PEGylation of Recombinant Human Deoxyribonuclease I Provides a Long-Acting Version of the Mucolytic for Patients with Cystic Fibrosis

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ABSTRACT: Inhalation of recombinant human deoxyribonuclease I (rhDNase) is a gold-standard therapy in the management of cystic fibrosis (CF). Yet, the rapid elimination of the mucolytic from the lungs requires its daily administration and rhDNase contributes to the high therapy burden of patients. Here, we present a long-acting version of rhDNase that could be delivered once weekly instead of once daily. Conjugation of rhDNase to a polyethylene glycol (PEG) chain sustained its presence and mucolytic activity within the murine lungs for more than 15 days. One single dose of PEGylated rhDNase was as effective as 1 daily dose of unconjugated rhDNase during 5 days to decrease the DNA content in the lungs of β-ENaC mice, a model of the CF lung disease. Moreover, once weekly administration of PEGylated rhDNase over 4 weeks decreased both the DNA content and the neutrophil counts in the lungs of β-ENaC mice. PEGylated rhDNase was stable to jet nebulization. Finally, multiple high-dose administrations of PEGylated rhDNase for up to three months did not cause any significant pulmonary or systemic toxicity, nor accumulation of the rhDNase or PEG moieties in biological fluids. PEGylation of rhDNase might offer a convenient long-acting mucolytic to patients with CF.

Keywords: Recombinant human deoxyribonuclease I, cystic fibrosis, polyethylene glycol, long-acting drugs, β-ENaC mice.

Abbreviations

AM, alveolar macrophages; BAL, bronchoalveolar lavage; CCL, chemokine ligand; CF, cystic fibrosis; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; EDTA, ethylenediaminetetraacetic; ENaC, transepithelial Na^+ channel; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IFN, interferon; IL, interleukin; Km, Michaelis constant; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NAL, nasal lavage; PBS, phosphate-buffered saline;

PEG, polyethylene glycol; rhDNase, recombinant human deoxyribonuclease I; SEC, size exclusion chromatography; SEM, standard error of the mean; Tris, trishydroxymethylaminomethane; Vmax, maximal reaction rate; WT, wild-type; β-ENaC, β-epithelial Na^+ channel–overexpressing.

1. Introduction

Cystic fibrosis (CF) is a severe genetic disorder associated with loss-of-function of the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein. More than 2,000 putative mutations have been identified[1]. The most prevalent mutation, found in approximately 70% of CF chromosomes, and in at least one allele in 90% of patients with CF, corresponds to a deletion of the phenylalanine residue at position 508 (F508del-CFTR) of the single polypeptide chain of 1480 amino acids[2]. The effects of the mutations on the synthesis, folding, transport, recycling and function of the CFTR protein are diverse and related to the severity of the disease.

The CFTR protein functions essentially as a transcellular transporter of chloride in exocrine epithelia, including the airways, the pancreatic and bile ducts, the reproductive tracts, the sweat and salivary glands[3]. The defect in ion transport causes dehydration of secretions with hyperviscous mucus and leads to chronic airway obstruction, pancreatic insufficiency and intestinal malabsorption. Although multi-systemic, CF primarily affects the respiratory system and respiratory involvement is responsible for the high morbidity and mortality of the disease due to the combination of retained pulmonary secretions, chronic infection and bronchiectasis.

The median life expectancy of patients with CF was less than 5 years in the United States in the 1950s but it is now above 40 years[4]. This fantastic improvement is the result of multiple factors including: (i) centralized care in referral centres, (ii) early multidisciplinary care before irreversible complications, and (iii) proactive and aggressive symptomatic treatment which combines mucolytics, bronchodilators, antibiotics, anti-inflammatories and nutritional and physiotherapy measures. Such lifelong symptomatic treatment is the heaviest reported in medicine for patients.

Indeed, it requires an average of 2 hours per day, excluding sport and physiotherapy. This time burden limits patient adherence to the treatment in the long term and thereby the outcome of the medications[5]. Moreover, the treatment is insufficient to circumvent the decline in pulmonary function, which conditions the prognosis.

Over the last decade, new developments in CF therapy have mainly focused on small molecules called CFTR modulators. CFTR modulators have the ability to enhance or even restore the functional expression of specific CF-causing mutations[6]. The potentiator Kalydeco™ and the tritherapy Trikafta™ result in substantial restoration of the lung function[7, 8]. Kalydeco™ (ivacaftor) was approved for patients bearing the G551D-CFTR mutation (5% of patients with CF) in 2012. Trikafta™ combines ivacaftor and two correctors, elexacaftor and tezacaftor. It was approved in October 2019 for people with CF aged 12 years and older who have at least one copy of the F508del mutation. Yet, CFTR modulators also present uncertainties and limitations. The clinical response is variable and ranges from no to high response[7]. The long-term efficacy and safety are unknown. The cost of treatment is very high: Kalydeco™ costs for instance €300,000 a year. Finally, the de-escalation of the treatment once CFTR modulators are added in routine remains to be proven.

Chronic pulmonary therapies are a major component of the symptomatic treatment regimen for individuals with CF. Among recommended pulmonary therapies, dornase alfa (Pulmozyme™) or recombinant human deoxyribonuclease I (rhDNase) is the most widely prescribed[4]. Respiratory secretions of patients contain a high number of neutrophils and their apoptosis releases large amounts of DNA. By cleaving the DNA in respiratory secretions, rhDNase strongly decreases their viscosity, thereby facilitating expectoration. As such, daily inhalation of rhDNase can break the vicious cycle of obstruction, inflammation and recurrent respiratory infections[9]. However, the current treatment with rhDNase presents limitations. First, rhDNase is cleared within a few hours from the lungs: when the dose of 2.5 mg is inhaled, a concentration of 3 µg/ml is measured in sputum immediately after inhalation and it is reduced to 0.6 µg/ml after two hours[10]. This rapid

elimination of rhDNase implies the requirement to deliver the protein once to twice daily. Second, although introduction of the e-flow rapid has significantly shortened the actual nebulization times, the current nebulization procedure still remains suboptimal and cumbersome as the entire process from setting up the device to cleaning easily takes 10 to 15 minutes. Therefore, rhDNase is considered to be one of the treatments that significantly contributes to the high therapy burden of patients with CF. In order to improve the treatment adherence and to preserve both the quality of life and life expectancy, it is essential to develop treatments that can be more conveniently delivered[11].

To address this unmet medical need, we designed a PEGylated rhDNase version that could provide a sustained presence of the mucolytic in the lungs and reduce its administration frequency. PEGylation is a well-established technology to increase the serum half-life of proteins and more than 15 PEGylated proteins are approved for clinical use by injection[12]. More recently, PEGylation has been shown to prolong the residence time of proteins in the lungs and to improve their local therapeutic efficacy in preclinical models of respiratory diseases[13, 14]. However, residence times in the lungs have not not exceeded 48h and no PEGylated protein for inhalation is currently available on the market.

We previously conjugated a large (≥20kDa) PEG chain to the N-terminal amino acid of rhDNase and demonstrated that PEGylated rhDNase retained the full biological activity of the enzyme *in vitro* as well as in CF sputa *ex vivo*[15, 16]. In the present study, we show that PEGylation of rhDNase tremendously extended its residency and mucolytic activity in the lungs of healthy mice for up to 20 days. In addition, we demonstrate that one single dose of PEGylated rhDNase provided a similar therapeutic activity as 1 daily dose of rhDNase during 5 consecutive days in the transgenic β-ENaC mouse model of the CF lung disease[17]. Along with this, PEGylated rhDNase was stable to jet nebulization and single or multiple administrations of high doses of PEGylated rhDNase over a period of up to three months did not cause any significant pulmonary or systemic toxicity in mice.

2. Results

2.1. PEGylation of rhDNase considerably prolongs its time of residence in the murine lungs

In a first step, we assessed the extension of the residence time of rhDNase in the lungs that could be provided by conjugation to PEG. For that purpose, we delivered 5 µg of PEGylated biotin-labelled rhDNase or the unconjugated biotin-labelled enzyme to the respiratory tract of mice by intratracheal instillation. We then measured rhDNase content in nasal lavage (NAL), bronchoalveolar lavage (BAL) and lung homogenate by ELISA on streptavidin-coated plates at different time points after delivery. Three different PEGylated rhDNase proteins varying in PEG size and architecture were studied: rhDNase conjugated to linear 20kDa PEG (PEG20-rhDNase), linear 30kDa PEG (PEG30-rhDNase) or two-armed 40kDa PEG (PEG40-rhDNase). These molecular weights were selected from high molecular weight PEGs already used clinically[12] with the objective of obtaining a maximum effect of PEG on extension of the residence time of rhDNase in the lungs.

Conjugation of rhDNase to PEG markedly prolonged the residence time of the enzyme in the lungs (Figure 1). Whereas the unconjugated protein was cleared from the lungs within a day, the PEGylated versions of rhDNase were present in the lungs for up to 20 days post-delivery. The residence time of PEGylated rhDNase in the lungs depended on the PEG size: the larger the PEG, the more prolonged rhDNase presence in the lungs. Three days post-delivery, the percentage of the delivered dose that remained present in the lungs was 0%, 28%, 35% and 38% for rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase, respectively. Figures 1B and 1C present the detailed data on the content of rhDNase in the BAL and lung tissue over time. Immediately after delivery to the lungs, all the proteins were mainly found in BAL rather than lung homogenate. However, from day 3, the PEGylated proteins were solely found in lung homogenates. The nasal cavity did not retain any of the proteins beyond 24 h (Supplementary Figure 1).

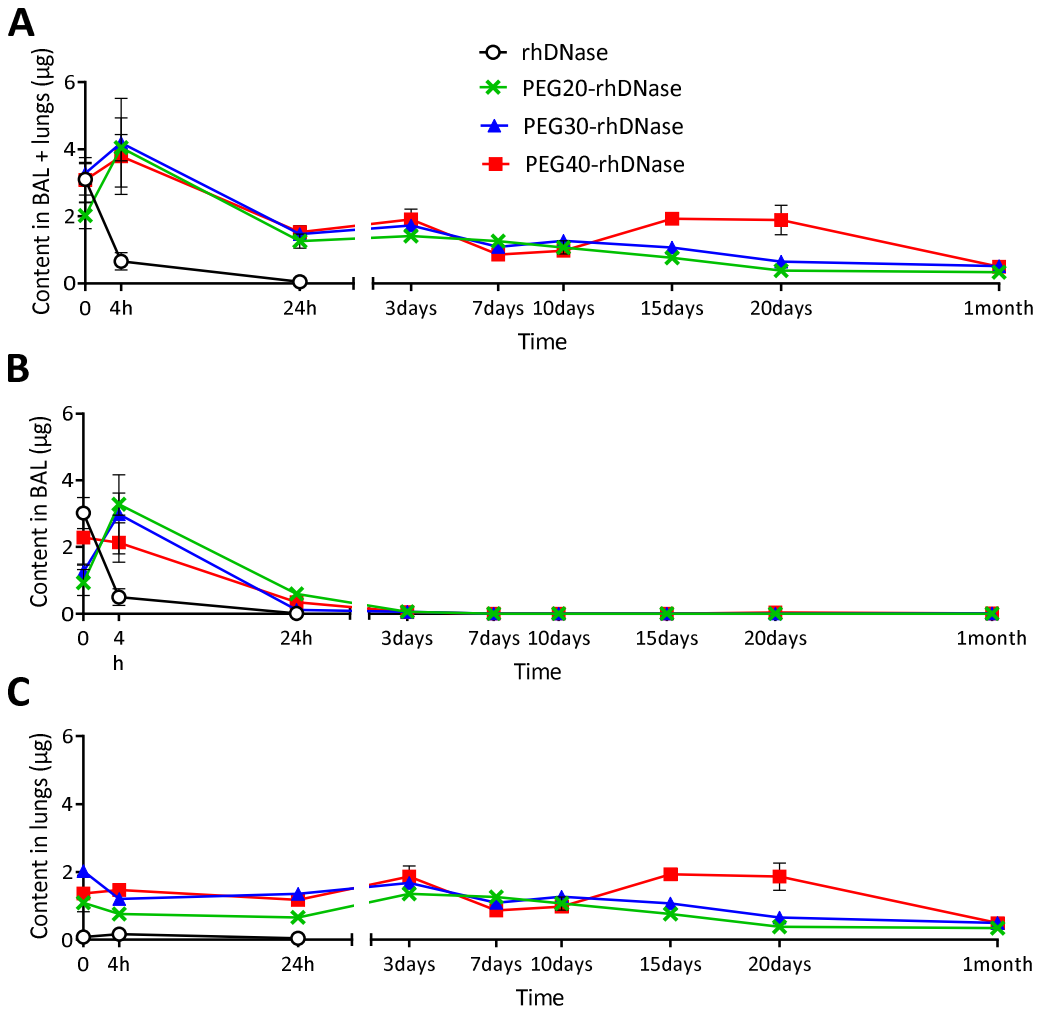


Figure 1. Quantities of biotin-labelled rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase recovered from the respiratory tract of NMRI mice at different time points following the intratracheal delivery of a 5 µg (0.14 nmol) biotin-labelled rhDNase dose. The PEG was not counted in the mass delivered and recovered from the respiratory tract. Therefore, the different mouse groups received the same number of moles of rhDNase compounds. **A.** bronchoalveolar lavage (BAL) + supernatant of lung homogenate (lungs), **B.** BAL and **C.** lungs. The data represent the mean values (± SEM) of 5 to 10 mice per time point.

2.2. PEGylated rhDNase remains active in the lungs for up to 20 days

To verify whether the increased residence time of the PEGylated conjugates in murine lungs is associated with a sustained enzymatic activity over time, we assessed the ability of the PEGylated

enzymes recovered from lung homogenates to degrade DNA in solution using rheology. A DNA solution of 0.75 mg/ml presents a viscosity of 2 mPa.s at 37°C. When this DNA solution is mixed with rhDNase, its viscosity rapidly decreases due to DNA cleavage (Supplementary Figure 2A). It should be noted that biotin labelling did not alter rhDNase enzymatic activity (Supplementary Figure 3). Based on the rhDNase concentrations obtained by ELISA, lung homogenates obtained at different time points and from different conjugates were diluted to a final rhDNase concentration of 0.25 µg/ml in the DNA solution. In this way, the activity of the different rhDNase conjugates could be compared and the loss of activity of each conjugate over time could be visualized.

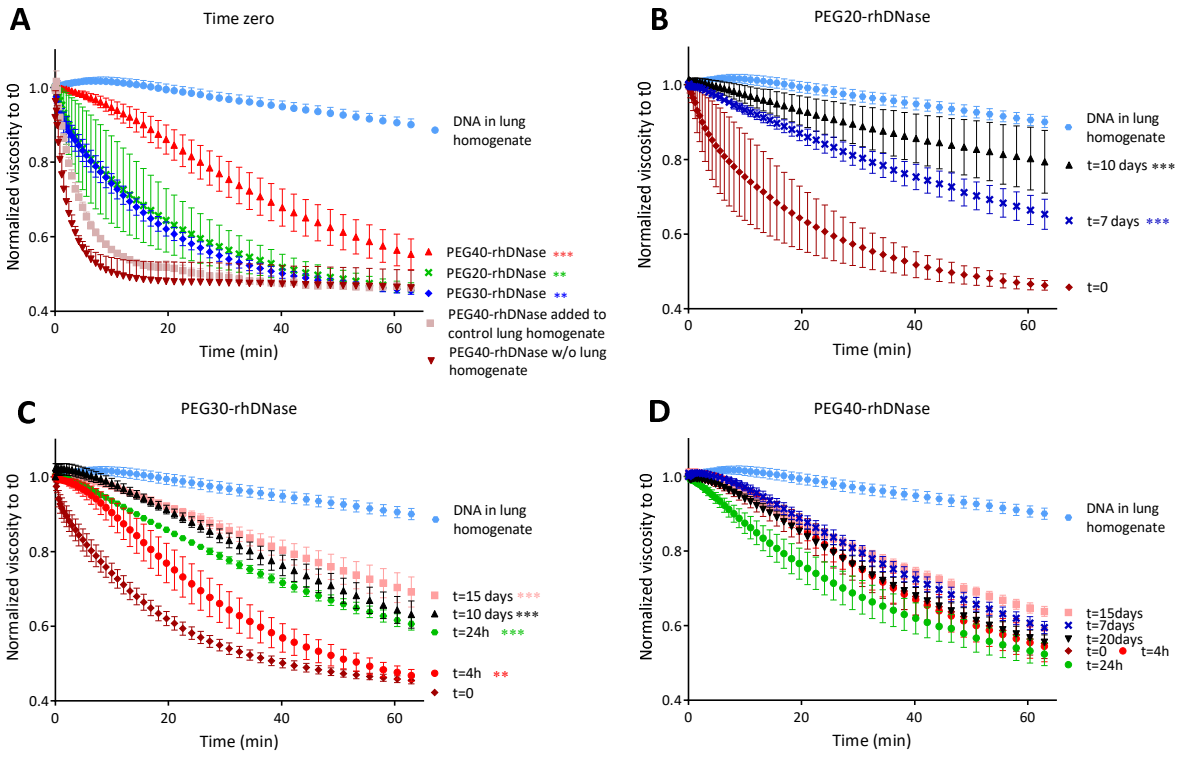


Figure 2. Activity assay of PEGylated biotin-labelled rhDNase compounds retrieved from lung homogenates using rheology of a DNA solution. (**A**) Activity of rhDNases retrieved from lung homogenates immediately after delivery compared with activity of PEG40-rhDNase added or not added to lung homogenate of control mice. Activity of PEG20-rhDNase (**B**), PEG30-rhDNase (**C**) and PEG40-rhDNase (**D**) retrieved from lung homogenates at different time points after delivery. In all rheology measurements, the concentrations of PEGylated rhDNases were adjusted to 0.25 µg/ml (without counting the PEG in the concentration) and the concentration of salmon testes DNA was 0.75 mg/ml. Each viscosity *vs*

time curve was normalized to its time zero viscosity. The data represent the mean values (± SEM) of 3 to 10 mice per time point and were compared using two-way ANOVA, Bonferroni post-test (*** $p < 0.001$, ** $p < 0.01$). The viscosity *vs* time curve of each protein retrieved from lung homogenates at time zero was compared to the curve of PEG40-rhDNase added to control lung homogenates (**A**). For each PEGylated protein, viscosity vs time curves were compared to the curve at time zero (**B**, **C** and **D**).

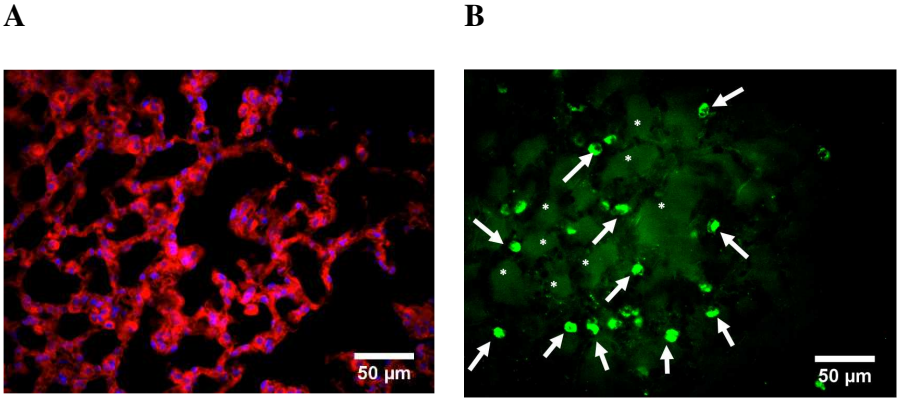
Figure 2A shows that each PEGylated rhDNase recovered from the lungs immediately after delivery (time zero) had lost part of its activity, whatever the PEG size, compared with PEGylated rhDNase that had not been delivered to the lungs. PEG40-rhDNase recovered from the lungs immediately after delivery decreased the least the viscosity of the DNA solution compared with PEG20-rhDNase and PEG30-rhDNase. Of note, before delivery to the lungs, each PEGylated rhDNase presented the same activity as unconjugated rhDNase in this assay (Supplementary Figure 2A).To check whether components of lung homogenates could decrease rhDNase activity, we measured the ability of PEGylated rhDNase to decrease the viscosity of a DNA solution in presence or absence of lung homogenate. The impact of lung homogenate on PEGylated rhDNase activity was minimal (Figure 2A).

Because the concentration of native rhDNase in lung homogenates was too low, its activity could not be evaluated in this assay. However, the concentrations of rhDNase and PEGylated molecules in BAL at time zero were all above 0.25 µg/ml and we thus also measured their activity in BAL (Supplementary Figure 4). PEGylated rhDNase compounds were all more active in BAL than in lung homogenate. PEG40-rhDNase was the least active in BAL as it was in lung homogenates. PEG20-rhDNase and PEG30-rhDNase presented the same activity and they were the most active in BAL as they were in lung homogenates. Unconjugated rhDNase presented an intermediate activity between that of PEG40-rhDNase and that of PEG20-rhDNase/PEG30-rhDNase.

PEG20-rhDNase and PEG30-rhDNase recovered from lung homogenates progressively lost their activity over time after delivery to the lungs (Figures 2B & 2C). However, PEG40-rhDNase retrieved from lung homogenates remained as active at later time points as at time zero (Figure 2D).

2.3. PEGylated rhDNase is mainly retained within the lung lumen rather than the lung parenchyma

High concentrations of DNA, the therapeutic target of rhDNase, are present in the respiratory secretions of patients with CF. Therefore, the mucolytic should remain within the lung lumen as long as possible to exert its therapeutic activity. However, from day 3, significant levels of PEG-rhDNase conjugates could only be measured in lung homogenates rather than BAL using ELISA (Figure 1). This means either that the PEGylated protein crossed lung epithelia and penetrated the lung parenchyma or, that it was firmly attached to respiratory epithelia and was not detached during the lavage procedure. In order to clarify this question, confocal laser scanning microscopy studies were carried out to localize the protein conjugated to PEG in the pulmonary tissue. Figure 3 shows a confocal image of the murine lungs 4 days after delivery of Alexa-fluor 488 labeled PEG30-rhDNase. The green-labeled PEGylated protein was clearly visible in the lung airspaces while it could not be distinguished in the lung parenchyma, supporting the sustained presence of the PEGylated protein within the lung lumen.



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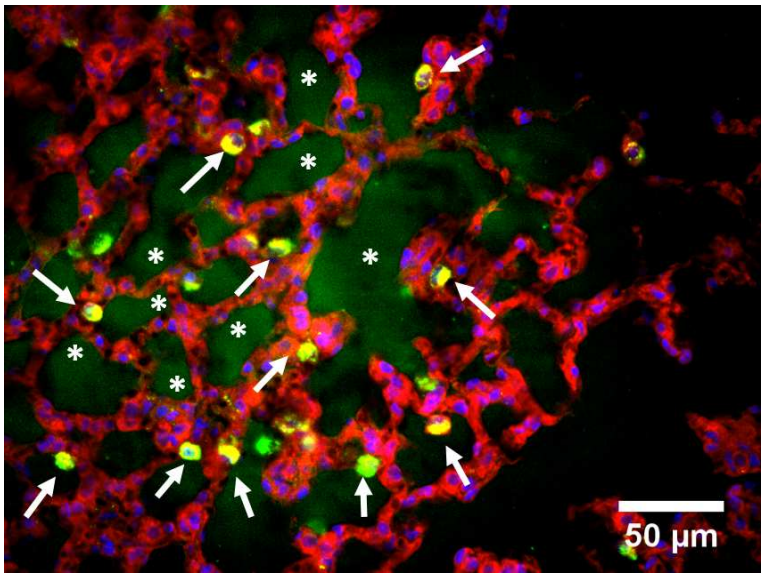

Figure 3. Localization of PEG30-rhDNase in mouse lungs by confocal imaging 4 days after intratracheal instillation. 37 µg (1 nmol) of Alexa488-PEG30-rhDNase (green) was administered to NMRI mice by intratracheal instillation (n=2). Four days later mice were sacrificed and lung slices were imaged with Cell Observer Spinning Disk (COSD, Zeiss, Germany). Images were recorded in green (Alexa488-PEG30-rhDNase), red (tissue, MitoTracker Red CMXRos), and blue (nuclei, Draq5). A) Lung section stained with red for tissue and blue (pseudo color) for nuclei; B) Alexa488-PEG30-rhDNase in green; C) merge of A and B: signal from Alexa488-PEG30-rhDNase is indicated by arrows in alveolar macrophages (AM) and stars in alveolar spaces. Images were processed by ImageJ (1.47v, NIH, USA). Scale bars are 50 µm.

2.4. One dose of PEG-rhDNase is as potent as 1 daily dose of rhDNase during 5 days to hydrolyze DNA in respiratory secretions of β-ENaC mice.

We evaluated the ability of conjugated *vs* non-conjugated rhDNase to reduce DNA content and to resolve pulmonary inflammation in β-ENaC mice. β-ENaC mice display increased Na^+ absorption and impaired water transport across airway epithelia as typically observed in human CF airways. They are characterized by airway mucus obstruction and chronic airway inflammation[17]. Our goal was to evaluate whether the administration frequency of PEGylated rhDNase could be reduced compared with unconjugated rhDNase in a murine model of the CF lung disease. Since rhDNase was cleared from the healthy murine lungs within 24h and PEGylated rhDNase remained for at least 15 days

(Figure 1), we delivered one dose of PEG30-rhDNase or PEG40-rhDNase to the lungs of β-ENaC mice and compared the therapeutic efficacy of this single dose 5 days later to that of rhDNase delivered every day during the same period of time (Figure 4A). The unit dose delivered per mouse for each of the rhDNase compounds was 10 µg or 435 µg/kg, which is approximately 10-times the human unit dose of 2.5 mg or 42 µg/kg (considering an average body weight of 60 kg for patients with CF[18]). Cellular markers of inflammation, cytokines and DNA were quantified in BAL at day 5.

As can be seen in Figure 4, β-ENaC mice presented a chronic neutrophil inflammation in their lungs. Neutrophils represented 20% of the cell population in BAL of β-ENaC mice while they represented 1% of the cell population in the BAL of wild-type (WT) normal homozygous littermates (Figures 4B & 4C). As a direct consequence of neutrophils presence in airways, the DNA content in BAL was increased in β-ENaC mice compared with WT littermates (Figures 4D & 4E). The lung levels of IL-6 and chemokine ligand 2 (CCL2) were increased in β-ENaC mice as well (Supplementary Figure 5).

The three rhDNase compounds tested significantly reduced DNA levels in BAL of β-ENaC mice while they had no effect in WT (Figures 4D & 4E). 328 ng/mL of DNA was found in the phosphate-buffered saline (PBS) group whereas this concentration decreased to 117 ng/ml, 150 ng/ml and 161 ng/ml for the rhDNase, PEG30-rhDNase and PEG40-rhDNase groups, respectively. One dose of PEGylated rhDNase thus provided a similar efficacy as 1 daily dose of rhDNase during 5 consecutive days to hydrolyze DNA in the BAL of β-ENaC mice. However, none of the rhDNase compounds had an impact on cellular inflammation markers and cytokines in BAL (Figures 4B-C & Supplementary Figure 5).

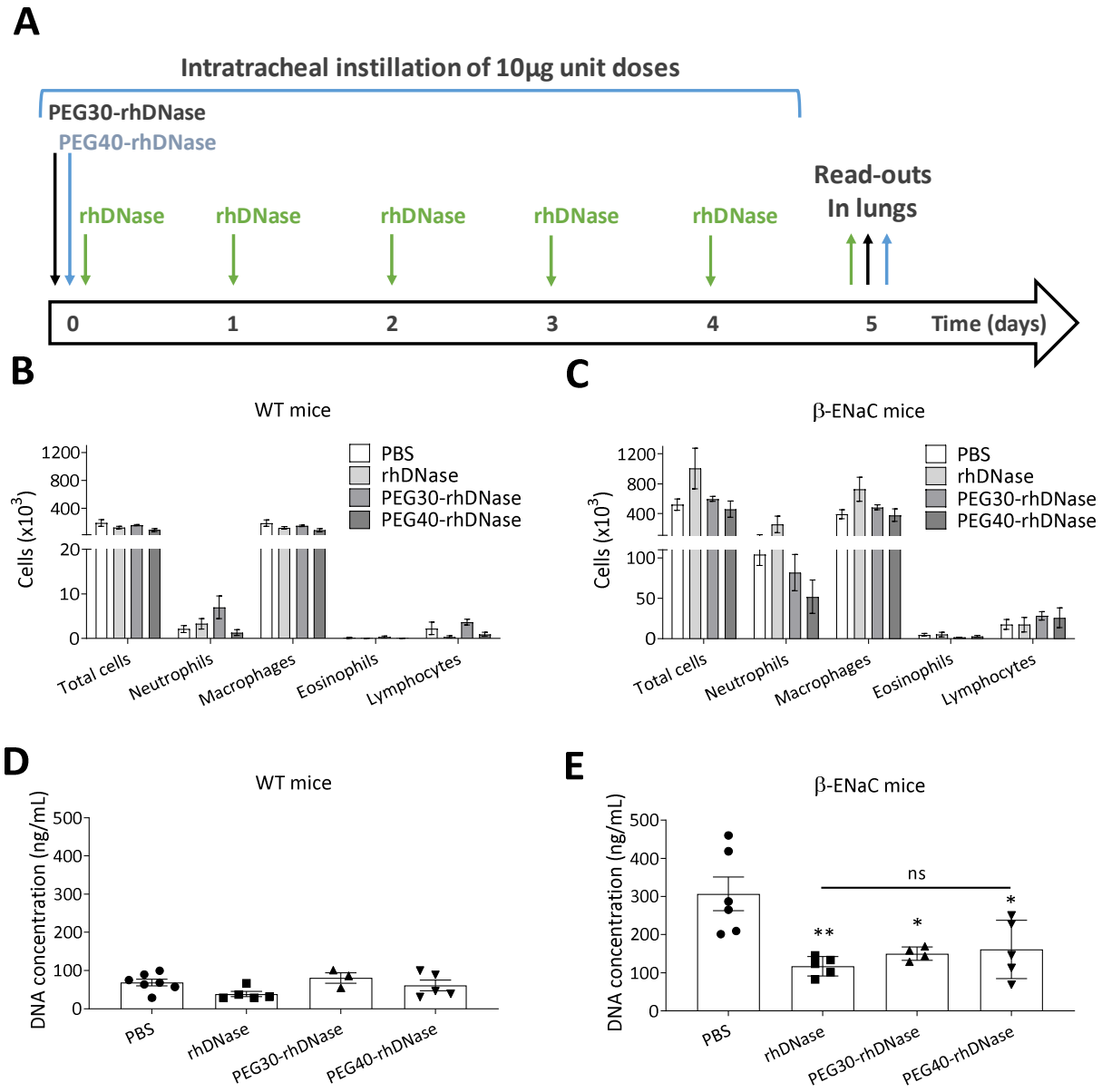

Figure 4. A. Timeline of the short-term therapeutic efficacy study in β-ENaC mice. One 10µg unit dose of PEG30-rhDNase or PEG40-rhDNase was intratracheally instilled in the lungs of β-ENaC mice and wild-type (WT) littermates and the therapeutic efficacy of this single dose was compared 5 days later to that of rhDNase delivered every day during the same period of time. The PEG was not counted in the mass of rhDNase compounds delivered. Control mice were intratracheally instilled with phosphate buffer saline (PBS) every day as were the PEG30-rhDNase and PEG40-rhDNase groups the days that followed PEG-rhDNase dosing. Cellular markers found in bronchoalveolar lavage (BAL) of WT (**B**) and β-ENaC (**C**) mice at day 5. DNA content in BAL in WT (**D**) and β-ENaC (**E**) mice at day 5. Data are expressed as mean values (± SEM) of 4 to 7 mice per experimental group and were compared using one-way ANOVA, Tukey's Multiple Comparison Test (* $p < 0.05$ and ** $p < 0.01$ vs the respective PBS group; ns, not significant).

The mice from all the groups, including the PBS control group, lost between 4% and 6% of their initial body weight during the course of the experiment (Supplementary Figure 5). This weight loss indicates that β-ENaC as well as WT mice slightly suffered from the everyday manipulation.

2.5. Once a week administration of PEG-rhDNase over 4 weeks results in lasting therapeutic efficacy

It has previously been suggested that rhDNase might present indirect anti-inflammatory properties[19]. Although rhDNase is primarily a mucolytic agent as it breaks down polymerized DNA, it might also indirectly reduce lung inflammation by improving mucociliary clearance. A potential indirect anti-inflammatory effect of PEGylated rhDNase will probably require a long treatment duration before becoming apparent in β-ENaC mice. Therefore, we performed an additional long-term therapeutic efficacy study in which PEGylated rhDNase was delivered once a week over 4 weeks to β-ENaC mice and WT littermates. As in the 5 days study, the readouts included DNA, cytokines and cellular markers of inflammation in BAL. They were analyzed one week after the last dosing (Figure 5A).

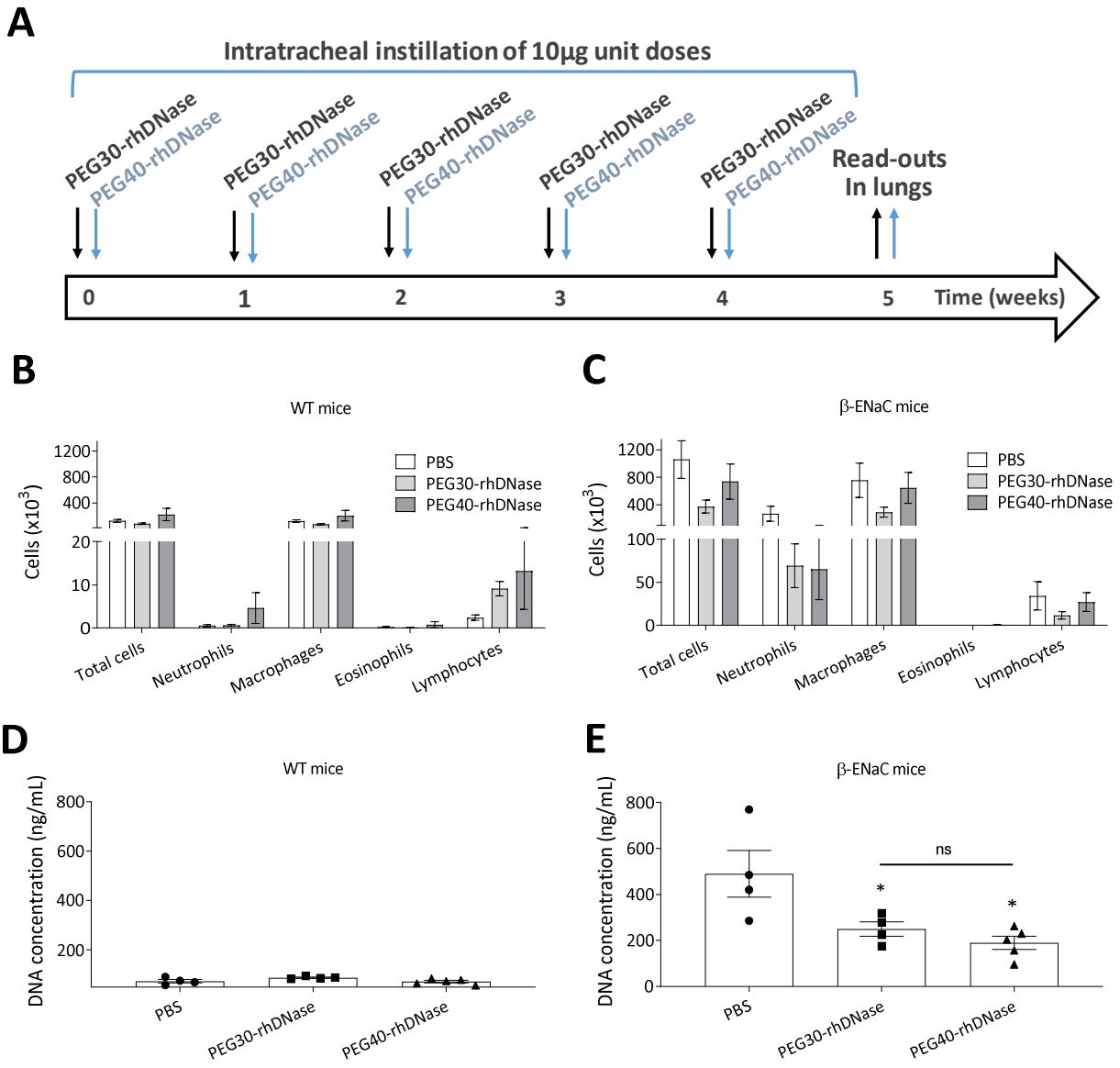

Figure 5. A. Timeline of the long-term therapeutic efficacy study in β-ENaC mice. One 10µg unit dose of PEG30-rhDNase or PEG40-rhDNase was intratracheally instilled in the lungs of β-ENaC mice and wild-type (WT) littermates every week during 4 weeks and the therapeutic efficacy of this once a week dosing was assessed 1 week after the last dose. The PEG was not counted in the mass of PEGylated rhDNase compounds delivered. Control mice were intratracheally instilled with phosphate buffer saline (PBS) every week. Cellular markers found in bronchoalveolar lavage (BAL) of WT (**B**) and β-ENaC (**C**) mice at week 5. DNA content in BAL in WT (**D**) and β-ENaC (**E**) mice at week 5. Data are expressed as mean values (± SEM) of 4 to 5 mice per experimental group and were compared using one-way ANOVA, Tukey's Multiple Comparison Test (* $p < 0.05$ vs the respective PBS group; ns, not significant).

DNA analysis in BAL of β-ENaC mice showed similar activity of PEG30-rhDNase and PEG40-rhDNase (Figure 5). A DNA concentration of 490 ng/ml was found in the BAL of the PBS group whereas this concentration decreased to 250 ng/ml and 190 ng/ml in the mouse groups treated once weekly with PEG30-rhDNase and PEG40-rhDNase, respectively (Figure 5E). Unlike β-ENaC mice, WT mice contained very low quantities of DNA in their lung lumen (less than 90 ng/ml; Figure 5D) and PEGylated rhDNase had no effect on these DNA levels.

Neutrophils counts decreased 4-times in β-ENaC mice following the 4-week treatment with PEG30-rhDNase or PEG40-rhDNase (Figure 5C). However, the large variability between mice prevented these decreases to be significant. Quantification of pro-inflammatory (IL-6, CCL2) cytokine markers in β-ENaC mice showed no significant difference among groups (Supplementary Figure 6). Mice from all the groups gained between 2% and 11% of their initial weight at the end of the 5 weeks experiment (Supplementary Figure 6).

2.6. PEGylated rhDNase is stable to jet nebulization

Since the mode of rhDNase administration to patients involves inhalation of a nebulized solution, we evaluated the stability of PEGylated rhDNase to nebulization. The biological activity of rhDNase and its PEGylated conjugates was determined after nebulization with an air-jet nebulizer using the methyl green assay. Jet nebulization did not result in any loss of activity of either native or, PEGylated rhDNase compounds (Figure 6 & Supplementary Figure 7). The curves of methyl green absorbance vs enzyme concentration before and after nebulization were superimposed for each compound. In addition, the specific activity after nebulization was calculated relative to native or PEGylated rhDNase before nebulization. There was no significant loss in enzymatic activity for any of the nebulized proteins (Figure 6 & Supplementary Figure 7).

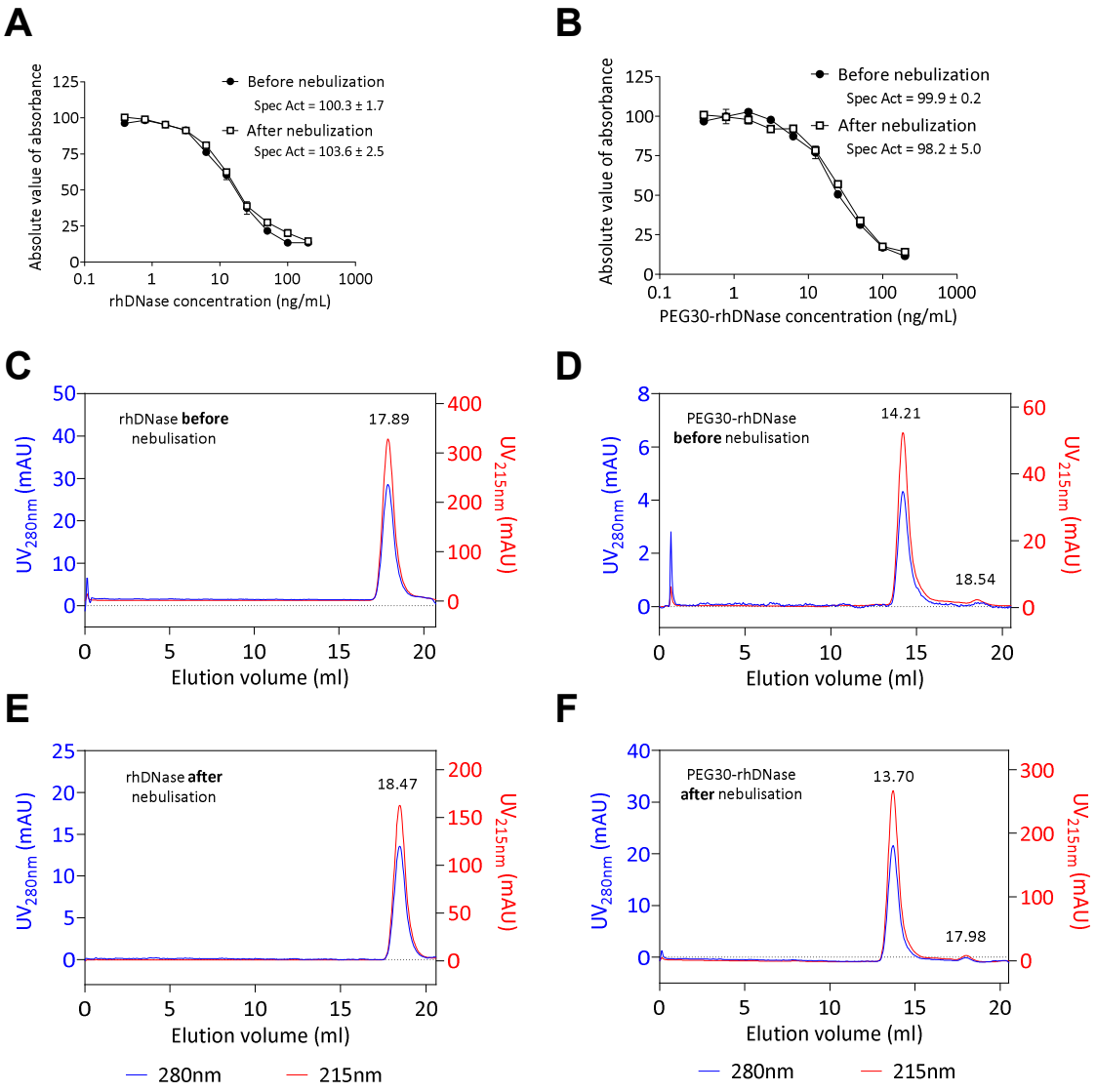

Figure 6. Evaluation of the stability of rhDNase (**A, C, E**) and PEG30-rhDNase (**B, D, F**) to nebulization. (**A-B**) Hydrolysis of DNA by rhDNase (**A**) and PEG30-rhDNase (**B**) was assessed using the methyl green activity assay. Data are expressed as % specific activity (± SEM) relative to the sample before nebulization, i.e., concentration of protein determined by activity assay divided by that based on UV absorption of the sample (n=3). Specific activities before and after nebulization were compared using an unpaired t-test and showed no loss of activity ($p > 0.05$). (**C-F**) Size-exclusion chromatography analysis to assess potential protein aggregation following jet nebulization of rhDNase (before, **C**, and after, **E**, nebulization) and PEG30-rhDNase (before, **D**, and after, **F**, nebulization). No additional peaks appeared at elution volumes smaller than native proteins, indicating the absence of aggregates.

The potential aggregation of rhDNase and PEGylated rhDNase caused by jet nebulization was monitored by measuring the absorbance at 350 nm and by size-exclusion chromatography (SEC). The absorbance at 350 nm of all rhDNase solutions recovered after nebulization was zero, showing the absence of any insoluble aggregates (Supplementary Table 1). SEC analysis prior to nebulization showed a peak of rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase at retention volumes of 17.9 ml, 15.1 ml, 14.2 ml and 13.6 ml, respectively (Figure 6 and supplementary Figure 7). After nebulization, no additional peak with lower retention volume than that of non-nebulized rhDNases was observed on SEC chromatograms for all proteins, indicating that no soluble aggregate was formed (Figures 6C-F, Supplementary Figure 7). Therefore, PEGylated rhDNase, like rhDNase, appeared stable to jet nebulization.

2.7. PEGylated rhDNase does not cause any lung toxicity after acute single-dose or repeat-dose administration.

Preclinical and clinical studies have demonstrated that rhDNase is a safe drug with rare adverse events[9, 20]. In order to investigate whether PEGylated rhDNase has a similar safety profile, a single-dose study and two repeat-dose toxicity studies of different durations were performed in healthy Swiss mice.

In our acute toxicity study, a dose of either 100 µg of rhDNase or PEG30-rhDNase (4 mg kg^{-1}) was delivered in a single administration via intratracheal instillation. A control group was dosed with drug vehicle formulation. An equal number of mice (6/group) was sacrificed on day 1 and day 14 after exposure for assessment of test article-related toxicity. Based on the lower respiratory tract deposition of PEGylated rhDNase obtained in our previous experiments, the delivered dose of 100 µg per mouse was estimated to result in a deposited dose of 0.25 mg/g lung weight, thereby representing an approximate safety margin of 50 times the Phase III clinical high-dose of 5 mg day-

[1] (supplementary Table 2)[20]. Moreover, this 100 µg dose corresponds to 10 times the therapeutic dose administered to β-ENaC mice in our therapeutic efficacy study.

In general, PEG30-rhDNase was found to be well tolerated and can be considered as essentially non-toxic following a single high-dose administration in mice. No evidence of respiratory distress or effects on clinical observations, body weight or organ weight were observed throughout this study (Figure 7 and supplementary Figure 8). Hematology analysis did not reveal any sign of an acute systemic inflammatory response to treatment with native or PEGylated rhDNase. Furthermore, no sign of an acute pulmonary inflammatory response was detected, neither 1 day nor 14 days after exposure. Indeed, the integrity of the lung cells and of the alveolar-capillary membrane, assessed by measuring respectively lactate dehydrogenase (LDH) activity and protein levels in BAL, were not affected (Figures 7A & 7B). In line with this, no significant difference in total BAL cell counts or in the absolute number of different types of BAL leukocytes were observed (Table 1), indicating that acute administration of native and PEGylated rhDNase does not result in an inflammatory cell infiltration in the lung tissue. With the exception of localized accumulation of alveolar macrophages in 1 of 5 drug vehicle-, 1 of 6 rhDNase- and 3 of 6 PEG30-rhDNase-treated animals sacrificed after 1 day, histological examination of the lung tissue did not reveal any evidence of a significant inflammatory response or tissue damage in response to native or PEGylated rhDNase (Figure 7C). After the recovery period of 14 days, the increased localized number of alveolar macrophages was no longer observed in mice treated with PEG30-rhDNase, although this increase persisted in 1 of 6 drug vehicle- and 1 of 5 rhDNase-treated mice.

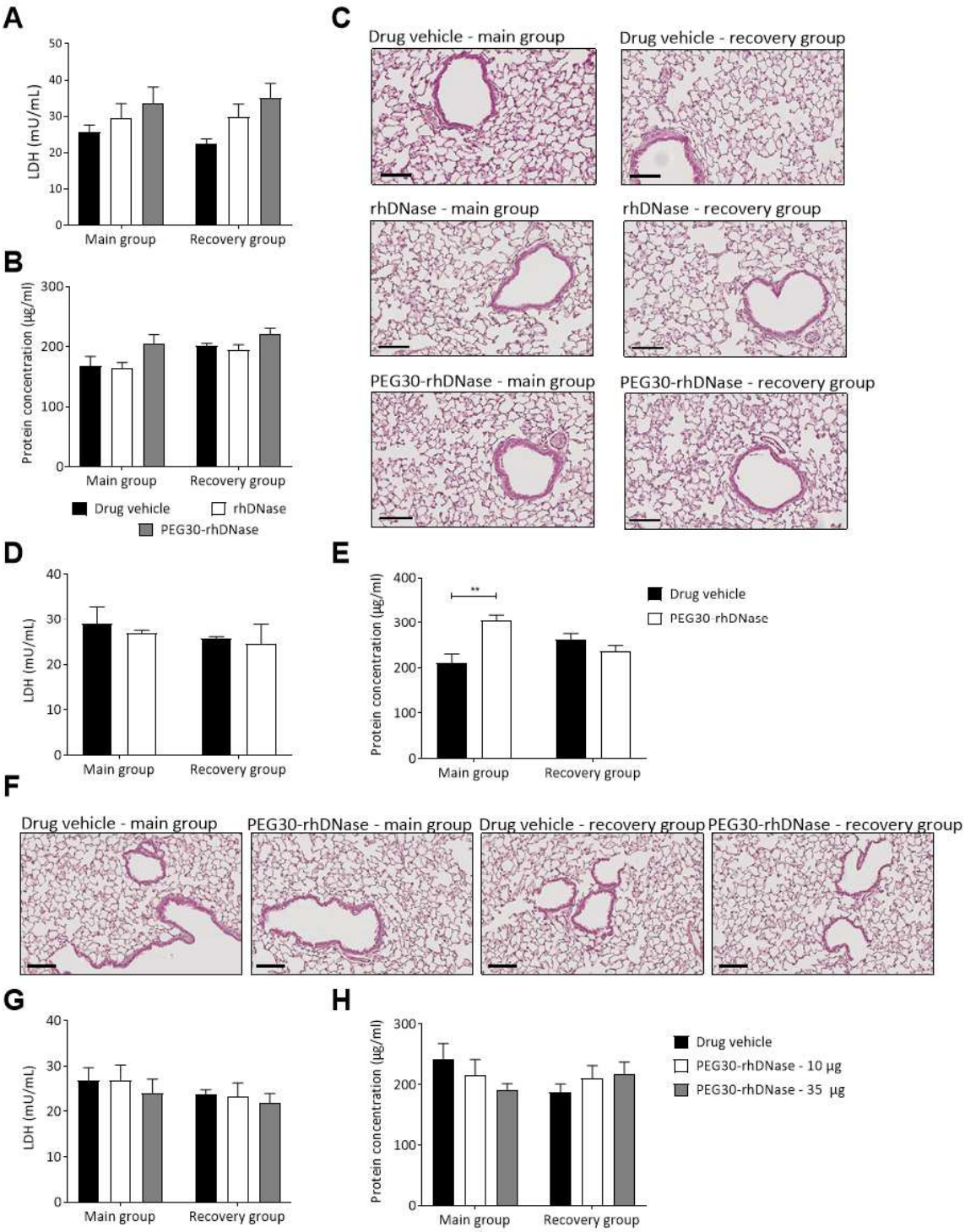

Figure 7. *In vivo* assessment of toxicity after a single-dose (**A**-**C**), sub-chronic (**D**-**F**) or chronic (**G**-**H**) dosing regimen in mice. Measurement of lactate dehydrogenase (LDH) activity (A, D, G) and protein concentration (**B**, **E**, **H**) in BAL fluid from main and recovery group mice. In main groups, mice were sacrificed 1 day after the last dosing and, in recovery groups, mice were sacrificed 14 (single-dose study) or 28 days (sub-chronic and chronic studies) after the last dosing. Data are presented as mean ± SEM of 3 to 7 mice per experimental group. Two-way ANOVA with Bonferroni multiple comparison post-test was used to assess statistical differences between experimental groups as well as between study

groups (main vs recovery, ** $p < 0.01$). Representative microphotographs of lung tissue stained with haematoxylin-eosin (**C**, **F**). Magnification 200x. Scale bar: 100 µm.

The sub-chronic toxicity profile of PEGylated rhDNase was investigated by delivering a dose of 100 µg of PEG30-rhDNase once a week via intratracheal instillation and this for four doses in total. A control group was exposed in a similar way to the drug vehicle. Up to 6 animals per group were sacrificed 1 day after the last administration and up to 4 animals per group after an additional 4-week treatment-free recovery period. No test-article related mortalities or changes in clinical observations, organ weight or body weight measurements were observed during and after the treatment period (Supplementary Figure 9). Although the concentration of proteins in BAL fluids was significantly higher in animals treated with PEG30-rhDNase as compared to the control, this increase was not accompanied by an increase in LDH activity or a change in BAL cells counts. In addition, the difference in protein concentration between treatment and control groups was reversible (Figure 7E). No significant pulmonary histologic lesions were observed after sub-chronic intratracheal administration of PEG30-rhDNase (Figure 7F). Hematology analysis did not show any sign of a systemic inflammatory response that could be the result of lung injury induced by PEG30-rhDNase treatment (Supplementary Figure 9).

Table 1: Cellular distribution in BAL from mice of the toxicity studies

Day	Treatment	Total cells	Macrophages	Neutrophils	Lymphocytes	Eosinophils
Single-dose toxicity study						
1	Drug vehicle	121 ± 15	114 ± 14	3.5 ± 1.1	2.8 ± 0.6	0.4 ± 0.2
	rhDNase	171 ±16	164 ± 15	3.0 ± 0.8	3.9 ± 0.6	0.2 ± 0.1
	PEG30-rhDNase	139 ± 27	134 ± 26	1.5 ± 0.3	3.5 ± 0.9	0.4 ± 0.2
14	Drug vehicle	102 ± 35	94 ± 32	4.1 ± 1.6	3.8 ± 1.9	0.3 ± 0.1
	rhDNase	144 ± 45	136 ± 43	4.1 ± 1.2	3.6 ± 0.7	0.4 ± 0.2

	PEG30-rhDNase	136 ± 22	129 ± 21	2.3 ± 0.6	4.6 ± 1.2	0.4 ± 0.1
Sub-chronic toxicity study						
1	Drug vehicle	75 ± 26	71 ± 25	1.1 ± 0.4	3.2 ± 0.8	0.06 ± 0.05
	PEG30-rhDNase	99 ± 25	93 ± 24	0.6 ± 0.2	5.2 ± 1.2	0.21 ± 0.03
28	Drug vehicle	117.1 ± 38.4	106.8 ± 35.3	1.91 ± 0.51	4.97 ± 1.49	0.11 ± 0.06
	PEG30-rhDNase	98.8 ± 11.5	89.9 ± 10.7	1.31 ± 0.24	4.15 ± 0.34	0.07 ± 0.04
Chronic toxicity study						
1	Drug vehicle	106 ± 16	101 ± 15	0.7 ± 0.1	4.1 ± 0.7	0.27 ± 0.06
	PEG30-rhDNase - 10 µg	134 ± 40	128 ± 39	0.9 ± 0.4	4.9 ± 1.5	0.27 ± 0.13
	PEG30-rhDNase - 35 µg	144 ± 36	136 ± 33	1.0 ± 0.5	7.3 ± 2.5	0.26 ± 0.13
28	Drug vehicle	58 ± 5	54 ± 4	1.7 ± 0.5	1.8 ± 0.3	0.21 ± 0.08
	PEG30-rhDNase - 10 µg	89 ± 13	83 ± 13	3.3 ± 0.8*	2.6 ± 0.4	0.21 ± 0.06
	PEG30-rhDNase - 35 µg	100 ± 13	95 ± 12	1.1 ± 0.2	3.5 ± 0.6	0.16 ± 0.07

Cell numbers are listed as actual numbers (x 10^3) and results are given as mean ± SEM of 3 to 7 mice per experimental group. Two-way ANOVA with Bonferroni multiple comparison post-test was used to assess statistical differences between experimental groups as well as between study groups. Asterisk (* $p < 0.05$) refers to statistical significance of the group with other main groups (1 day) and with PEG30-rhDNase - 35 µg of recovery group (28 days).

To assess whether a long-term repeated exposure to PEGylated rhDNase was associated with the appearance of adverse toxicological effects, a chronic toxicity study was performed. In this study, PEG30-rhDNase or drug vehicle was administered once a week for 12 consecutive weeks to three groups of mice (≥ 12/group): a control group receiving drug vehicle, a low dose group receiving 10 µg protein and a high dose group receiving 35 µg protein. Half of the mice of each group were sacrificed 1 day or 28 days after administration of the final dose, and local as well as systemic toxic effects were evaluated. Throughout the chronic exposure study, no evidence of respiratory distress or changes in behavior became apparent. Furthermore, no sign indicating a major systemic

inflammatory response induced by PEG30-rhDNase treatment could be observed in animals necropsied after the 12-week exposure period or after the additional 4-week treatment-free recovery period. Indeed, no treatment-related effects on organ weights could be detected (Supplementary Figure 10). Still, it should be noted that high-dose mice displayed a 10% reduction in body weight gain as compared to the control group at the end of the 12-week treatment period. In addition, hematology analysis showed a significant but reversible increase in basophil counts for both treatment regimens. Potential pulmonary toxicity induced by chronic PEG30-rhDNase exposure was evaluated by assessing the cellular composition, LDH and protein levels in BAL fluids. As can be seen in Figure 7, treatment with PEGylated rhDNase did not have a significant effect on these parameters, neither 24 hours nor 28 days after exposure. While the low-dose recovery group showed a significant infiltration of neutrophils as compared to the low-dose group sacrificed 1 day after 12 weeks of exposure, the control or high-dose recovery groups did not display such a tendency (Table 1).

2.8. PEG does not accumulate in the lungs after repeated administrations over a three-month period

To verify whether local or systemic accumulation of PEG30 and/or rhDNase occurred following exposure to PEG30-rhDNase, the concentrations of the protein and PEG moieties were measured using ELISA in lung tissue, BAL fluid, alveolar macrophages and serum samples collected from mice used in the above described toxicity studies.

In agreement with Figure 1, unconjugated rhDNase was almost completely cleared from the lungs 24h post-exposure of a single dose since only approximately 1% of the administered dose could be recovered. In contrast, 10-fold higher concentration of PEGylated rhDNase could be measured in lung samples 24h after a single high-dose administration. Sub-chronic administration of 100 µg PEG30-rhDNase doubled the amount of protein that could be recovered from the lungs

as compared to the single dose administration study (Figure 8A). Still, almost no protein could be detected in recovery mice. In our chronic exposure study, lung concentrations of the rhDNase moiety were decreased to near baseline levels after a 28 days recovery period. Interestingly, the levels of the PEG30-moiety show a similar decrease over time in all our studies although its clearance from the lungs is somewhat delayed as compared to the rhDNase moiety. Whereas similar levels of PEG and rhDNase were recovered from BAL, much higher levels of PEG were detected in lung homogenates as compared to rhDNase (Supplementary Figure 11).

Confocal imaging of labeled PEG30-rhDNase clearly showed that a portion of the delivered protein was captured by alveolar macrophages (Figure 3). Therefore, we quantified the amount of PEG30 and rhDNase moieties contained inside alveolar macrophages. As can be seen in Figure 8C and 8D, both the rhDNase moiety and the PEG30 moiety of PEG30-rhDNase could clearly be detected, although at low levels, in alveolar macrophage samples obtained 24h post-exposure in the single-dose study as well as in the two repeat-dose studies. However, while no measurable rhDNase concentrations could be obtained in any of the recovery groups, the PEG30 moiety could still be detected although at a lower level than 24h post-exposure. The amount of non-PEGylated rhDNase recovered from alveolar macrophages 24h after a single administration was significantly decreased as compared to its PEGylated counterpart.

Measurable serum concentrations of rhDNase were obtained 24h after a single high-dose administration. However, no detectable amounts of the rhDNase-moiety of PEG30-rhDNase could be detected in the single-dose study nor, in the multi-dose toxicity studies (Figure 8E). Low systemic absorption of the PEG30-moiety could be observed in the single-dose and repeat-dose toxicity studies. The sub-chronic dosing regimen resulted in a significant but reversible systemic accumulation of PEG30. Furthermore, PEG30 serum levels were significantly increased in high-dose mice of the chronic exposure study as compared to the low-dose group. This effect was again reversible after a treatment-free recovery period (Figure 8F).

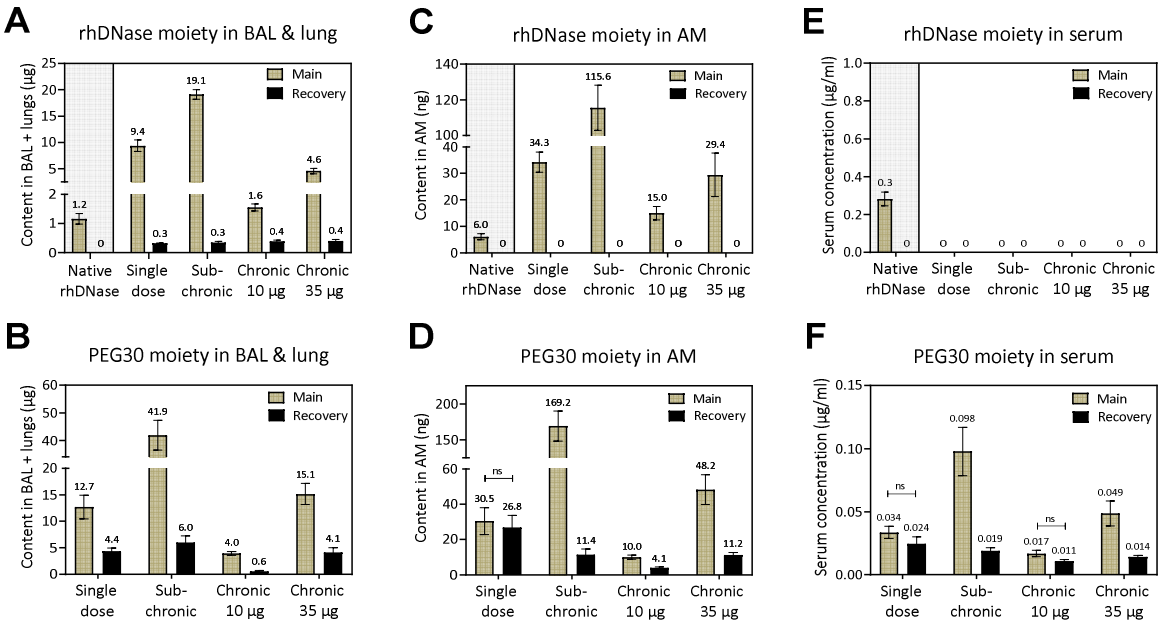

Figure 8. Quantification of the rhDNase and PEG moieties of PEG30-rhDNase measured in BAL fluid, lung homogenates, alveolar macrophages (AM) and serum samples of the toxicity studies using ELISA. The data represent mean values of 3 to 7 mice per experimental group and error bars indicate SEM. Main *vs* recovery group quantities were compared using multiple unpaired t-tests. Unless indicated otherwise, a significant difference was observed between main and recovery group. Not significant, ns.

3. Discussion

Patients with CF have to deal with an enormous daily therapy burden. Therefore, there is a significant need for new symptomatic treatment options that are more convenient and improve patient adherence. Among symptomatic therapies, Pulmozyme™ is a gold-standard but its daily administration contributes to the large therapy burden of patients with CF. Here, we present a PEGylated long-acting version of the mucolytic that could be delivered once a week, instead of once or twice a day. By reducing the administration frequency of rhDNase, patients will benefit from an improved quality of life. We can also expect an increased patient adherence and thereby an improved outcome of the therapy and a prolonged life expectancy.

PEG attachment to rhDNase resulted in an impressive extension of its residence time (≥ 15 days) in the lungs of mice (Figure 1). Such an improvement in residence time has not been observed previously for any PEGylated protein delivered to the lungs in preclinical models. PEGylation of recombinant human alpha1-antitrypsine with a 20kDa PEG sustained the presence of the conjugate in the lungs of mice for 48h, whereas the non-PEGylated counterpart was cleared within 24h[13]. PEGylation of an anti-IL-17A Fab' antibody fragment with 2-armed 40kDa PEG increased its residence time in the lungs of mice, rats and rabbits[21]. Following intratracheal administration, the unconjugated Fab' was cleared from the lungs within 24h while large quantities of the PEGylated Fab' remained present up to 48h. Interferon (IFN)α2b conjugated to linear 12kDa PEG (Pegintron®) was better retained in the lungs of rats following intratracheal instillation than unconjugated IFNα2b (IntronA®)[22]. Eight hours after delivery, 54% of the 12kDa PEG-IFNα2b dose were recovered from the lungs compared to 19% for IFNα2b. Thirty hours after delivery, negligible amounts of the two proteins were retrieved from the lungs. The exact mechanisms explaining the tremendous residence time of PEGylated rhDNase in the lungs remain to be determined. It might be related to the high physico-chemical stability of the native protein combined with additional protection conferred by PEG against clearance mechanisms in the lungs[23]. Conjugation to PEG doubles rhDNase molecular weight, thereby decreasing the transport of the enzyme across respiratory epithelia towards the bloodstream, as confirmed by our data (Figure 8). It has also been reported that PEGylation of proteins improves their resistance towards proteolysis[24]. Moreover, PEGylation has been suggested to decrease protein uptake by alveolar macrophages and respiratory epithelial cells[14, 25].

The larger the PEG attached to rhDNase, the longer the residence time of the active enzyme in the murine lungs. While PEG20-rhDNase and PEG30-rhDNase could be detected in the lungs up to 15 days post-delivery, PEG40-rhDNase was detected up to day 20 (Figure 1). In addition, PEG20-rhDNase lost most of its activity during its residency in the lungs while PEG40-rhDNase was as active in the lungs right after delivery as at day 20 post-delivery (Figure 2). Interestingly, Gursahani *et al.*

measured the residence time of 0.55 to 20kDa PEG alone in the lungs of rats and showed that the residence time correlated with the size of PEG as well[26]. The PEG quantity retrieved in BAL decreased exponentially over time and PEGs ≤5kDa were mostly cleared within 2 days whereas small quantities of 20kDa PEG were still present in the lavage at day 3. Measurement of PEG concentrations in lung homogenates indicated that very small PEGs (≤ 1kDa) were totally cleared from the lung tissue within 24h but that 10 to 15% of the delivered dose of PEG ≥ 2kDa remained associated with the lung tissue for at least 7 days. Different mechanisms could explain the impact of PEG size on the residence time of rhDNase in the lungs. First, as already stated above, there is an inverse relationship between the size of macromolecules and their absorption rate into the systemic circulation[27]. Second, rhDNase stability towards proteolysis in the lungs might increase with PEG length[28]. Yet, the activity of the PEGylated rhDNase compounds in lung homogenates was lower right after delivery compared with their activity before delivery and PEG40-rhDNase was the least active in lung homogenates. The components of lung homogenates had no major impact on PEGylated rhDNase activity (Figure 2A). Moreover, PEGylated rhDNase was more active in BAL than in lung homogenates retrieved immediately after delivery. Therefore, our hypothesis is that the procedure of lung homogenization and sample preparation might cause activity loss of PEGylated rhDNase compounds and PEG40-rhDNase might be the least stable during this procedure.

In line with Gursahani *et al.*, PEGylated rhDNase was mainly found in BAL rather than lung homogenate at early time points (up to 24h) and only in lung homogenate at later time points (from day 3; Figures 1A & 1B)[26]. Although this might suggest that PEGylated rhDNase had penetrated the lung parenchyma, confocal laser scanning microscopy studies rather indicated that the PEGylated enzyme was predominantly located in lung airspaces (Figure 3). The sustained presence of the PEGylated protein within the lung lumen is necessary to exert its mucolytic activity on respiratory secretions. This localization is also consistent with a limited transport across respiratory epithelia resulting from the large size of the PEGylated protein. The fact that the bronchoalveolar lavage

procedure could not detach PEGylated rhDNase from the lung tissue might result from the mucoadhesive character of PEG. We had previously shown similar behavior with PEGylated antibody fragments which were poorly retrieved by BAL. The addition of Triton, a surfactant that dismantles the mucus gel, to the lavage solution increased by two-fold the recovery rate of PEGylated antibody fragments[14].

One single dose of PEGylated rhDNase provided similar efficay as 1 daily dose of unconjugated rhDNase during 5 consecutive days to decrease the DNA content in BAL of β-ENaC mice (Figure 4). In addition, once a week administration of PEGylated rhDNase over 4 weeks decreased both the DNA content and neutrophils counts in BAL, although the decrease in neutrophil counts did not reach statistical significance (Figure 5). β-ENaC mice are characterized by airway mucus obstruction and chronic airway inflammation and have been proposed as a murine model of the CF airway disease[17]. β-ENaC mice mimic the increased Na^+ absorption and impaired water transport across airway epithelia that are observed in human CF airways. The ENaC activity is in fact negatively regulated by the CFTR protein and this control is lost in CF. β-ENaC mice are a good alternative to CF mice homozygous for the F508del mutation in the CFTR protein because CF mice do not exhibit the severe pathology of the established human CF lung disease[29]. The trend towards a decreased neutrophil counts that we observed in the long-term study in β-ENaC mice is in good agreement with a 3-year clinical trial on Pulmozyme™[19]. The percentage of neutrophils, elastase activities and IL-8 concentrations in BAL fluid significantly increased in untreated patients with CF while they remained constant in the rhDNase-treated group. The authors thus inferred a positive influence of rhDNase on airway inflammation, an effect that was considered secondary to the improved clearance of retained secretions.

As unconjugated rhDNase, PEGylated rhDNase was stable to jet nebulization (Figure 6). Air-jet nebulizers were the first devices approved by the Food and Drug Administration and European Medicines Agency for the pulmonary administration of rhDNase[30]. Such nebulizers, powered by

compressed air, create droplets of the drug formulated in aqueous solution[31]. The process used to generate the droplets results in exposure of the protein to stresses such as high shear rates and air-water interfaces[32]. Therefore, nebulization might cause protein denaturation and aggregation at air-water interfaces. In addition, droplets that are not sufficiently small impact the jet breeze wall and return to the solution, so that the protein undergoes several nebulization cycles. PEGylated rhDNase did not aggregate during jet nebulization and it maintained its full enzymatic activity. Many proteins are unstable to jet nebulization and form protein aggregates[33, 34]. The stability of rhDNase to jet nebulization is therefore unusual and is an indication of its high physicochemical stability. In general, PEGylation protects proteins against aggregation as a result of the hydrophilic nature of PEG[35]. Mesh nebulization is softer than jet nebulization because droplets of the right size are formed in one step with no recirculation[34]. Mesh nebulization is now also approved for inhalation of native rhDNase and it is the preferred nebulization method for many proteins currently under development[32].

Overall, one single or multiple administrations of high doses of PEG30-rhDNase over a period of up to three months did not cause any significant pulmonary or systemic toxicity *in vivo*. No evidence of respiratory distress or changes in clinical observations were observed throughout the studies. Hematology analysis did not reveal any sign of an acute systemic inflammatory response. Furthermore, no sign of an acute pulmonary inflammatory response was detected (Figure 7, Table 1). However, histological examination of the lung tissue indicated localized accumulations of alveolar macrophages in the single high-dose toxicity study but this increase in alveolar macrophages disappeared after the recovery period. Also, an accumulation in alveolar macrophages did not appear in the sub-chronic toxicity study. The presence of increased numbers of alveolar macrophages is a common observation after delivery of pharmaceutical material to animal lungs and is considered to be an adaptive and non-adverse response[36]. The concentration of total proteins in BAL fluids was significantly higher in animals treated with PEG30-rhDNase as compared to the

control in the sub-chronic toxicity study (Figure 7). Yet, this difference was reversible and might in part be explained by the administration of large quantities of PEGylated rhDNase, itself a protein.

The PEG30 and rhDNase moieties did not appear to accumulate in the lungs or systemically following exposure to PEG30-rhDNase. In our sub-chronic and chronic toxicity studies, lung concentrations of the rhDNase moiety decreased to near baseline levels after the 28 days recovery period (Figure 8). The levels of the PEG30-moiety show a similar decrease over time although its clearance from the lungs was slightly delayed as compared to the rhDNase moiety. Whereas similar levels of PEG and rhDNase were recovered from BAL, much higher levels of PEG were detected in lung homogenates as compared to rhDNase, suggesting that the protein moiety partially degraded in lung homogenates while the PEG moiety is not biodegradable but cleared unchanged. Both the rhDNase moiety and the PEG30 moiety of PEG30-rhDNase could be detected in alveolar macrophage samples obtained 24h post-exposure (Figure 8). However, while no measurable rhDNase concentrations could be obtained in any of the recovery groups, the PEG30 moiety could still be detected although at a lower level than 24h post-exposure. The pulmonary clearance of PEGylated rhDNase thus appears partly orchestrated by alveolar macrophages. No detectable amounts of the rhDNase-moiety of PEG30-rhDNase could be detected in mouse sera of the single-dose and repeat-dose toxicity studies. However, the sub-chronic and chronic dosing regimens resulted in a significant but reversible systemic accumulation of PEG30. PEG30-rhDNase is thus slowly absorbed in the systemic circulation and the resulting low levels are then gradually cleared from the body[25].

PEGylated rhDNase presented a two-weeks residence time in the murine healthy lungs and one single dose of PEG30-rhDNase or PEG40-rhDNase presented a similar efficacy as 1 daily dose of unconjugated rhDNase during 5 consecutive days in β-ENaC mice (Figures 1 & 4). Yet, it is not straightforward to foresee how these data will extrapolate to humans. However, we expect still longer residence times of PEGylated rhDNase in the human lungs than the murine lungs. Freches *et*

al. compared the residence time of an anti-IL-17A Fab' antibody fragment conjugated to a two-armed 40kDa PEG in the lungs of mice, rats and rabbits[21]. They showed that PEGylated Fab' was progressively eliminated from the lungs over 48h with a tendency towards a longer residence time in higher species. Several clearance pathways in the lungs have been shown to be slower in humans compared to rodents. The time for mucus turnover is a few times longer in humans than in rodents[37]. The absorption rate of compounds from the airway lumen into the bloodstream is also several times slower in humans than in rodents[38, 39]. Therefore, as demonstrated in β-ENaC mice (Figure 5), we expect the PEGylated enzyme to require only once a week inhalation to be therapeutically efficacious in patients with CF. Offering patients an inhaled product that would be administered as infrequently would be really pioneering in the field because the dosing frequency of inhaled marketed drugs currently varies from once to 4-times daily.

4. Conclusions

In this article, we presented a long-acting PEGylated version of rhDNase that could be delivered once weekly instead of once or twice daily as native rhDNase. Conjugation of rhDNase to 20kDa, 30kDa or 40kDa PEG sustained the presence of the protein within the murine lungs for more than 15 days while the unconjugated enzyme was cleared within a day. The larger the PEG, the more prolonged rhDNase residency in the lungs. The prolonged presence of PEGylated rhDNase in the murine lungs was associated with a sustained mucolytic activity. PEG40-rhDNase remained active in the lungs over time whereas PEG20-rhDNase and PEG30-rhDNase progressively lost their activity and PEG20-rhDNase lost it the most. The PEGylated enzyme was predominantly located in the airspaces of the lungs where it can optimally exert its therapeutic activity. One single dose of PEG30-rhDNase or PEG40-rhDNase was as effective as 1 daily dose of unconjugated rhDNase during 5 consecutive days to decrease the DNA content in the lung airspaces of β-ENaC mice. Moreover, once

a week administration of PEG30-rhDNase or PEG40-rhDNase over 4 weeks decreased both the DNA content and the neutrophils counts in the lungs of β-ENaC mice, although the decrease in neutrophils counts did not reach statistical significance. PEGylated rhDNase showed a stability to jet nebulization comparable to that of native rhDNase, suggesting that nebulization could be considered as a delivery method for potential future clinical application of PEGylated rhDNase. Finally, single or multiple administrations of high doses of PEGylated rhDNase over a period of up to three months did not cause any significant pulmonary or systemic toxicity in mice. Moreover, the PEG and the rhDNase moieties did not appear to accumulate in the lungs or systemically following sub-chronic or chronic exposure to PEGylated rhDNase. To conclude, these data are promising and pave the way towards future development of PEGylated rhDNase as a long-acting version of the mucolytic with reduced therapy burden for patients with CF.

5. Experimental Section

Materials: Pulmozyme™ was from Genentech, Inc. (South San Francisco, CA, USA). Linear 20kDa and 30kDa methoxy PEG (PEG20 and PEG30, respectively) propionaldehydes and two-arm 40kDa methoxy PEG (PEG40) propionaldehyde were obtained from NOF Corporation (Tokyo, Japan). Unless otherwise stated, chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Production and purification of PEGylated rhDNase: PEGylation on the N-terminal leucine residue of rhDNase was achieved by reductive alkylation at acidic pH using methoxy PEG propionaldehyde, as previously described[15]. Briefly, rhDNase (1 mg/ml) was dialyzed against 1 mM $CaCl_2$, 0.1 M CH_3COONa at pH 5 (PEG20 and PEG30) or pH 5.5 (PEG40) at 4°C during 6 h (Spectra/Por molecular porous membrane tubing MWCO 6–8 kDa, Spectrum). Dialyzed rhDNase was then added to a vial containing methoxy PEG propionaldehyde at a [PEG]:[protein] molar ratio of 16:1 (PEG20 and PEG30) or 8:1 (PEG40). Once the PEG was dissolved, sodium cyanoborohydride (19.6 ml of a 173 mM solution in water per mg of rhDNase) was added to the reaction mixture. The reaction was

pursued under stirring at room temperature for 4 days. The solution was then dialyzed against 20 mM $N(CH_2CH_2OH)_3$, 5 mM NaCl, 1 mM $CaCl_2$, pH 7.5 (buffer A) prior to purification by anion-exchange chromatography (Resource Q, 1 ml column; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) on an ÄKTA™ purifier 10 system (GE Healthcare Bio-Sciences AB). A salt gradient elution was used. Buffer A was 20 mM $N(CH_2CH_2OH)_3$, 5 mM NaCl,1 mM $CaCl_2$, pH 7.5 and buffer B was 20 mM $N(CH_2CH_2OH)_3$, 250 mM NaCl,1 mM $CaCl_2$, pH 7.5. The collected fractions containing the PEGylated species were gathered, concentrated using Vivaspin 15R sample concentrator (10,000 molecular weight cut-off, Sartorius, Stonehouse, Gloucestershire, UK) and stored in 150 mM NaCl, 1 mM $CaCl_2$ at 4°C as marketed rhDNase. Specific mono-PEGylation of rhDNase on its N-terminus was previously confirmed by our laboratory using SDS-PAGE, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and peptide mapping[15]. This protocol was adapted to produce and purify lipopolysaccharide (LPS)-free PEGylated rhDNase for the toxicity studies in mice (Supplementary material). A DNA hyperchromicity assay of rhDNase[40] confirmed that the Michaelis-Menten kinetics of the rhDNase conjugated to linear 30kDa PEG were not different from the native enzyme (Supplementary method and Supplementary Figure 2B).

Animals: Specific-pathogen-free female NMRI mice (7 to 12 weeks old; Elevage Janvier, Le Genets-St-Isle, France) were used for the *in vivo* disposition and confocal laser scanning microscopy studies. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Université catholique de Louvain (Permits number: 2012/UCL/MD/006 and 2016/UCL/MD/019).

Female Swiss mice (8 weeks old; RjOrl: SWISS, Elevage Janvier) were used for toxicity studies. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Université catholique de Louvain (Permits number: 2016/UCL/MD/019).

Transgenic mice overexpressing the β-subunit of the amiloride-sensitive transepithelial Na^+ channel (β-ENaC) and their WT littermate controls were used in the therapeutic efficacy studies.

The mouse model was built in the C57Bl6 genetic background[41] and a colony was bred and housed at the animal care facility of UCLouvain following recommendations of the Federation of European Laboratory Animal Science Associations (FELASA)[42]. Offspring were genotyped by polymerase chain reaction of genomic DNA at 21 days of age. Experiments were performed in sex-matched mice aged from 9 to 16 weeks. The studies and procedures were approved by the ethics committee for animal research of the Université catholique de Louvain (2017/UCL/MD/015) and in agreement with the European Community regulations for animal use in research (CEE n° 86/609).

Animals were kept on a 12:12h light/dark cycle. Mice were given free access to tap water and standard diet throughout the experiments. All studies were performed under anesthesia and all efforts were made to minimize animal discomfort.

Kinetics of in vivo disposition in the respiratory tract: Female NMRI mice were anaesthetized using ketamine/xylazine (90/10 mg/kg) intraperitoneal injection. Biotin-labelled rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase were administered intratracheally. rhDNase was biotin-labeled using EZ-Link Sulfo-NHS-LC-Biotin reagent (Thermo Fisher Scientific, Rockford, IL, USA). Briefly, rhDNase or PEG-rhDNase solutions at a concentration between 0.4 and 1.3 mg/ml in 150 nM NaCl, 1 mM $CaCl_2$ were exchanged against PBS by dialysis (dialysis membranes 6-8,000 MWCO; Spectra/Por dialysis membrane; Spectrum laboratories, Inc. Rancho Dominguez, CA, USA). The appropriate volume of biotin at 4.4 mg/ml in water was then added to the protein solution at a molar ratio biotin:protein of 10:1 and the reaction mixture was kept on ice for 2 h. The bulk of free biotin was subsequently removed by dialysis against 150 mM NaCl, 1 mM $CaCl_2$ at 4°C overnight. Biotin-labelled rhDNase compounds were only used for the studies of *in vivo* disposition in the respiratory tract. A laryngoscope (Penn-Century Inc., Philadelphia, USA) was used to correctly place the bended and blunt needle of a 100 µl precision micro-syringe (Hamilton, Bonaduz, Switzerland) into the trachea. Mice were placed on their back with a tilt angle of 45°. Twenty-five µl of a solution containing 5 µg of biotinylated protein (without counting the PEG moiety in the mass delivered) in

PBS were delivered followed by a 200 µl air bolus using a 1 ml syringe having an angled 18 gauge needle with a blunt tip.

At various pre-determined times (immediately, 4 h, 24 h, 3 days, 7 days, 10 days, 15 days, 20 days and 1 month) following protein administration, the mice were sacrificed by cervical dislocation. A nasal lavage (NAL) was performed by cannulating the trachea towards the nasal cavities and instilling 1 ml Hanks' balanced salt solution (HBSS; Gibco™, Life Technologies, Gent, Belgium) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The fluid emerging from the nostrils was collected. A BAL was then performed. One ml of HBSS containing protease inhibitors was injected into the trachea, left for 10 to 30 s, followed by withdrawal and re-injection of twice 0.5 ml of the fluid. Then, all the BAL liquid was removed from the lungs. Afterwards, the lungs were removed and ground to release the proteins using a tissue grinder (Polytron; Kinematica, Luzern, Switzerland). For all the proteins, tissue processing was carried out using Triton® X-100 (Merck Millipore, Darmstadt, Germany) diluted at 1:1000 in HBSS containing protease inhibitor. Triton allows to increase the recovery of proteins conjugated to large PEG chains from biological samples[14]. The lungs were ground in 0.2 mL of HBSS containing protease inhibitors and Triton® then an additional 0.8 ml of HBSS was added. The tubes were rinsed twice with 1 ml HBSS containing protease inhibitors and Triton®. A total of 3 ml was collected for the lung homogenate. BAL and tissue homogenate samples were then centrifuged at 4500 g at 4 °C for 10 min. The supernatants were stored at -20 °C until they were assayed for protein content by ELISA.

Quantification of rhDNase compounds: rhDNase and PEGylated conjugates were quantified by ELISA. Briefly, a 96-well plate was coated using streptavidin (5 µg/mL) in 1% $NaHCO_3$ solution, pH 9.6 at 4°C overnight and blocked with 1% proteins from milk powder in PBS-Tween 0.1% at 37°C for 1 h. NAL, BAL and lung supernatant samples as well as standards (rhDNase, PEG20-rhDNase, PEG30-rhDNase or PEG40-rhDNase) were incubated at 37°C for 1 h. A first rabbit polyclonal antibody to human rhDNase (Bio-connect, Huissen, The Netherlands) then a goat anti-rabbit IgG conjugated to

horse radish peroxidase (Abcam, Cambridge, UK) were used for development. Revelation was conducted using tetramethylbenzidine solution (Thermo Fisher Scientific, Rockford, IL, USA) and stopped with 2 M H_2SO_4.

Rheology: Rheology assays were performed on a 0.75 mg/ml salmon testes DNA solution in 25 mM HEPES at pH 7.5. 62.5 µl of a 6 mg/ml DNA solution was incubated at 37°C for 30 min with an average volume of 320 µl (depending on the calculated volume of lung homogenate to be added) of 25 mM HEPES, 4 mM $CaCl_2$, 2 or 4 mM $MgCl_2$, 0.2% bovine serum albumin, 28 mM KCl solution at pH 7.5. Then, lung homogenate containing rhDNase or PEGylated rhDNase (average volume of 117.5 µl) was added to reach a final concentration of 0.25 µg/ml. The total final volume was 500 µl. Viscosity measurements were performed during 30 to 60 min at 37°C using a Modular Compact Rheometer MCR102 (Anton Paar, Gent, Belgium). A 50 mm stainless steel cone plate with a 0.5° angle (Anton Paar, Gent, Belgium) was used. A solvent trap containing distilled water prevented dehydration of samples during rheological analysis. The experiments were performed in rotational mode at 1000 rpm. The results were recorded by RheoCompass™ software (Anton Paar).

Confocal laser scanning microscopy: Labeling of rhDNase with Alexa Fluor®488 was performed using Alexa Fluor®488 protein labeling kit (ThermoFisher Scientific, Rockford, IL, USA). One nmol of Alexa488-PEG30-rhDNase (37 µg, without counting the PEG in the mass delivered) was delivered to the lungs of mice by intratracheal instillation as described above. Four days following administration, mice were sacrificed by cervical dislocation and the lungs withdrawn. The lungs were cut into thin slices and stained for confocal laser microscopy. The tissue was stained with MitoTracker® Red CMXRos (2µM; Thermo Fisher Scientific, Rockford, IL, USA) and nuclei with Draq5™ (30µM; Thermo Fisher Scientific). Images were acquired by Cell Observer Spinning Disk (COSD, Zeiss, Germany) and were recorded in green (488nm), red (561nm), and far-red (blue pseudo color, 635nm) channels for Alexa488-PEG30-rhDNase, tissue, and nuclei respectively. ImageJ (1.47v, NIH, USA) was used to process images.

Therapeutic efficacy: β-ENaC and WT mice were anaesthetized using ketamine/xylazine (90/10 mg/kg) intraperitoneal injection. Ten µg of rhDNase, PEG30-rhDNase or PEG40-rhDNase (without counting the PEG moiety in the mass delivered) in 25 µl PBS were delivered to mice by intratracheal instillation, as described above.

Short-term study: One dose of PEG30-rhDNase or PEG40-rhDNase was delivered to mice at day zero and compared with 5 doses (1 dose/day during 5 days) of rhDNase. Mice were anaesthetized every day and 25 µl of PBS were administered to the PEG30-rhDNase and PEG40-rhDNase groups from day 1 to 4. Mice were sacrificed at day 5 (Figure 4A).

Long-term study: One dose of PEG30-rhDNase or PEG40-rhDNase was delivered to mice once a week during 5 weeks. Mice were sacrificed 7 days following the last dose (Figure 5A).

Collection and analysis of samples: The mice were sacrificed by cervical dislocation. A BAL was performed and then the lungs were removed and processed as described above. Cells pellets were collected after centrifugation of BAL for cellular markers. BAL and lung supernatants were frozen at -20°C for future analyses.

Cellular markers were quantified in BAL. One hundred µl of ammonium-chloride-potassium lysing buffer were added to each BAL cells pellet. Samples were then incubated at 4°C for 10 min. 500 µl of 10% fetal bovine serum (Gibco™, Life Technologies, Gent, Belgium) was added and samples were centrifuged at 1200 rpm, 4°C for 10 min. The supernatant was discarded and cell pellets were re-suspended in 500 µl of 10% fetal bovine serum to proceed with total cell counts. Ten µl were mixed with 10 µl trypan blue solution and counted using TC20™ automated cell counter (Bio-Rad, Hercules, CA, USA). Cytospin™ (Thermo Fisher Scientific) was then performed to get 30,000 cells on Superfrost plus® microscope slides (Thermo Fisher Scientific). Fixation of cells was carried out using a staining set Diff-Quik® (Medion Diagnostics, Düdingen, Switzerland). Macrophages, neutrophils, lymphocytes and eosinophils were counted using Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany).

DNA contained in BAL was quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific). The protocol was adapted from the manufacturer method. Briefly, samples and standards were diluted in 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM trishydroxymethylaminomethane (Tris) buffer at pH 8.0 and the same volume of PicoGreen® reagent (diluted 1:200 in Tris-EDTA buffer) was added. Samples and standards were incubated in darkness for 5 min in a black 96-well plate. Fluorescence was measured with a SpectraMax® M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) with 485 nm excitation and 530 nm emission filters.

The murine pro-inflammatory cytokines, IL-6 and CCL2, were quantified in BAL by ELISA. A 96-well plate was coated using anti-mouse IL-6 monoclonal antibody (eBioscience, San Diego, CA, USA) in 40 mM glycine solution, pH 9.4 at 4°C overnight and blocked with 10% fetal calf serum in PBS at 37°C for 1 h. BAL samples as well as standards (mouse IL-6) were incubated at 37°C for 2 h. Anti-mouse IL-6 biotin conjugated monoclonal antibody (eBioscience) was added and the plate was incubated at 37°C for 90 min. Avidine- horse radish peroxidase (Biolegend, San Diego, CA, USA) was used for development (37°C, 30 min). Revelation was conducted using tetramethylbenzidine solution and stopped with 2 M H_2SO_4. The ELISA plate was read at 450 nm. CCL2 was assessed using a standard ELISA (Mouse CCL2/JE/MCP-1 Duoset ELISA; Biotechne, Minneapolis, MN, USA). All biochemical analyses were performed in duplicate.

Nebulization of rhDNase and PEGylated conjugates: rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase were nebulized using an air-jet nebulizer (Sidestream®, Teleflex Medical, Wayne, PA, USA with Respironics Prota-neb®, Philips, Amsterdam, The Netherlands). One ml of rhDNase or PEGylated rhDNase at 0.15 mg/ml in 150 mM NaCl and 1 mM $CaCl_2$ was placed in the nebulizer chamber and allowed to nebulize for 15 min. The droplets generated were then collected in 1.5 ml Eppendorfs hermetically connected to the nebulizer outlet (to prevent evaporation) through an L-shaped (90°) connecting tube ensuring the collection of generated

aerosols by coalescence on Eppendorfs walls. The collected solution was analyzed using a colorimetric assay and size exclusion chromatography (SEC).

Colorimetric determination of rhDNase activity: The methyl green assay was adapted from Sinicropi's paper[43]. A solution of 2 mg/ml of DNA isolated from salmon testes was prepared in Buffer A (25 mM HEPES, 1 mM EDTA, pH 7.5) and mixed for 3–4 days at room temperature until the solution was homogenous. The solution was stored at 4°C. A 0.4% solution of Methyl Green (Sigma-Aldrich) was prepared in Buffer B (20 mM acetate-NaOH, pH 4.2). The solution was extracted with chloroform to remove traces of crystal violet until the organic layer was colorless. The upper aqueous layer was separated, stirred in the hood for 2–3 h to evaporate the excess chloroform and the solution was stored at 4°C. The DNA Methyl Green substrate was prepared by gently mixing 77% (v/v) of 2 mg/ml DNA, 4.6% of 0.4% Methyl Green and 18.4% of Buffer C (25 mM HEPES, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.1% bovine serum albumin, 0.01% thimerosal, 0.05% Tween20, pH 7.5). The solution was stored at 4°C and incubated at room temperature overnight before the assay. Samples (9 serial 2-fold dilutions of rhDNase, PEG20-rhDNase, PEG30-rhDNase or PEG40-rhDNase) were prepared in Buffer C. Each well of a 96-well plate was filled with 100 µl of samples and 100 µl of DNA-Methyl Green substrate. The plate was sealed and incubated for 6 h at 37°C. The absorbance at 650 nm was measured at the end of the incubation. The specific activity after nebulization was determined relative to a standard curve of rhDNase or PEGylated rhDNase before nebulization run on the same plate. Data are expressed as % relative specific activity, i.e., concentration of protein determined by activity assay divided by that based on UV absorption of the sample.

Absorbance at 350 nm: Absorbance at 350 nm was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) in order to evaluate the presence of insoluble aggregates of rhDNase, PEG20-rhDNase, PEG30-rhDNase or PEG40-rhDNase following nebulization. Briefly, one µl of protein solution was loaded on the apparatus. Then, absorbance at 350 nm was

measured. For each sample, measurement was performed three times. Average of the three absorbances was finally reported.

Size exclusion chromatography (SEC): SEC was performed on an ÄKTA™ purifier 10 system using a Superose 6 Increase 10/300 GL column (GE Healthcare Bio-Sciences). A pre-filtrated and degassed solution of 150 mM NaCl, 1 mM $CaCl_2$ was used for elution. Each sample was analyzed twice and absorbance was measured at 280 nm and 215 nm.

Toxicity studies: In the single-dose as well as in both repeat-dose toxicity studies, mice were anaesthetized intraperitoneally with a ketamine/xylazine solution (90/10 mg/kg) before delivering 25 µl of solution (drug vehicle, rhDNase or PEG30-rhDNase) by intratracheal instillation.

Single-dose study: One dose of 100 µg rhDNase or PEG30-rhDNase (in 25 µl of drug vehicle; without counting the PEG moiety in the mass delivered) was administered and mice were sacrificed 24h (main group) or 14 days (recovery group) post-exposure. The control group was treated with a single dose of drug vehicle, i.e., 150 mM NaCl and 1 mM $CaCl_2$. Organ weight, hematology and histology data were obtained from half of the mice, while BAL and lung samples were collected from the other half of mice.

Sub-chronic study: A dose of 100 µg PEG30-rhDNase (without counting the PEG moiety in the mass delivered) or 25 µl of drug vehicle was delivered to mice once a week during four consecutive weeks. Mice were sacrificed 1 day (main group) or 28 days (recovery group) after the last dose. Similar to the single dose study, mice were again divided into two groups, one group for BAL and lung collection and another group for organ collection, hematology and histological analysis of lung tissue.

Chronic study: Mice were intratracheally challenged with 10 µg or 35 µg of PEG30-rhDNase (without counting the PEG moiety in the mass delivered) once per week for twelve consecutive weeks and sacrificed 1 day (main group) or 28 days (recovery group) after receiving the final dose. Control mice were treated in a similar way with drug vehicle. In contrast to the single-dose and sub-

chronic studies, histology of the lung tissue was not performed and all analyses were performed on the same group of mice.

Collection and analysis of samples: Blood collected by orbital bleeding was transferred to an EDTA-coated tube and/or an Eppendorf tube for subsequent hematology analysis or serum preparation, respectively. Hematological analysis was performed within 2h of blood collection using a MS9-5 analyzer (Melet Schloesing laboratories). Serum samples were prepared by incubating tubes overnight at 4°C in an upright position to allow clotting to occur. Next, tubes were centrifuged at 2000 x g for 10 min and the supernatants stored at -80°C. After cervical dislocation, BAL was collected (4 lavages with 1 ml HBSS added with 1 mM $CaCl_2$ but without protease inhibitors) and lungs were removed as described above. However, since polyoxyethylene derivatives are known to interfere with the PEG ELISA, lung tissue processing of toxicity samples was carried out using HBSS containing 0.1% SDS instead of Triton X-100. While BAL and lung supernatants were frozen for further analysis, half of the BAL cell pellet of the four lavages was immediately used for total and differential cell counts as described above. The other half of the cell pellet was stored at -20°C and subsequently used for the preparation of lysed alveolar macrophages using M-PER cell lysis reagent (Thermo-Scientific) according to the manufacturer's instructions. For mice used for histology, lungs were insufflated with 4% paraformaldehyde, embedded in paraffin and a haematoxylin-eosin staining was performed on histological lung slides.

Total protein levels (Pierce™ BCA Protein Assay Kit; Thermo Fisher Scientific) and LDH activity (LDH assay kit, Sigma Aldrich) were measured in BAL supernatants of the first lavage according to the manufacturer's instructions. The amount of PEG30 in BAL (first lavage), lung supernatants, alveolar macrophages and serum samples was quantified using a PEGylated protein ELISA kit (Enzo Life Science) and the level of rhDNase was quantified using an in-house ELISA using a rabbit monoclonal anti-rhDNase capture antibody (Sino-Biological; Eschborn, Germany) and a rabbit

polyclonal anti-rhDNase detection antibody labelled with biotin (Bio-connect, Huissen, The Netherlands).

Statistical Analysis: All results are presented as mean values ± standard error of the mean (SEM). Sample sizes are indicated in figure legends. Values in Figures 2, 4, 5 and 7 and Table 1 were compared using one- or two-way analysis of variance followed by Bonferroni or Tukey multiple comparison post-test. Unpaired t-tests were employed for statistical analysis of the data in Figures 6 and 8. All tests were performed using the GraphPad Prism software (CA, USA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Acknowledgments

We thank Cristina Loira-Pastoriza, Jordane Gougué and Camille Bélot for technical assistance, Sabrina Noel for help in the selection of cytokines to analyze in β-ENaC mice and Danielle Freches (Laboratoires SMB; Brussels, Belgium) for advice in the design of the toxicity studies. We thank Marcus Mall (Charité-Universitätsmedizin Berlin, Germany) for providing β-ENaC mice and wild-type normal homozygous littermates to start colonies at UCLouvain. This work was supported by research grants from the Belgian Cystic Fibrosis Association, Special Funds for Research of UCLouvain, Laboratoires SMB and the Proof of Concept program of the Belgian Walloon Region (Grant 1780012). Rita Vanbever is Research Director of the Fonds National de la Recherche Scientifique (FNRS, Belgium). Sohaib Mahri was supported by the European Commission, Education, Audiovisual and Culture Executive Agency (EACEA), Erasmus Mundus programme, NanoFar doctorate (EMJD NanoFar - ref.520170-1-2011-1-FR-ERA MUNDUS-EMJD).

Conflicts of interest

The authors report no conflict of interest.

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