 h and both CFTR and NHERF1 expression were measured in Western Blots (Fig. 5a). Figure 5b shows that the *P. aeruginosa*-dependent down-regulation of CFTR expression was reduced by approximately 28 % in cells transfected with siRNA against NHERF1 compared to the almost 85 % CFTR down-regulation in the infected scrambled control. To test possible effects of *P. aeruginosa* infection on NHERF1 phosphorylation and on CFTR down-regulation, HBE cells were transfected with NHERF1 cDNA mutated to alanine in both serine 271 and serine 301. As can be seen in Fig. 5c and d, transfection with the cDNA of NHERF1 S271A/S301A increased CFTR expression by about 27 % (not significant) and very strongly reversed the PAO1-dependent reduction in CFTR expression from an approximate 70 % reduction to a 30 % reduction. In contrast, transfection with the WT NHERF1 cDNA while increasing basal CFTR expression similarly to the mutant clone (approximately 24 %) did not have any effect on the PAO1-dependent reduction in CFTR (74±9 %). Altogether, these results strongly suggest that the reduction of CFTR expression by *P. aeruginosa* infection is, at least partially, dependent on NHERF1 phosphorylation.

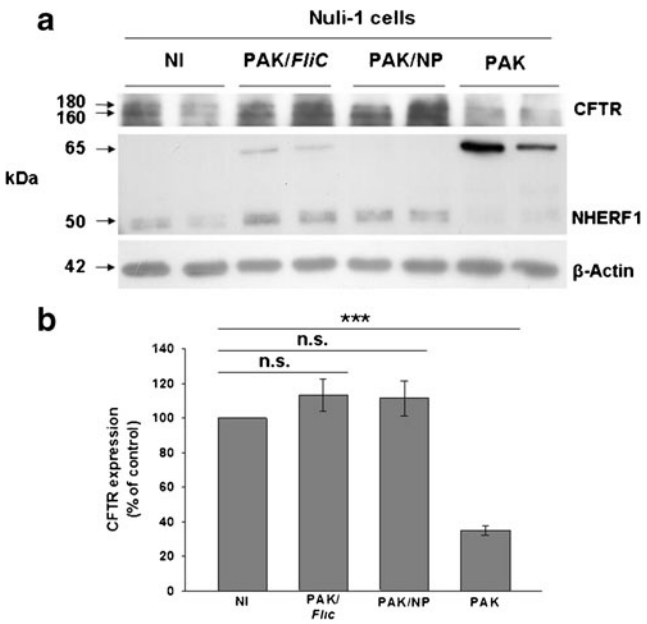

Fig. 3 Pili and flagellum of *P. aeruginosa* are responsible for NHERF1 mobility shift. NuLi-1 cells were infected with PAK, a wt, non-mucoid, piliated, motile strain; or PAK/NP, a non-mucoid, non-piliated, motile strain; or PAK/*fliC* a non-motile derivative of PAK for 4 h and the lysates from the infected cells were analyzed by Western blot (**a**). Results are representative of three independent experiments. Histogram shows the quantitative measurements of protein bands standardized to their relative actin bands and expressed as mean ± SEM of the percentages of the non-infected control (*NI*) (**b**)

Genetic deletion of NHERF1 reduced the effects of *P. aeruginosa* on CFTR expression

We next aimed to verify these in vitro results in experiments in vivo using wt and NHERF1−/− (KO) mice. These mice were infected with *P. aeruginosa* for 4 h, after which the lungs were excised, and Western Blots were performed on lysates as reported in Materials and methods.

As shown in Fig. 6b, the absence of NHERF1 expression in the KO mice reduced basal levels of CFTR by about 71.8± 7.9 % compared to the wt mice (Fig. 6a), verifying the role of NHERF1 expression observed in the in vitro experiments. Furthermore, *P. aeruginosa* infection increased the relative expression of phosphorylated NHERF1 from approximately 25 % of the total NHERF1 expression in the lungs of non-infected wt mice to approximately 40 % of the total NHERF1 expression in infected lungs. Concomitantly, *P. aeruginosa* infection dramatically reduced basal CFTR expression in infected lungs of NHERF1 wt mice by 85.9±18.5 % and in the lungs of NHERF1 KO mice by only 48.4±5.8 % (Fig. 6c), confirming that NHERF1 expression is crucial.

NHERF1 KO mice showed signs of inflammation under basal condition which were aggravated by *P. aeruginosa* infection

It has been suggested that individuals with CF have a predisposition to inflammation even in the absence of infection, and that this may be due to a reduction of CFTR in the apical membrane. We therefore examined the lungs of the mice involved in this study in order to determine the level of inflammation and the effect of *P. aeruginosa* infection in both NHERF1 wt and KO mice. Animals were sacrificed 4 h after challenge as previously reported [7–9]. Lung histology was

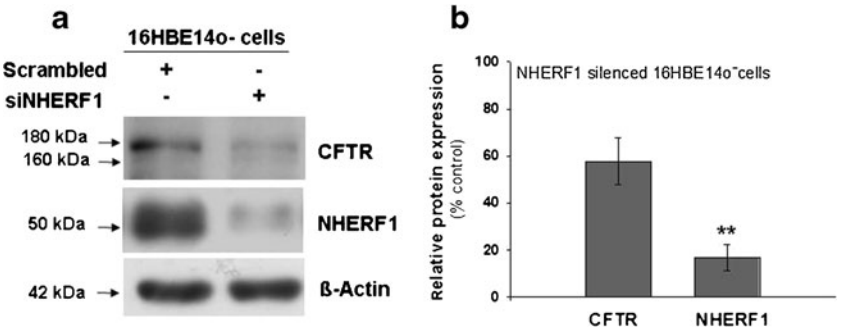

Fig. 4 Effect of transient transfection of siRNA against NHERF1 on CFTR expression in airway cells. 16HBE14o-cells were transiently transfected with siRNA against NHERF1 or scrambled oligonucleotides for 72 h and the expression of CFTR and NHERF1 were assayed by Western blot as described in Materials and methods (**a**). Histogram shows the quantitative measurements of protein bands standardized to their relative actin bands and expressed as mean ± SEM of the percentages of the non-infected control (*NI*). Results are representative of five independent experiments. Significance in Student's *t* test between scrambled and siRNA against NHERF1: $^{**}p<0.01$ (**b**)

performed as described in Materials and methods. In wt mice, exposure to *P. aeruginosa* resulted in the presence of inflammatory cell infiltrates (mainly eosinophils) and type II pneumocyte hyperplasia (Fig. 7b).

Interestingly, the lung of NHERF1 KO mice already showed per se a more severe presence of diffuse alveolar septal edema and inflammatory cell infiltrates (eosinophils and macrophages) in peribronchial and perivascular regions (see Fig. 7c).

Furthermore, exposure of the NHERF1 KO mice to *P. aeruginosa* elicited inflammatory cell infiltrates (eosinophils and neutrophils) mainly in the perialveolar regions, as

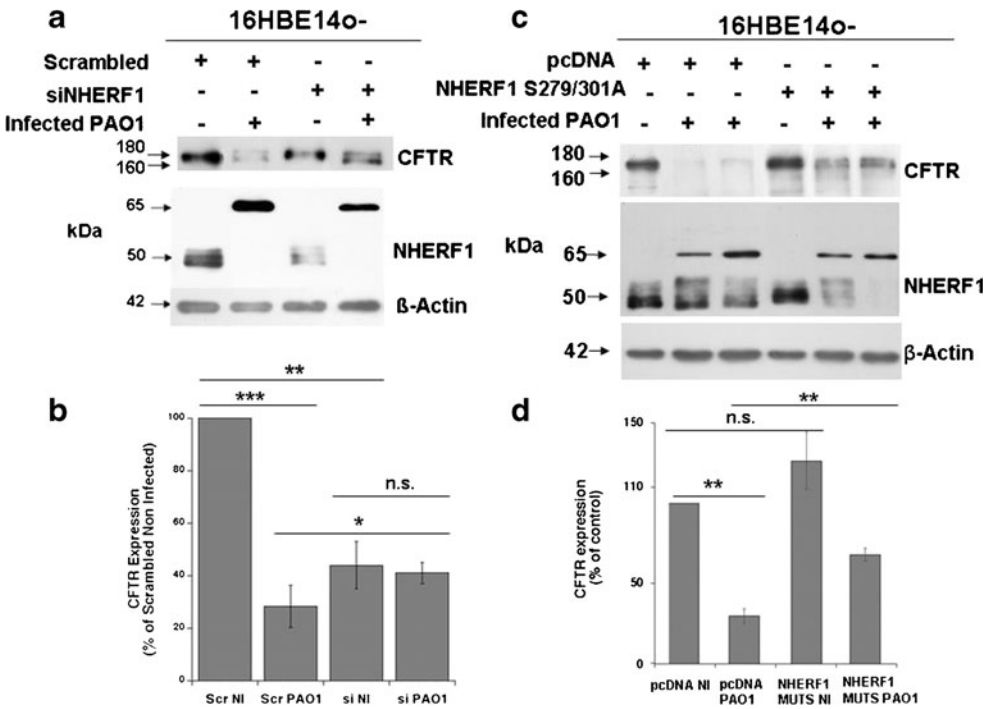

Fig. 5 Transient knock-down of NHERF1 or *NHERF1 S271A/S301A mutation* reduced the negative effects of *P. aeruginosa* infection on CFTR expression. 16HBE14o- cells transfected with either siRNA against NHERF1 or scrambled control, or with cDNA of NHERF1 mutated S271A/S301A were infected with *P. aeruginosa* for 4 h. Cells were then lysed and analyzed in Western Blot using an antibody directed against NHERF1 or CFTR (**a** and **c**). In panel **c**, *lanes 2 and 3* and *lanes 5 and 6* are duplicates of each other. The intensities of CFTR bands were quantified and are represented in the histogram as the mean ± SEM of the percentage value of each treatment compared to the scrambled Not Infected controls (Scr NI) or pc DNA Not Infected control (**b** and **d**). Results are representative of three independent experiments. **b** Significance in ANOVA test between Scr NI and scrambled PAO1 infected, Scr PAO1: $^{***}p<0.001$; or between Scr NI and not infected siRNA against NHERF1, si NI: $^{**}p<0.01$; or between PAO1 infected siRNA against NHERF1, si PAO1 and Scr PAO1: $^{*}p<0.05$. The difference between si PAO1 and si NI was not significant (*n.s.*). ANOVA analysis of the entire data set showed the global differences to be highly significant. **d** Significance in ANOVA test between pcDNA NI and pcDNA PAO1: $^{**}p<0.01$; or between pcDNA NI and NHERF1 muts NI and pcDNA NI was not significant (*n.s.*); or between pcDNA PAO1 and NHERF1 muts PAO1: $^{**}p<0.01$. ANOVA analysis of the entire data set showed the global differences to be highly significant

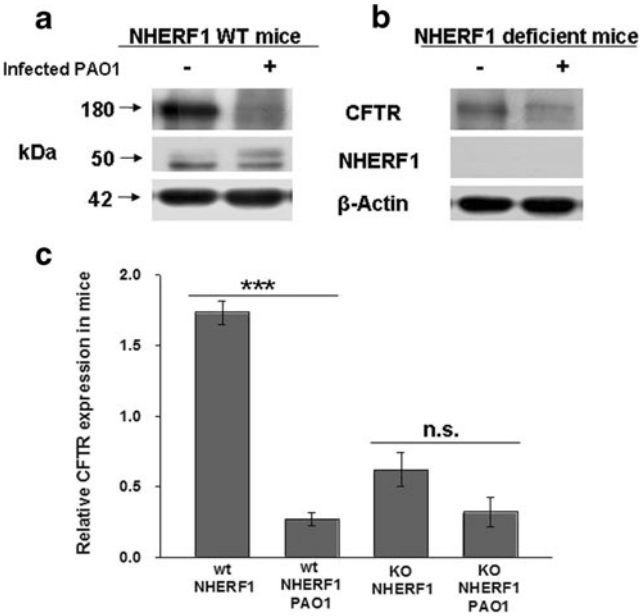

Fig. 6 Genetic deletion of NHERF1 decreased the *P. aeruginosa* induced reduction of CFTR expression in vivo. wt (n=7) or NHERF1 knockout (n=4) mice were infected with *P. aeruginosa* for 4 h as described in Materials and methods. As control, both wt (n=10), and NHERF1 null (n=5), mice were treated i.t. with sterile PBS. The lungs were excised and the lysates were carried on SDS-PAGE and assayed by Western Blot (**a** and **b**). The intensities of CFTR bands were quantified and are represented in the histogram as mean ± SEM (**c**). Significant difference: ***p<0.001 between not infected and PAO1 infected wtNHERF1 mice. The difference between not infected and PAO1 infected KO NHERF1 mice was not significant (*n.s.*)

shown in Fig. 7d that were stronger than in infected wt mice as shown in the panels at higher magnification of Fig. S2. This suggests that NHERF1 could be a novel component of the signalling network involved in the expression of inflammatory genes in murine lung.

Discussion

Changes in the phosphorylation status by specific kinases and phosphatases are one of the major mechanisms for regulating protein activity and function. NHERF1 is phosphorylated both constitutively and by physiological stimuli. This phosphorylation modifies both the binding ability/specificity of the PDZ domains through serine 77 [36, 37] and through serine 162 [23, 29]. The formation of homodimers occurs via the constitutive phosphorylation of serine 289 [15], serine 279 and serine 301 by cdc2 kinase [16]. The stimulus-driven phosphorylation involves serines 339 and 340 [12, 36]. These studies provided the first evidence for a potential negative regulation of the assembly and function of multiprotein NHERF1 complexes by the phosphorylation of NHERF1. The association among CFTR C-terminus, NHERF1, ezrin and the actin cytoskeleton has been proposed to tether CFTR on the membrane [30], making NHERF1 a potential target for bacteria to use to alter CFTR expression.

The major observations in this study are that both in vitro and in vivo infection of respiratory cells with *P. aeruginosa* induced an increase of molecular weight of NHERF1 with a reduction of NHERF1 basal expression (Fig. 1a and b) and that this band contains phosphorylated and ubiquitinated NHERF1 (Fig. 2a and b). Importantly, this event is associated

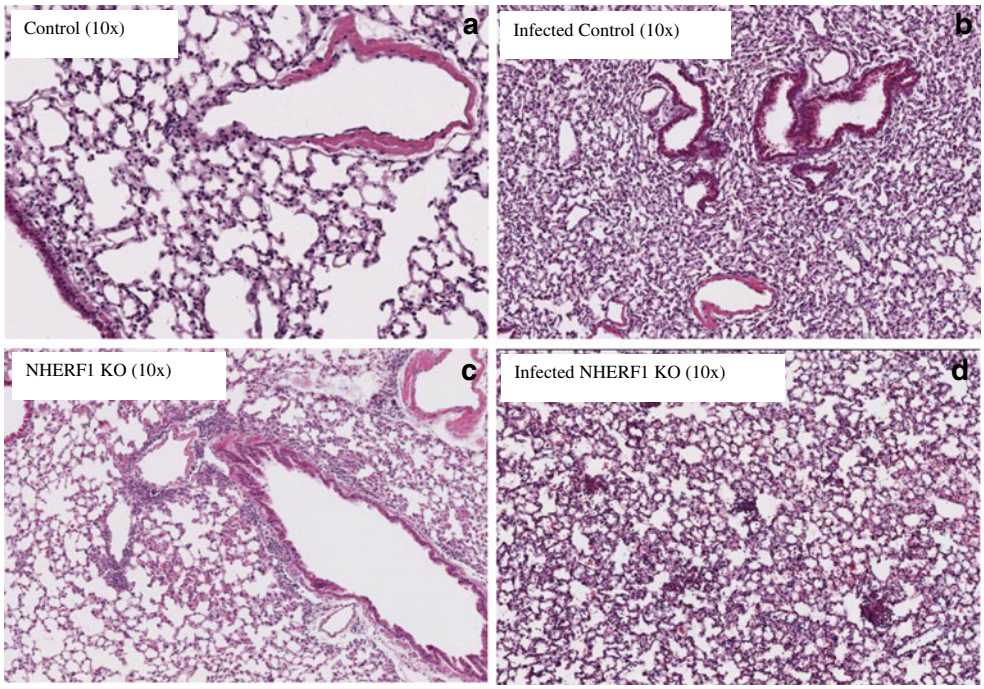

Fig. 7 NHERF1 KO mice showed signs of inflammation under basal condition which were aggravated by *P. aeruginosa* infection. The lungs of two mice for group investigated were excised, fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin. **a** Airway histology from wt mice (control). **b** Airway histology from wt mice after infection with *P. aeruginosa* for 4 h (infected control). **c** Airway histology from non infected NHERF1 knockout mice (NHERF1 KO). **d** Airway histology from NHERF1 knockout mice infected with *P. aeruginosa* for 4 h (infected NHERF1 KO)

with a reduction of the expression of CFTR (Fig. 1). In vitro, both the knock-down of NHERF1 (siRNA, Fig. 5a and c) and the phospho-dead mutations (S271A/S301A, Fig. 5b and d) of NHERF1 greatly reduced the negative effect of *P. aeruginosa* infection on CFTR expression. These finding were confirmed in in vivo NHERF1 knockout mice where the KO of NHERF1 expression also reduced the negative effect of *P. aeruginosa* infection on CFTR expression (Fig. 6). These results suggest that the observed PAO1-dependent phosphorylation of NHERF1 does, indeed, play a role on CFTR expression upon PAO1 infection and suggests that its role in driving the formation of multiprotein complex(es) that stabilize CFTR in plasma membrane may be due to its post-translational modifications rather than on its absolute quantity.

We also demonstrated that another laboratory strain of bacteria, PAK, induced the same effects of PAO1, while PAK strains without flagellum or pili partially or completely abolished the increase of molecular weight of NHERF1 (Fig. 3). Thus, the multiple interactions of *P. aeruginosa* with epithelial cell receptors may induce parallel transmembrane signaling events that could cooperate to determine the observed NHERF1 modification and CFTR endocytosis.

Indeed, bacterial pathogens operate by attacking crucial intracellular pathways in their hosts. They usually target more than one intracellular pathway and often interact at several points in each of these pathways to fully commandeer them [3, 20]. In this respect, the first studies on the mechanism underlying the *P. aeruginosa* infection-dependent reduction of CFTR expression showed that infection inhibits the endocytic recycling of CFTR chloride channels [32] through a released bacterial toxin termed, Cif (CFTR inhibitor factor), which regulates the host protein (USP10) involved in ubiquitination and lysosomal degradation of CFTR [4]. Here, we found that acute infection with *P. aeruginosa*, also resulted in dramatic reduction of CFTR expression both in vitro and in vivo (Figs. 1 and 6), and also induced a hyper-phosphorylation of NHERF1, which was confirmed by the effect of AP on extracts of infected NuLi-1 as shown in Fig. 2a.

However, a certain amount of NHERF1 was resistant to this AP treatment and might represent either a different group of phosphorylated residues or, since we found the high molecular weight band of NHERF1 to also be ubiquitinated, it could represent the presence of ubiquitin molecules bound to NHERF1. The role of both these processes in regulating NHERF1 function and trafficking needs to be further evaluated in the future. Indeed, it is now clear that ubiquitylation also mediates various non-degradative molecular functions, such as protein trafficking, protein–protein interactions and regulation of signal-transduction events [1, 17, 24]. Further, the presence of ubiquitin molecules bound to NHERF1 explained the larger mobility shift observed, since the phosphorylation of NHERF1 alone determines a mobility shift of not more than 4 kDa [15, 16, 23, 36].

Altogether, these data suggest that *P. aeruginosa* regulates, in part, CFTR membrane stability and endocytic recycling through modulation of NHERF1 phosphorylation either on the known phosphorylation sites or on new sites that are still undescribed. Further studies using mass spectrometry (MS) analysis will be necessary to identify the amino acids of NHERF1 that undergo phosphorylation when *P. aeruginosa* interacts with the airway cells.

It is known that *P. aeruginosa*, upon interaction with pattern PRRs expressed in respiratory cells, induces a proinflammatory response involving the participation of the MAPKs p38 and ERK [2, 6,13, 26, 33]. These kinases induced by the early interaction could influence the phosphorylation and/or ubiquitination state of NHERF1 suggesting that NHERF1 could also play a role in the pro-inflammatory response. In support of this possibility, the airway histology performed on the lungs utilized in this study indicated the presence of inflammatory cells even in non-infected NHERF1 KO mouse lung and that upon exposure to *P. aeruginosa* the immune response was stronger in NHERF1 KO mice than in infected wt mice (Fig. 7 and Fig. S2). These preliminary results provide interesting indications that will require a large series of dedicated experiments to determine if NHERF1 could be one of components of the signalling network involved in the expression of inflammatory genes in murine lung and in its absence, which is associated with a reduction of CFTR expression, could play a critical role in the pathogenesis of CF. Indeed, this idea fits very well with emerging evidence suggesting the "sterile inflammation" in CF lungs, in a view where mutant CFTR may itself contribute to the hyper inflammatory response observed in the lung of CF patients even in the absence of bacterial infection [5, 18, 19, 28, 34]. It has been recently shown a new pathway by which neutrophil elastase degraded CFTR protein through calpain enzymatic activation [22]. This could be an additional mechanism to perpetuate the inflammatory phenotype together with earlier effects of the interaction of *P. aeruginosa* with epithelial receptors and the secretion of virulence factor, Cif [4, 25].

In conclusion, our results lend support to the proposed hypothesis that *P. aeruginosa* infection inhibits the endocytic recycling of CFTR via the modification of phosphorylation state of NHERF1 and its ubiquitylation and this could be an obstacle to the efficient use of protentiator/corrector therapies currently under study. Therefore, further understanding the molecular mechanisms regulating the *P. aeruginosa*-dependent relation between NHERF1 and CFTR in CF cells are crucial to permit the development of better therapeutic strategies to restore the plasma membrane functional expression of CFTR and reduce the detrimental inflammatory status in the lungs of patients affected by CF also in the presence of *P. aeruginosa*.

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