Modified h*CFTR* mRNA restores normal lung function in a mouse model of cystic fibrosis

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Abstract: Being a classic monogenic disease, gene therapy has always been a promising therapeutic approach for Cystic Fibrosis (CF). However, numerous trials using DNA or viral vectors encoding the correct protein resulted in a general low efficacy. In the last years, chemically modified messenger RNA (cmRNA) has been proven to be a highly potent, pulmonary effective drug. We thus explored the expression of human (h)CFTR encoded by h*CFTR* cmRNA *in vitro*, analyzed by flow cytometry and Western Blot and its function with a YFP assay. Very similar effects could be observed *in vivo* when h*CFTR* cmRNA was assembled with Chitosan-coated PLGA to nanoparticles (NPs) and intratracheally (i.t.) or intravenously (i.v) injected, the latter one as an alternative administration route to circumvent the clogged airways of CF patients. This significantly improved lung function, which suggests that h*CFTR* cmRNA-NPs are a promising therapeutic option for CF patients independent of their *CFTR* genotype.

Introduction: Cystic fibrosis (CF), the most common life-limiting autosomal-recessive disease in Caucasian populations (1/2,500 newborns), affects more than 80,000 people world-wide (1). It is caused by different mutations within the gene encoding for the CF transmembrane and conductance regulator (CFTR). Those mutations result in impaired

anion secretion and hyper-absorption of sodium ions across epithelia (2, 3). Chronic lung disease and slow lung degradation is the major factor contributing to both the mortality and a strongly reduced quality of life (4, 5). With currently available therapies, the mean survival is between 35 and 45 years (6, 7). Since the CFTR gene was first cloned in 1989, many efforts have been made to deal with the mutations at a cellular and genetic level (8). Gene therapy approaches made it quickly to the clinic aiming to deliver viral CFTR encoding vectors [such as adenoviruses (Ad) or adeno-associated viruses (AAV)] to CF patients (9, 10). However, none of the clinical studies and current treatments seem to provide sufficient human (h)CFTR expression to prevent the ultimately lethal CF symptoms in the respiratory tract of CF patients. Furthermore, repeated administration of viral vectors or DNA lead to the development of unwanted immune reactions, mainly due to viral capsids and vector-encoded proteins (11-13).

Newly designed viral vectors circumvent those problems and can be administered repeatedly, but from a clinical perspective the field is still in need of a therapeutic tool that combines efficient expression in lungs and other (affected) organs and cells, while avoiding immunogenicity and genotoxicity completely (14-16). Recently, *in vitro* transcribed (IVT) chemically modified messenger RNA (cmRNA) came into focus, which has the potential to combine the mentioned advantages in a single-stranded molecule (17-19). Chemically modified mRNA has been tested for repeated administration, without developing immune responses or losing efficacy, presenting h*CFTR* cmRNA complexed with biodegradable chitosan-coated PLGA nanoparticles (NPs) as a promising therapeutic for the treatment of CF patients (1, 18, 20). Versatile delivery options of mRNA ensures unique possibility to utilize it in early infants as well as in

adults, independent of the underlying *CFTR* mutation. To best of our knowledge, we provide the first *in vivo* studies delivering h*CFTR* cmRNA to the lungs of CFTR deficient mice by intravenous (i.v.) and intratracheal (i.t.) administration, complexed with NPs. We hereby demonstrate a proof of concept of cmRNA-NP-mediated, ELISA quantified hCFTR expression in the lungs of *Cftr*$^{-/-}$ mice, leading to significantly reduced chloride secretion and, more importantly, restored normal lung function parameters.

Materials and Methods

mRNA production: h*CFTR* was PCR amplified from pcDNA3.hCFTR with the fusion of *Kpn*I and *Eco*RI restriction sites and cloned into a polyA-120 containing pVAX (pVAX.A120, www.lifetechnologies.com) by sticky-end ligation using the mentioned restriction sites. For control experiments, DsRED reporter protein was sub-cloned into pVAX.A120 vector from its original vector pDsRED (www.clontech.com). For *in vitro* transcription (IVT), the plasmids were linearized downstream of the poly-A tail with *Xho*I (www.neb.com). IVT reaction was carried out using MEGAscript T7 Transcription kit (www.ambion.com) with an anti-reverse CAP analog (ARCA) at the 5' end (www.trilink.com). To produce modified mRNA, the following chemically modified nucleosides were added to the IVT reaction in the indicated ratios: uridine-tri-phosphate (UTP) and cytidine-tri-phosphate (CTP) were fully replaced by N1-Pseudo-UTP and 5-Methyl-CTP, abbreviated to cmRNA$^{h\mathit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ and partly replaced by the incorporation of 25% 2-Thio-UTP and 25% 5-methyl-CTP, respectively, abbreviated to cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$(www.trilink.com). The h*CFTR* and *DsRed* mRNA

were purified using the MEGAclear kit (www.ambion.com) and analyzed for size and concentration using a RNA NanoChip 6000 for Agilent 2100 Bioanalyzer (www.agilent.com).

Mammalian cell culture and transfection: Human bronchial epithelial (HBE) and cystic fibrosis epithelial (CFBE) cell lines were maintained in Minimum Essential Medium (MEM, www.biochrom.com) supplemented with 10% (v/v) heat-inactivated Fetal Calf Serum, L-Glutamine (2 mM) and Penicillin –Streptomycin (50 U/ml). Cells were incubated at 37 ºC in a humidified atmosphere containing 5% CO_2 until they reached 80-90% confluency. Cell lines were washed with cold sterile PBS and detached by trypsin-EDTA. Trypsinisation was stopped by adding MEM medium with serum. Cells were collected and spun down at 500 x g for 5 minutes before resuspension in fresh MEM. One day before transfection 250,000 cells/well/1 ml were plated in 12-well plates and grown overnight in MEM without antibiotics. At a confluency of 70-90%, cells were then transfected with 1000 ng mRNA encoding hCFTR using lipofectamine 2000 (www.invitrogen.com) following the manufacturer's instructions and after changing the media to the reduced serum media, Opti-MEM (www.thermofisher.com). After 5 h, cells were washed with PBS and serum-containing MEM was added. Cells were kept for 24 h and 72 h before further analyses.

Flow cytometry analyses: All flow cytometry analyses were performed using a Fortessa X-20 (www.bdbioscience.com). For detection of hCFTR protein in HBE and CFBE cell lines, cells were transfected as described above and subsequently prepared

for intracellular staining using a Fixation/Permeabilization Solution Kit as directed in the manufacturer's instruction (www.bdbioscience.com). As primary antibody mouse anti-human hCFTR clone 596 (1:500, kindly provided by the cystic fibrosis foundation therapeutics Inc.) has been used. As secondary antibody served Alexa Fluor 488 goat anti-mouse IgG (1:1,000, www.lifetechnologies.com). At least 20,000 gated cells per tube were counted. Data were analyzed with FlowJo software, version 10.

YFP-based functional assay: CFTR activity following transient transfection of h*CFTR* (c)mRNA in A549 cells was determined using the halide-sensitive yellow fluorescent protein YFP-H148Q/I152L (21). CFTR deficient A549 cells stably expressing the YFP were plated in 96-well microplates (50,000 cells/well) in 100 µl of antibiotic-free culture medium and, after 6 h, transfected with either plasmids carrying the coding sequence for CFTR or different h*CFTR* (c)mRNA. For each well, 0.25 µg of mRNA or plasmid DNA and 0.25 µl of Lipofectamine 2000 were pre-mixed in 10 µl of OPTI-MEM (www.invitrogen.com) to generate transfection complexes that were then added to the cells. After 24 hours, the complexes were removed by replacement with fresh culture medium. The CFTR functional assay was carried out 24, 48, or 72 h after transfection. For this purpose, the cells were washed with PBS and incubated for 20-30 min with 60 µl PBS containing forskolin (20 µM). After incubation, cells were transferred to a microplate reader (FluoStar Galaxy; www.bmg.labtech.com) for CFTR activity determination. The plate reader was equipped with high-quality excitation (HQ500/20X: 500 ± 10 nm) and emission (HQ535/30M: 535 ± 15 nm) filters for yellow fluorescent protein (www.chroma.com). Each assay consisted of a continuous 14-s fluorescence

reading (5 points per second) with 2 s before and 12 s after injection of 165 µl of a modified PBS containing 137 mM NaI instead of NaCl (final NaI concentration in the well: 100 mM). To determine iodide (I^-) influx rate, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope. After background subtraction, cell fluorescence recordings were normalized for the initial average value measured before addition of I^-. For each well, the signal decay in the final 11 s of the data caused by YFP fluorescence quenching was fitted with an exponential function to derive the maximal slope that corresponds to initial influx of I^- into the cells (21). Maximal slopes were converted to rates of variation of intracellular I^- concentration (in mM/s) using the equation: $d[I^-]/dt = K_I[d(F/F_0)/dt$. Where K_I is the affinity constant of YFP for I^-, and F/F_0 is the ratio of the cell fluorescence at a given time vs. initial fluorescence (21).

Whole blood assay: Blood from three different, healthy donors was taken and collected in EDTA collection tubes (www.sarstedt.com). For each treatment group 2 ml of EDTA-blood was transferred into 12-well plates and treated accordingly. R-848 (Resiquimod, www.sigmaaldrich.com) was added at a concentration of 1 mg/ml to the respective blood positive controls. (Un-)modified h*CFTR* mRNA and pDNA (15 µg each) were complexed to NPs at a ratio of 1:10. Samples were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. At 6h and 24h, 1ml of whole blood was transferred into micro tubes containing serum gel (www.sarstedt.com) and spun down at 10,000 x g for 5 min to obtain serum. Sera were stored at -20 °C for further cytokine measurement analyses.

Animal experiments: All animal experiments were approved by the local ethics committee and carried out according to the guidelines of the German Law for the Protection of Animals (file number: 35/9185.81-2 / K/16). *Cftr*$^{-/-}$ mice (CFTR^{tm1Unc}) were purchased from Jackson Laboratory (www.jax.org) at an age of 6 to 8 weeks and were maintained under standardized specific pathogen-free conditions on a 12 h light-dark cycle. Food, water as well as nesting material were provided *ad libitum.* Prior to i.t. spray applications, mice were anesthetized intraperitoneally (i.p.) with a mixture of medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl (50 µg/kg). *Cftr*$^{-/-}$ mice received 20 µg or 40 µg of h*CFTR* (c)mRNA or equivalent of 20 µg or 40 µg (calculated using nmols) h*CFTR* pDNA encapsulated in chitosan-coated PLGA nanoparticles [Chitosan (83% deacetylated (Protasan UP CL 113) coated PLGA (poly-D,L-lactide-co-glycolide 75:25 (Resomer RG 752H) nanoparticles; short: NPs] by intratracheal (i.t.) spraying (n=4), and intravenous (i.v.) injection (n=4-7) into the tail vein. Mock treated control *Cftr*$^{-/-}$ mice received 20 µg *DsRed* mRNA complexed to NPs (n=5) by i.t. delivery or just 200 µl of NPs by both i.v. and i.t. delivery. For both interventions, (c)mRNA-NP and pDNA-NP complexes were administered in a total volume of 200 µl. Mice received two injections on a three day interval (day 0 and day 3). Detailed description of the i.t. procedures are explained in previous published study (22). After 6 days mice were sacrificed for further end point analyses. To assess immune responses to (un-)modified h*CFTR* mRNA and h*CFTR* pDNA , C57/BL6 mice (n=4 per group) were

189 treated as described for *Cftr*$^{-/-}$ mice. As positive controls served mice that received *E.*
190 *coli* mRNA-NPs (20 µg) intravenously. C57BL/6 mice received one injection of 20 µg
191 mRNA complexed to NPs. After 6 h, 24 h and 72 h mice were sacrificed and blood was
192 collected to obtain serum.

193 **Pulmonary mechanics:** Lung function for each group was evaluated using a
194 FlexiVent® (www.scireq.com). Prior to tracheostomy, mice were anaesthetized
195 intraperitoneally as described above. After anaesthesia, a 0.5 cm incision was
196 performed from the rostral to caudal direction. The flap of skin was retracted, the
197 connective tissue was dissected, and the trachea was exposed. The trachea was then
198 cannulated between the second and third cartilage rings with a blunt-end stub adapter.
199 The mouse was connected to the FlexiVent® system and respiratory mechanics were
200 measured.
201
202 **Salivary assay:** Prior to tracheostomy, anaesthetized mice were injected with 50 µl of 1
203 mM acetylcholine (ACh) in the cheek to stimulate production of saliva. The fluid was
204 collected via glass capillaries and a chloride assay was performed using the Chloride
205 (Cl^-) Assay Kit according to the manufacturer's protocol (www.sigmaaldrich.com).
206 Briefly, saliva was diluted at a ratio of 1:100 with water in a total volume of 50 µl and
207 subsequently 150 µl chloride reagent was added. After 15 min incubation at room
208 temperature in the dark, absorbance was measured at 620 nm using an Ensight
209 Multimode plate reader (www.perkinelmer.com).
210

Western blot analysis: Protein lysate isolated from cell lines was separated on Bolt NuPAGE 4-12% Bis-Tris Plus gels and a Bolt Mini Gel Tank (all from www.lifetechnologies.com). Immunoblotting for hCFTR was performed by standard procedures according to the manufacturer's instructions using the XCell II Mini-Cell and blot modules (www.lifetechnologies.com). After blocking with Blocking buffer (5% Nonfat Dry milk, www.cellSignaling.com) for 1 h at room temperature, primary antibodies against hCFTR (clone 596, 1:1,000) or mouse anti-GAPDH (1:5,000, www.scbt.com) were incubated overnight; horseradish peroxidase–conjugated secondary antibodies (1:5000, anti-mouse from www.dianova.com) were incubated for 1 h at room temperature. Blots were processed using ECL Prime Western Blot Detection Reagents (www.gelifesciences.com). Semiquantitative analysis was performed using the ImageJ software.

Real-time RT-PCR: After i.t. or i.v. injection of differently modified h*CFTR* cmRNA the lungs were isolated at day 6 (experimental end point) homogenized and lysed with tubes of the Precellys Ceramic Kit 1.4/2.8 mm at 5,000 rpm for 20 s in a Precellys Evolution Homogenizer for subsequent RNA-isolation (all from www.peqlab.com). Reverse transcription of 50 ng RNA was carried out using an iScript cDNA synthesis kit (www.bio-rad.com). Detection of hCFTR mRNA was performed by SYBR-Green based quantitative Real-time PCR in 20 µl reactions on a ViiA7 (www.lifetechnologies.com). In all involved procedures we strictly followed the MIQE protocols for RealTime experiments (23). Pre- and post-reaction rooms were strictly separated. Reactions were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95°C and 2 min at

50°C (annealing and extension), followed by standard melting curve analysis. The following primer pairs were used: hCFTR fwd TGTACGGCTACAGGGGAA, hCFTR rev GCCGATAGGCAGATTGTA; house-keeping gene 18S rRNA fwd GGGAGCCTGAGAAACGGC, 18S rRNA rev GACTTGCCCTCCAATGGATCC.

Enzyme-linked immunosorbent assays (ELISAs): To detect protein levels of hCFTR after i.t. or i.v. injection of differently modified h*CFTR* cmRNA, the lungs were isolated at day 6 (experimental end point). A human CFTR ELISA kit was used (www.elabscience.com). Protein was isolated in 600 µl RIPA-buffer and 5 µl protease inhibitor cocktail using the Precellys Ceramic Kit with a bead size of 1,4/2,8 mm (www.sigmaaldrich.com). Tissue was homogenized in a Precellys Evolution Homogenizer at 6,500 rpm for 10 s for a total of three cycles, each interrupted by a 15 s break (www.peqlab.com). Subsequently, supernatants were kept on ice and additionally homogenized 10 times with a 20G needle and incubated for 20 min (www.bdbioscience.com). Lysates were spun down for 20 min at 13,000 x g and 4°C. Supernatant was collected and stored at -20°C for further use. Prior to hCFTR ELISA detection, protein concentration was measured using the Pierce BCA protein assay kit (www.thermofisher.com). For each sample an equal amount of 15 µg whole protein lysate was used. For cytokine measurement, blood from mice and donors was taken to obtain serum and tested for IFN-α and TNF-α production as directed in the manufacturer's instructions (www.bdbioscience.com).

Statistics: All analyses were performed using the Wilcoxon-Mann-Whitney test with Graphpad Prism Version 6 (www.graphpad.com). Most of the data are represented as mean ± SD; box plot data are represented as mean ± minimum to maximum values $P \leq 0.05$ (two-sided) was considered statistically significant.

Results

h*CFTR* (c)mRNA and hCFTR protein quantification *in vitro*

To evaluate the influence of chemical nucleoside modification on h*CFTR* mRNA, we first conducted a set of *in vitro* analyses to characterize the efficacy and functionality of hCFTR protein expression. First, we compared the expression profile of plasmid-encoded hCFTR, unmodified h*CFTR* mRNA and two well-defined nucleoside modifications which have been described to exert state-of-the-art stability/expression *in vitro* or in lung-specific cell contexts *in vivo* (1, 24-26). Flow cytometry analyses 24 h after transfection of human cystic fibrosis bronchial epithelial (CFBE) cells showed hCFTR positive cells ranging from 15.8% after h*CFTR* pDNA transfection to 23.7% after unmodifed h*CFTR* mRNA transfection and up to 33.6% and 49.6% after $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ and $cmRNA^{hCFTR}_{m1\Psi_{1.0}/m5C_{1.0}}$ transfection, respectively. At 24 h all transfection rates (hCFTR-positive cells, marked as black dots) and hCFTR median fluorescence intensities (MFIs, marked as columns) of unmodifed h*CFTR* mRNA,

cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$, and cmRNA$^{hCFTR}_{m1\Psi_{1.0}/m5C_{1.0}}$, were significantly higher compared to pDNA ($P \leq 0.05$; Figure 1A, left lower panel).

Total hCFTR expression, defined as median fluorescent intensity (MFI) multiplied by the transfection efficiency, was significantly higher of cmRNA$^{hCFTR}_{m1\Psi_{1.0}/m5C_{1.0}}$, unmodified h*CFTR* mRNA and cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ at 24 h compared to pDNA ($P \leq 0.05$; Figure 1A, left upper panel). In contrast, after 72 h all three h*CFTR* (c)mRNAs expressed significantly lower compared to h*CFTR* pDNA transfected cells, reflected both in percentage of positive cells, MFI and in total hCFTR expression ($P \leq 0.05$; Figure 1A, both right panels). Expression of cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ and cmRNA$^{hCFTR}_{m1\Psi_{1.0}/m5C_{1.0}}$ compared to unmodified h*CFTR* mRNA after 72 h was also significantly lower ($P \leq 0.05$).

To confirm and substantiate those findings, we performed Western blot analyses of protein lysates taken from transfected CFBE cells at 24 h and 72 h post treatment (Figure 1B). As a positive control served protein lysate from untransfected HBE cells, and GAPDH was used to normalize band intensities. At 24 h h*CFTR* pDNA transfected CFBE cells showed an average of 22.8% of the protein expression of hCFTR observed in HBE cells, which increased 4.1-fold to 94.0% at 72 h (Figure 1B). This drastic increase of hCFTR expression after pDNA transfection goes well in line with the observations in flow cytometry as does the quick onset of hCFTR expression after h*CFTR* (c)mRNA transfection at 24 h (Figure 1B). However, relative to the 24 h time-point, hCFTR expression either remained nearly static (unmodified h*CFTR* mRNA

resulted in 33.8% and 34.7% expression at 24 h and 72 h, respectively), decreased (cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ resulted in 45% and dropped to 29.3% hCFTR expression at 24 h and 72 h, respectively) or increased (cmRNA$^{h\mathit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$, 46.4% at 24 h and raised to 63.3% at 72 h). Ultimately, the expression of h*CFTR* mRNA *in vitro* was strongly dependend on its chemical modification, with cmRNA$^{h\mathit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ resulting in the most robust hCFTR expression.

hCFTR (c)mRNA functionality test *in vitro*

For functional analysis of the (c)mRNA-encoded CFTR channel, we performed a YFP-based functional assay using CFTR null A549 cells which stably express halide-sensitive YFP-H148Q/I152L (25). Quenching of the YFP signal induced by hCFTR channel-mediated I^- influx is reciprocally proportional to hCFTR channel function (21, 27). Figure 1C shows the quenching efficacy after transfection of 250 ng h*CFTR* (c)mRNA, for three different time points, normalized to mock transfected cells. In pDNA transfected cells, the quenching efficacy was significantly higher after 48 h and stayed high even after 72 h ($P \leq 0.05$), while unmodified as well as modified h*CFTR* mRNA transfected cells revealed a single peak quenching at 48 h ($P \leq 0.05$), which was undetectable at 72 h, which is in line with expression patterns seen in Figure 1A and Figure 1B.

316 **h*CFTR* (c)mRNA and hCFTR protein quantification in lungs after application *in***
317 ***vivo***

318 We tested for the localization of h*CFTR* (c)mRNA complexed with nanoparticle in the
319 lungs after i.t. or i.v. application via RT-qPCR, quantified the hCFTR protein expression
320 with hCFTR ELISA and then evaluated its immunogenicity depending on modification.
321 Accordingly an experimental setup has been established (Figure 2A) with
322 comprehensive treatment schemes and unambigous main outcome parameters (Figure
323 2B). In contrast to the *in vitro* data, when 40 µg cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ was i.v. injected
324 into the mice, this resulted in a ~3.8-fold higher accumulation of that mRNA in the lung
325 as compared to 40 µg cmRNA$^{h\mathit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ and h*CFTR* pDNA ($P \leq 0.05$, Figure 2C).
326 More importantly, we wanted to analyze if there is a significant increase in hCFTR
327 protein levels in the lungs of treated mice by hCFTR ELISA (Figure 2D). These analyses
328 confirmed that mice treated with 40 µg cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ i.v. had a highly
329 significant increase of hCFTR in the lungs of treated mice vs control mice *(*$P \leq 0.01$;
330 Figure 2D). Moreover, we tested the effects of an increased amount of
331 cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ and h*CFTR* pDNA i.t. to 80 µg, which initially seemed to have a
332 low deposition (Figure 2D), but again showed a clear and significant increase of hCFTR
333 protein compared to control mice (Figure 2D) ($P \leq 0.05$). All the mock controls used in
334 hCFTR Elisa has proved to be not significantly different from negative control.
335

h*CFTR* (c)mRNA immunogenicity *in vivo* in mice after i.v. application and *ex vivo* in an adapted human whole blood assay*

*all *in vivo* experiments were performed with nanoparticles if not stated otherwise

Due to lack of a reliable method to detect immune responses that therapeutic mRNAs may trigger in a living orgamisms, we focussed on two different approaches. First, we applied different compounds such as nanoparticles and R-848 (Resiquimod, a strong TLR7 and TLR8 agonist) and modified or unmodified mRNA i.v. or i.t. to mice to monitor their immune reaction at three different time points. All compounds, mRNAs and application routes are color-coded in Figure 2A. Surprisingly, applying 40 µg unmodified h*CFTR* mRNA or h*CFTR* cmRNA (with any modifications used) did not lead to detectable responses of key cytokines IFN-α or TNF-α (detected by ELISA) at all three time points (Figure 2E) (28, 29). Nanoparticles alone (used in all *in vivo* experiments) showed no immune response over the detection limit. However, as expected the positive control (*E. coli* extract total RNA) resulted in a significant increase of IFN-α and TNF-α at 6 h and a trend increase of IFN-α at 24 h, while an effect at 72 h was not detectable (Figure 2E).

In contrast to that, different results were obtained when we used a more complex assay based on human whole blood. Interestingly, the negative control groups (blood only and NP only) did not raise IFN-α values above the detection limit (Figure 2F, red dotted lines), while one sample of TNF-α was already measureable in human blood untreated or treated only with NPs. That is the reason why we adapted the graphical presentation of Figure 2F as we already did in Fig. 2D, using a blue colored area that represents the variance of the negative controls, which are biological replicates. The

positive control (R-848) lead to a strong and significant production of both IFN-α (6 h and 24 h, respectively; $P \leq 0.05$) and TNF-α (6 h and 24 h, respectively; $P \leq 0.05$) (Figure 2F).

Human whole blood transfected with h*CFTR* cmRNAs showed a very similar result in cytokine expression as observed for negative controls: the IFN-α levels did not reach the detection limit of the ELISA; TNF-α responses were not statistically significant at 6 h and 24 h, respectively (Figure 2F). Unmodified h*CFTR* mRNA resulted in a significant increase of IFN-α (30.6 ± 3.0 pg/ml and 16.6 ± 3.5 pg/ml at 6 h and 24 h, respectively; $P \leq 0.05$) while the TNF-α levels were in line with the negative control. While h*CFTR* pDNA triggered high TNF-α responses at 6 h and 24 h (785.5 ± 256.80 pg/ml and 336.29 ± 182.68 pg/ml respectively; $P \leq 0.05$), lower but detectable IFN-α responses after 6 h and 24 h (17.24 ± 4.43 pg/ml and 21.82 ± 1.21 pg/ml) could be observed. Due to both a significantly lower expression of unmodified h*CFTR* mRNA *in vitro* (Figure 1A and 1B) and higher immune responses of unmodified h*CFTR* mRNA depicted in Figure 2F, we focused on h*CFTR* cmRNAs and *hCFTR* pDNA in the following therapeutic studies.

Therapeutic effect of h*CFTR* (c)mRNA *in vivo* in mice after i.t. and i.v. application*

*all *in vivo* experiments were performed with nanoparticles if not stated otherwise

After the expression- and immuno-profiling, we investigated the therapeutic potential of cmRNA in a mouse model of Cystic Fibrosis. In order to test the efficacy of h*CFTR* cmRNA, *CFTR* knock-out mice have been used in several experimental settings that

are explained and color-coded in Figure 3A. First, we performed a well established functional test, measuring the mouse saliva chloride concentration (30). The saliva chloride concentration detected in *Cftr*$^{-/-}$ mice (4084 ± 236.8 ng/µl) was significantly higher compared to *Cftr*$^{+/+}$ mice (748.8 ± 96.9 ng/µl, $P \leq 0.01$; Figure 3B). The treatment with either cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ i.v. or i.t. (80µg) significantly lowered the chloride concentrations in the saliva of *Cftr*$^{-/-}$ mice more than 52% and 36% repectively ($P \leq 0.05$; Figure 3B). However cmRNA$^{h\mathit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ and *hCFTR* pDNA treated mice (i.v.) only provided a 22% reduction, although increased amount *hCFTR* pDNA treatment (i.t.) resulted in 30% reduction of chloride concentration in saliva of *Cftr*$^{-/-}$ mice ($P \leq 0.05$; Figure 4B).

To assess the impact of h*CFTR* cmRNA on lung function, we evaluated clinically relevant parameters using the FlexiVent® lung function measurement system. We observed significant differences between Mock controls / *Cftr*$^{-/-}$ and healthy wild-type mice for all parameters measured ($P \leq 0.05$; Figure 3C-E and $P \leq 0.01$; Figure 3F). Applying 40 µg of cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ i.v. significantly lowered the resistance ($P \leq$ 0.01; Figure 3D). Furthermore, i.v. administration of cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ significantly increased the compliance from 0.02 ± 0.01 ml/cmH_2O (*Cftr*$^{-/-}$ mice) to 0.03 ± 0.01 ml/cmH_2O ($P \leq 0.01$), reaching equivalent values to those measured in *Cftr*$^{+/+}$ mice

399 (Figure 3C) . In the i.t. treated groups, a significant improvement of resistance and
400 compliance could be detected when the cmRNA$^{h\textit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ dose was increased to
401 80 µg (0.86 ± 0.18 cmH_2O.s/ml and 0.04 ± 0.01 ml/cmH_2O, respectively; P ≤ 0.05;
402 Figures 3C-D). However 40 µg of cmRNA$^{h\textit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ i.v. lowered the resistance but
403 not as effectively as cmRNA$^{h\textit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ (*P* ≤ 0.05; Figure 4D) and *hCFTR* pDNA
404 (80µg) i.t. treated mice also produced significant improvement of resistance and
405 compliance (P ≤ 0.05, Figure 4C-D). Furthermore the i.v. application of 40 µg
406 cmRNA$^{h\textit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ or *hCFTR* pDNA did not alter compliance significantly (Figure
407 4C).
408 Tissue elastance (peripheral lung mechanics), that is, energy conservation in the alveoli,
409 was significantly improved for cmRNA$^{h\textit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ i.v. treated group (*P* ≤ 0.01;Figure
410 3F) and i.t. application of 80 µg cmRNA$^{h\textit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ (*P* ≤ 0.05; Figure 3E) compared
411 to *Cftr*$^{-/-}$ mice, in which tissue elastance was detected with a value of 53.61 ± 10.67
412 cmH_2O/ml. cmRNA$^{h\textit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ and *hCFTR* pDNA, (i.v. and i.t.) treatment produced
413 a improved tissue elsastance but failed to reach the effect of i.v treatement of
414 cmRNA$^{h\textit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ (*P* ≤ 0.05; Figure 4E and Figure 3E).

$FEV_{0.1}$ (human equivalent of FEV_1) of *Cftr*$^{+/+}$ mice defined as projecting 100% forced exhale volume, the i.v. injection of 40 µg cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ or i.t. application of 80 µg cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ improved the $FEV_{0.1}$ by 23% ($P \leq 0.01$) and 19% ($P \leq 0.05$) respectively compare to untreated *Cftr*$^{-/-}$ (Figure 3F). Only i.v. injection of 40 µg cmRNA$^{h\mathit{CFTR}}_{m1\Psi_{1.0}/m5C_{1.0}}$ provide a $FEV_{0.1}$ improvement of 14% which is satistically significant ($P \leq 0.05$; Figure 4F). However i.v. or i.t. administration of *hCFTR* pDNA showed no significant improvement of $FEV_{0.1}$. Taken together, these results demonstrate significant lung function improvement in all relevant lung function parameters of *Cftr*$^{-/-}$ mice treated with h*CFTR* cmRNA.

Discussion

Although much progress has been achieved since the discovery of the *CFTR* gene 25 years ago, there is still a substantial need to restore robust CFTR function in patients suffering from cystic fibrosis (31). With the recent approvals of the small molecule agents ivacaftor and lumacaftor, science has paved a possible way to overcome the hurdles caused by the disease-conferring gene. Those treatments can be more or less effectively applied to patients bearing *CFTR* mutations delF508 (Lumacaftor-ivacaftor/Orkambi) and G551D (ivacaftor) (32-35). However, lung function as one of the main outcome parameters probably having the most significant influence on life quality of CF patients, is rarely tested in preclinical models. In fact, actual effects of (modern)

existing drugs on lung function, with forced expiratory volume in one second (FEV_1) as a key parameter, are quite low (36). Here by using h*CFTR* (c)mRNA, we are presenting a proof of concept for a viable and potent therapeutic alternative. We have vigorously tested mRNA therapy with focus on *in vivo* lung function normalization while avoiding any possible, unwanted immune reponses for a possibility of repeated dosing. The unique formulation utilized, can be used both topically (intratracheally) and systemically (via i.v. injection), having in both cases a profound effect on normalizing the lung function parameters, including compliance, resistance and $FEV_{0.1}$ of treated *Cftr*$^{-/-}$ mice to values obtained from *Cftr*$^{+/+}$mice.

In vitro, using h*CFTR* cmRNA, CFTR protein expression in CFBE cells was increased up to 5.5-fold compared to unmodified h*CFTR* mRNA, which is consistent with previous studies obtained by us and others (2, 26, 37). Incorporation of naturally occurring nucleosides has been shown to suppress inhibitory effects on translation by avoiding detection by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) TLR3, TLR7 and TLR8 (28, 29). Those receptors play a crucial role in the detection, processing and degradation of mRNA. Interestingly, depending on the mRNA modification, kinetics of hCFTR expression varies upon the different nucleosides used. In fact, we did not observe an increased quenching efficacy after 72h in CFTR null A549 cells, which would corroborate our findings from western blot analyses. Although there is a significant increase in I^- influx by functional hCFTR channels at 48h post transfection, both modified hCFTR mRNAs showed similar activity. Consequently, we assume that upon different cell lines, kinetics by which the

457 hCFTR protein is expressed varies. Earlier studies support our notion that different
458 modified mRNAs can have an impact on the translational effect between distinct cell
459 lines (26, 29).

460 To better determine the clinical potential of CFTR-encoded cmRNA we compared not
461 only different modifications *in vivo* but also two different routes of administration.
462 Applying cmRNA i.t., has been shown to significantly prolong survival in a surfactant
463 protein-B mouse model (22). Given the fact that in patients suffering from CF one of the
464 key barriers is the airway mucus layer in which inhaled particles are more likely to get
465 trapped and removed, we sought to apply h*CFTR* cmRNA/pDNA complexed to NPs by
466 i.v. injection as an alternative administration route. Systemic delivery via lipid modified
467 polymeric nanoparticles has been already shown to target the lungs efficiently (38). In
468 this study, by applying h*CFTR* cmRNA consecutively, both modifications were
469 successfully delivered to the lungs with the i.v. route being more efficient at doses of
470 40 µg (2 mg/kg) per treatment. Intriguingly, in contrast to the results obtained *in vitro*,
471 $cmRNA^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ showed a significantly higher CFTR protein expression with
472 higher accumulation of h*CFTR* cmRNA in lung cells. Assuming differences of cmRNA-
473 encoded transgene expression between distinct cell lines, it is plausible to consider
474 such differences between *in vitro* versus *in vivo* applications, which is by far more
475 complex. In this respect, the higher amount of $cmRNA^{h\mathit{CFTR},s2U_{0.25}/m5C_{0.25}}$ found in
476 lung cells after i.v. injection, might be due to the fact that its nucleoside composition is
477 more favorable to evade PRRs, thus being less degraded. However, regardless of
478 cmRNA kinetics we also observed differences in the delivery route of h*CFTR*

cmRNAs/pDNA-NPs. Our data suggests i.v. injection to be more efficient in delivering such complexes to the lung than topical administration. Tests of h*CFTR* cmRNA-NP's capacity of mucus penetration are in planning phase. The upper airways are lined with mucus and mucociliary movements clear foreign particles immediately. In addition, the main barriers in the deeper areas are the alveolar lining, scavenger transporters and alveolar macrophages (39, 40). We therefore concluded, that the dosing by which cmRNA-NPs were delivered i.t. was not sufficient to reach the lung cells efficiently. Indeed, increasing the amount by doubling the dose (to 80 µg) for each treatment showed a significant increase in hCFTR expression.

To exclude immune reactions caused by either NPs or the (c)mRNA itself, we conducted extensive immune assay tests *in vivo*. Except for the positive control (*E. coli* total mRNA) we could not detect any immunostimulatory effect *in vivo* that could arise from NPs or the (un-)modified h*CFTR* mRNAs. These results confirm our previous studies in which we showed that NPs as well as modified mRNA could be administered safely to the lungs without any substantial increase in cytokines, or inflammatory-related cells such as macrophages or neutrophils (22). Systemic delivery has also been reported to have no impact on proinflammatory cytokine secretion (24). To better mimic the *in vivo* human conditions, we performed an *ex vivo* whole blood assay (WBA) which offers a more complex environment to test for immune responses. This assay has already been used in a number of preclinical settings and Coch and colleagues could demonstrate that it has the potential to reflect broad aspects of the *in vivo* cytokine release caused by oligonucleotides (41). Indeed, we could show that the small

molecule resiquimod (serving as a positive control by activating TLR7 and TLR8) lead to a substantial release of IFN-α and TNF-α. Plasmid-encoded hCFTR as well as unmodified h*CFTR* mRNA also showed elevated cytokine levels probably due to the activation of innate immune receptors (28, 29). In contrast, incorporation of modified nucleosides into h*CFTR* mRNA abolished such responses, with no detectable amounts of IFN-α. This is in concert with previously published data, demonstrating cmRNA's limiting immune responses, mainly by evading detection from receptor such as TLRs, RIG-1, MDA-5 or PKR (28, 37). Interestingly, even though TNF-α could be detected, it rather shows donor-dependency than effects deriving from NPs and/or h*CFTR* cmRNA with cytokine levels being all within the variance of negative controls. Although it mirrors only the blood compartment and does not reflect the more complex *in vivo* situation, the WBA can give a prediction of how cytokines are released in the human system in response to systemically applied (c)mRNA prior to clinical testing.

Eventually, we determined the impact of h*CFTR* cmRNA and Plasmid-encoded hCFTR on relevant physiological outcomes such as the saliva chloride concentration as well as important lung function parameters to evaluate its therapeutical effect. Sweat chloride concentration has become an accepted method as a diagnostic readout to assess treatment effects of CF patients (42) . As an analog, chloride concentration of β-adrenergic stimulated salivary glands of CFTR knock-out mice can be investigated as it complies with findings in CF patients (30) . In this study, we could show a substantial difference in salivary Cl^- content of h*CFTR* cmRNA and *hCFTR* pDNA treated mice – both, i.v and i.t. – compared to their untreated counterpart. With end point-analysis, a

523 significant decrease in Cl^- to nearly 60 % was observed, indicating a restoration of
524 CFTR in the duct compartment of salivary glands and thus leading to an improved Cl^-
525 absorption. Previous studies estimated that a restoration of CFTR activity to 50 %
526 could lead to sweat chloride levels to near normal levels in CF patients. Given that, it is
527 possible that h*CFTR* cmRNA treatment has the potential to improve CFTR activity to
528 levels that are at least similar to those in patients with a mild CF phenotype (43).

529 To support our notion of improved CFTR activity, we additionally performed extensive
530 lung function measurements using state-of-the-art technology to provide detailed *in*
531 *vivo* information on different lung function parameters. *Cftr*$^{-/-}$ mice have been criticized
532 as a proper model for cystic fibrosis as it does not reflect the typical lung phenotype
533 seen in CF patients (44). However, the reason behind that seems to be in how deeply
534 lungs or other affected organs had been investigated. A layer of material can be
535 observed with characteristics of an acid mucopolysaccharide on the bronchiolar
536 surface and is also evident in alveoli by using scanning electron microscopy in *Cftr*$^{-/-}$
537 mice, which is not evident in *Cftr*$^{+/+}$ mice (45). Recent studies could clearly
538 demonstrate reduced airway compliance and increased resistance in comparison to
539 wild-type mice (46, 47). Indeed, we observed significantly higher and lower levels
540 regarding resistance and compliance, respectively, in *Cftr*$^{-/-}$ controls and mock-treated
541 *Cftr*$^{-/-}$ mice compared to homozygous wild type mice (*Cftr*$^{+/+}$) mice and demonstrated
542 that treatment with h*CFTR* cmRNA-NPs improved compliance and resistance
543 significantly equal to those seen in healthy *Cftr*$^{+/+}$ mice. FEV1 (Forced exhale volume)

544 percentage (for mouse or small animal FEV $_{0.1}$) is related to survival in CF and most
545 important physiological parameter for CF patients. Previous study demonstrated that
546 patients with a %FEV1 <30 had a 2-year mortality over 50% and hence it is regularly
547 exhamined in clinical setup (48). Our study provide a significant improvement of $FEV_{0.1}$
548 due to treatement with h*CFTR* cmRNA-NPs. Interestingly *hCFTR* pDNA when
549 administrated via i.t. route improve other parameter of lung function measurements did
550 amend $FEV_{0.1}$ but not as significantly as h*CFTR* cmRNA-NPs.

551 In addition, intrinsic mechanical properties of the parenchyma are altered in *Cftr*$^{-/-}$ mice
552 with increased tissue elastance (47, 49). Such differences in peripheral lung mechanics
553 indicate an energy loss due to frictional, resistive forces and that more work will be
554 required to expand the lungs (elastance) (49). As for the central lung mechanics,
555 peripheral measurements confirmed i.v.-treated groups to be more effective in
556 targeting lung cells than topical application, thereby decreasing tissue elastance. We
557 also observed i.t. administration of 80 µg $cmRNA^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ to positively
558 compensate most of lung fuction parameters. Overall, we could demonstrate that
559 certain protocols, applying h*CFTR* cmRNA either i.v. or i.t. efficiently restored lung
560 function values equal to those of wild type. Suggesting a more evenly distribution
561 through arteries and the bronchial circulation by i.v.-injection, especially for newborns
562 and young infants, this route and formulation could lead to a very potent therapy. By
563 providing functional CFTR early in life, the lungs could be protected from irreversable
564 damage. Nevertheless, when applied intratracheally - which mimicks deep inhalation of
565 a spray or powder formulation usually the primary application route in adults - an

adjustment in dose and/or formulation (e.g. cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ increased to 80 µg) might easily abrogate any negative effect of the *Cftr*$^{-/-}$ genetic background on lung function.

Taken together, this study is the first proof of concept of efficient application of h*CFTR* cmRNA NPs *in vivo* to restore lung function in a *Cftr*-deficient mouse model. Importantly, we could neither detect immune responses *in vivo* nor in a more defined setting *ex vivo*. Applying h*CFTR* cmRNA to *Cftr* knock-out mice could efficiently restore lung function to levels of healthy control mice. In addition, our study compared - apart from two well-known mRNA modifications and h*CFTR* pDNA - also two different delivery routes, demonstrating that systemic administration of cmRNA targets lung cells more efficiently at lower dosages. This study provides a proof of concept for alternative treatment of patients suffering from CF. h*CFTR* cmRNA transcript supplementation may be broadly applicable for most *CFTR* mutations, not only in adults but already in the postnatal state, thereby protecting the lungs from exacerbations from the very beginning of life.

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Figure Legends

Fig. 1: (c)mRNA-mediated expression and function of h*CFTR in vitro* (**A**) Percentage of hCFTR positive CFBE cells and total expression of hCFTR 24 h and 72 h after transfection with 1 µg h*CFTR* pDNA or (chemically modified) h*CFTR* mRNAs, detected by flow cytometry. *, $P \leq 0.05$ versus unmodified h*CFTR* mRNA; §, $P \leq 0.05$ vs. pDNA. (**B**) Western Blots, semi-quantifying human CFTR in the cell cultures used in (**A**), normalized to GAPDH and put relative to CFTR levels in HBE cells. *, $P \leq 0.05$ versus CFBE controls at 24 h and §, $P \leq 0.05$ versus CFBE controls at 72 h.(**C**) Quenching efficacy of pDNA or mRNA encoded h*CFTR* in A549 cells relative to untransfected CFBE controls was measured at 24 h, 48 h and 72 h post-transfection. *, $P \leq 0.05$ versus untransfected controls; MFI, median fluorescence intensities. All other bar graph data are depicted as means ± SDs while box plots data are depicted as the means ± minimum to maximum values.

Fig. 2: *In vivo* study plan, expression of modified h*CFTR* mRNA and hCFTR protein in mouse lungs and immunogenicity in mice and human whole blood.

(**A**) All mouse groups, particles and particle combinations depicted in the study plan (**B**) and utilized in (**C-F**) are color-coded for their treatment schemes, including dosage and application routes. (**C**) Relative amounts of differently modified h*CFTR* mRNAs in the lungs, applied i.v. or i.t., then determined by RT-quantitative PCR, compared to 40µg cmRNA $^{\text{h}CFTR}_{\text{s2U}_{0.25}/\text{m5C}_{0.25}}$ i.t. injection (*, $P \leq 0.05$); n = 4-7 mice per group. (**D**) ELISA, detecting specifically human CFTR, was performed on lung preparations at day 6 (endpoint); the same n = 4-7 mice per group as in (**C**) were used. *, $P \leq 0.05$, **, $P \leq 0.01$ versus untreated *CFTR* knock-out mice. (**E**) Mice were i.v. or i.t. injected with a mix of (c)mRNA and NPs at a 1:10 ratio, and ELISAs were performed post-i.v./i.t.-injection at three different time points. n.d., not detectable. (**F**) 2 ml whole blood,

each from three different healthy human donors, were incubated with either R848 (1 mg/ml) or 3.82 pmol pDNA or 7.91 pmol (c)mRNA (providing the same total number of nucleic acid molecules) and NPs at a 1:10 ratio; after 6 h and 24 h the immune response was determined by ELISA in the sera; * and §, $P \leq 0.05$ versus control at 6 h and 24 h, respectively. The red dotted lines in (**D-F**) mark the detection limit as specified in the respective ELISA kit. The blue areas in (**D, F**) represent the variance of the negative controls which are biological replicates. n.d., not detectable. All bar graph data are depicted as the means ± SD and box plots data are represented as the means ± minimum to maximum values.

Fig. 3: *In vivo* lung function measurements in h*CFTR* mRNA treated *CFTR* knock-out mice.

All mouse groups utilized in (**B-F**) are color-coded for their treatment schemes (**A**), including dosage and application routes. **B**) Functional test of reconstituted CFTR channel compared to *Cftr* knock-out mice (black), positive controls (violet), and percentages relative to the positive control; n = 4-7 mice per group; 3 mock controls were included (white); boxes represent the means ± minimum and maximum values. (**C-F**) Precision *in vivo* lung function measurements covering all relevant outcome parameters on mice treated twice (see **A**) and measured 72 hours after the 2nd installment; n = 4-7 mice per group. Data represent the means ± SD on compliance, resistance, tissue elastance and Forced Expiratory Volume in 0.1 seconds ($FEV_{0.1}$). *, $P \leq 0.05$, **, $P \leq 0.01$ versus untreated *Cftr* knock-out mice.

Fig. 4: *In vivo* lung function measurements in h*CFTR* pDNA treated *CFTR* knock-out mice.

All mouse groups utilized in (**B-F**) are color-coded for their treatment schemes (**A**), including dosage and application routes. **B**) Functional test of reconstituted CFTR channel compared to

Cftr knock-out mice (black), positive controls (violet), and percentages relative to the positive control; *n* = 4-7 mice per group; 3 mock controls were included (white); boxes represent the means ± minimum and maximum values. (**C-F**) Precision *in vivo* lung function measurements covering all relevant outcome parameters on mice treated twice (see **A**) and measured 72 hours after the 2nd installment; *n* = 4-7 mice per group. Data represent the means ± SD on compliance, resistance, tissue elastance and Forced Expiratory Volume in 0.1 seconds ($FEV_{0.1}$). *, $P \leq 0.05$, **, $P \leq 0.01$ versus untreated *Cftr* knock-out mice.

Figure 1

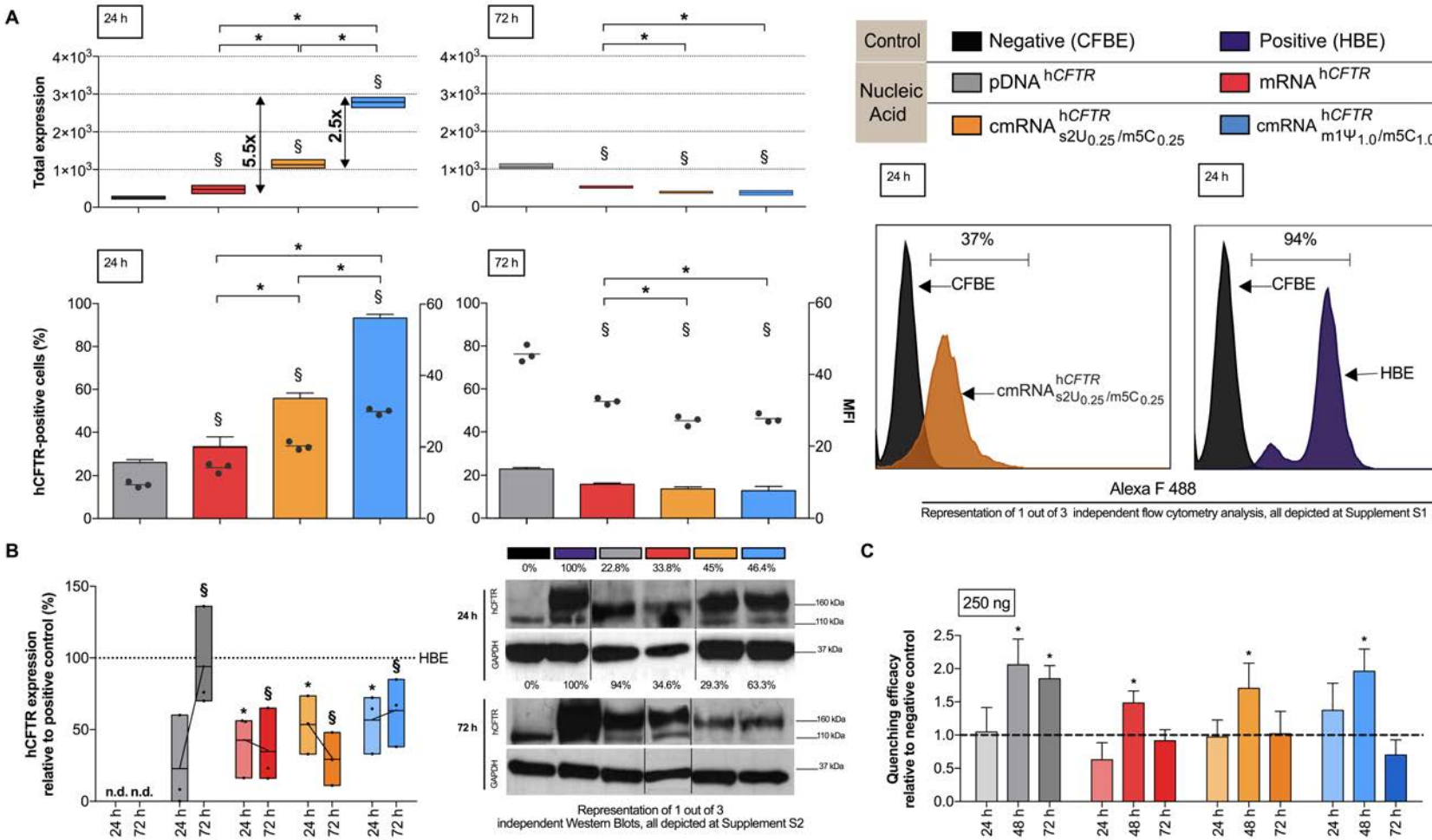

A
24 h
72 h
Total expression
5.5x
2.5x
hCFTR-positive cells (%)
MFI
Control
Nucleic Acid
Negative (CFBE)
Positive (HBE)
pDNA hCFTR
mRNA hCFTR
cmRNA hCFTR s2U0.25/m5C0.25
cmRNA hCFTR m1Ψ1.0/m5C1.0
37%
94%
CFBE
HBE
Alexa F 488
Representation of 1 out of 3 independent flow cytometry analysis, all depicted at Supplement S1
B
hCFTR expression relative to positive control (%)
HBE
n.d. n.d.
0%
100%
22.8%
33.8%
45%
46.4%
94%
34.6%
29.3%
63.3%
hCFTR
GAPDH
160 kDa
110 kDa
37 kDa
Representation of 1 out of 3 independent Western Blots, all depicted at Supplement S2
C
250 ng
Quenching efficacy relative to negative control
24 h
48 h
72 h

Figure 2

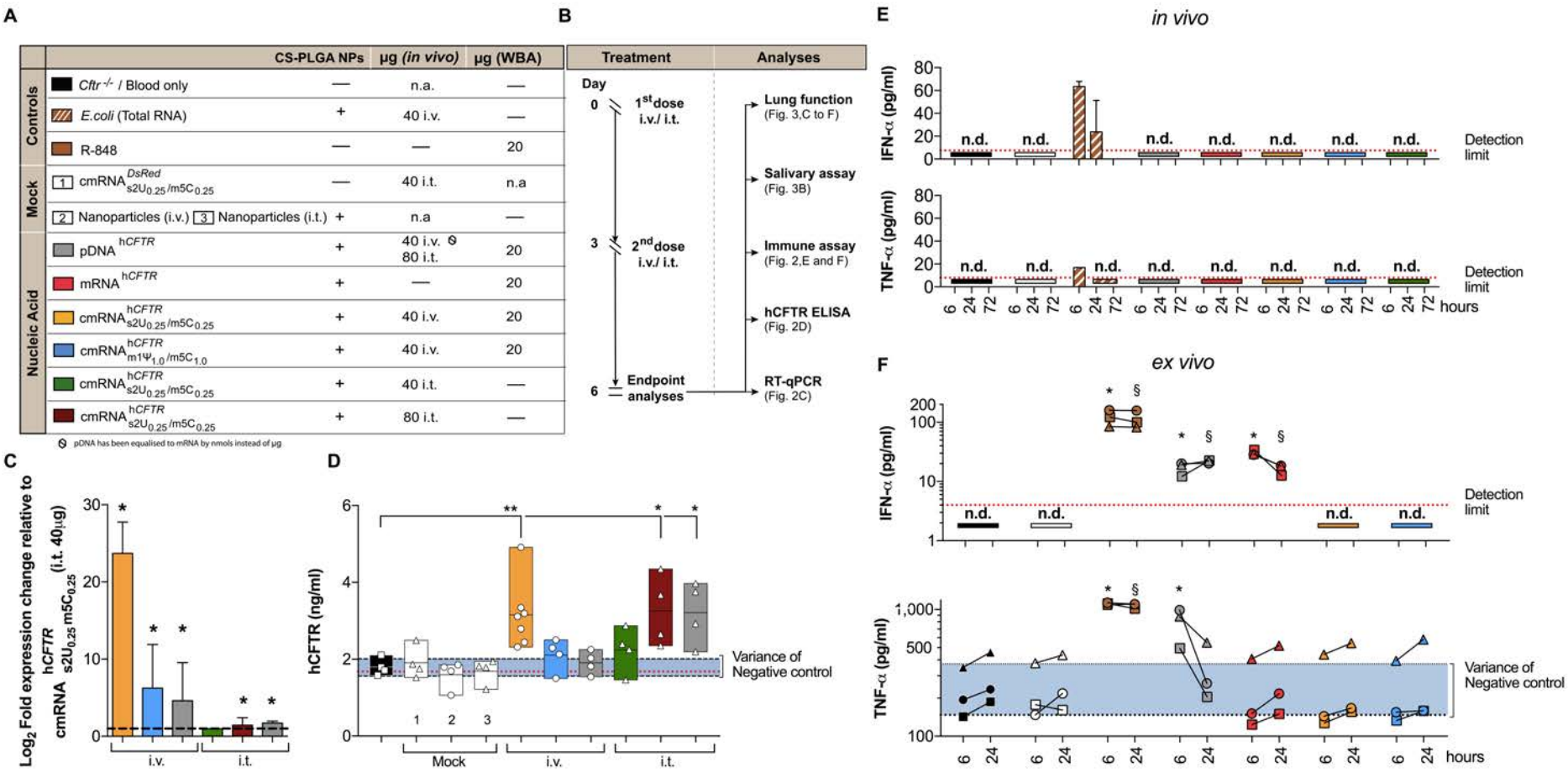

A
CS-PLGA NPs
µg (in vivo)
µg (WBA)
Controls
Cftr -/- / Blood only — n.a. —
E.coli (Total RNA) + 40 i.v. —
R-848 — — 20
Mock
1 cmRNA DsRed s2U0.25/m5C0.25 — 40 i.t. n.a
2 Nanoparticles (i.v.) 3 Nanoparticles (i.t.) + n.a —
Nucleic Acid
pDNA hCFTR + 40 i.v. ϴ 80 i.t. 20
mRNA hCFTR + — 20
cmRNA hCFTR s2U0.25/m5C0.25 + 40 i.v. 20
cmRNA hCFTR m1Ψ1.0/m5C1.0 + 40 i.v. 20
cmRNA hCFTR s2U0.25/m5C0.25 + 40 i.t. —
cmRNA hCFTR s2U0.25/m5C0.25 + 80 i.t. —
ϴ pDNA has been equalised to mRNA by nmols instead of µg
B
Treatment
Analyses
Day
0 1st dose i.v./ i.t.
3 2nd dose i.v./ i.t.
6 Endpoint analyses
Lung function (Fig. 3,C to F)
Salivary assay (Fig. 3B)
Immune assay (Fig. 2,E and F)
hCFTR ELISA (Fig. 2D)
RT-qPCR (Fig. 2C)
C
Log2 Fold expression change relative to hCFTR cmRNA s2U0.25 m5C0.25 (i.t. 40µg)
i.v.
i.t.
D
hCFTR (ng/ml)
Mock
i.v.
i.t.
Variance of Negative control
E
in vivo
IFN-α (pg/ml)
TNF-α (pg/ml)
n.d.
Detection limit
hours
F
ex vivo
IFN-α (pg/ml)
TNF-α (pg/ml)
Detection limit
Variance of Negative control
hours

Figure 3

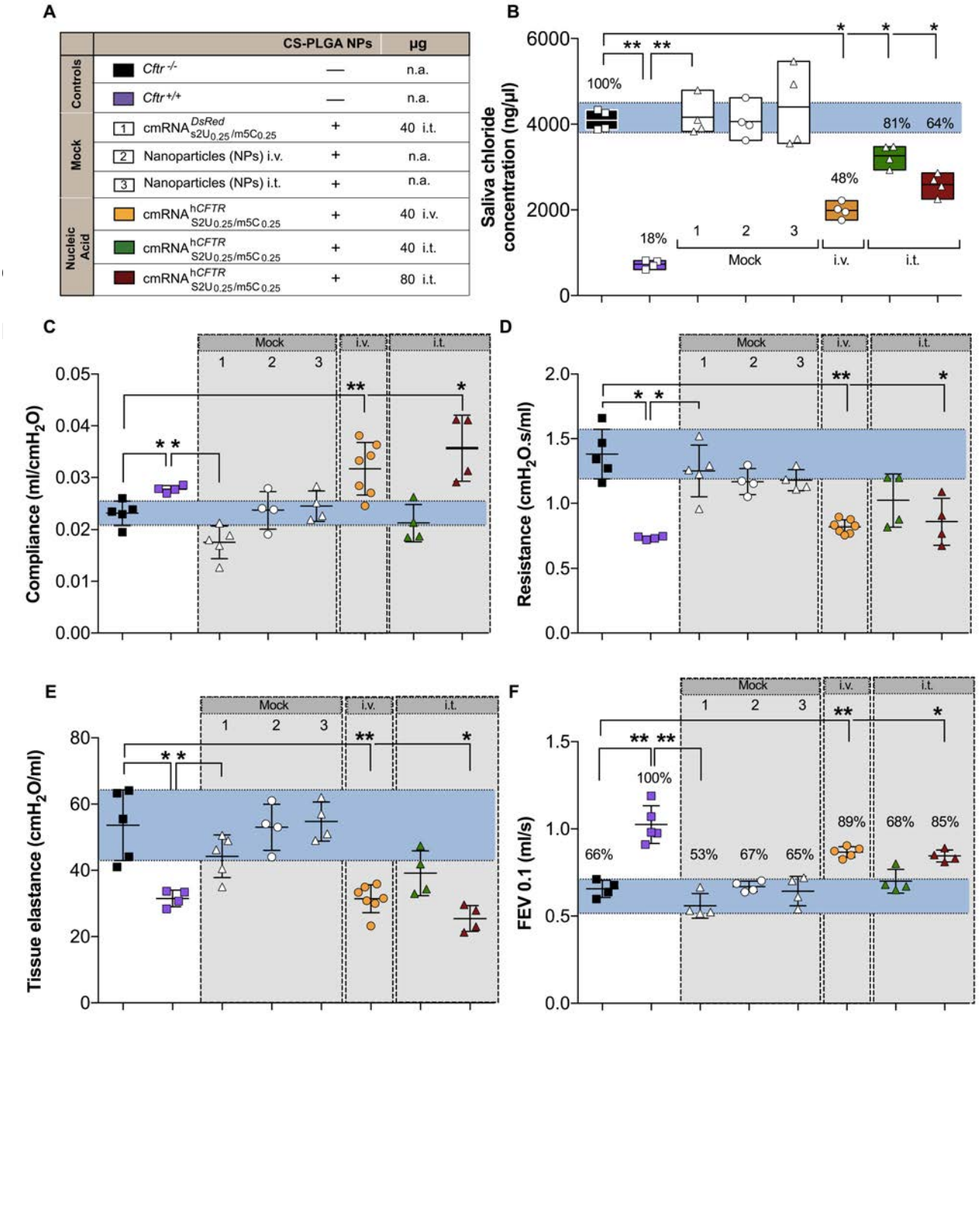
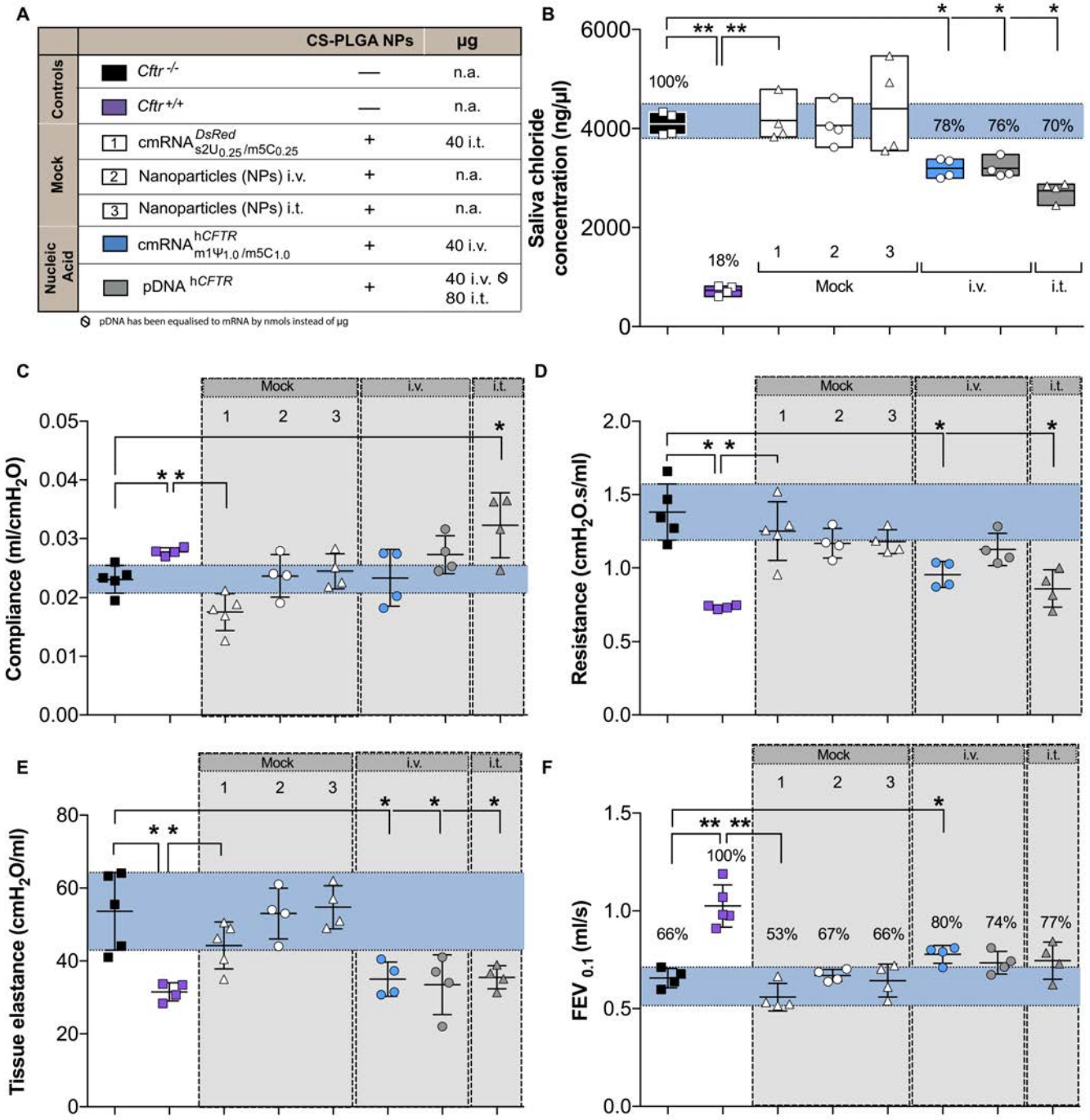
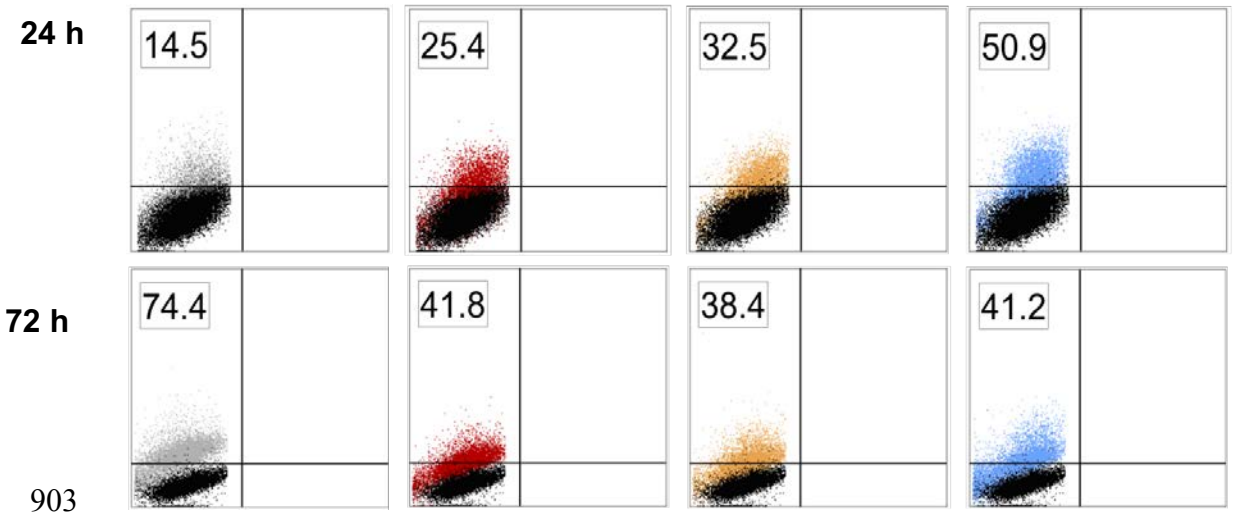


Figure 4

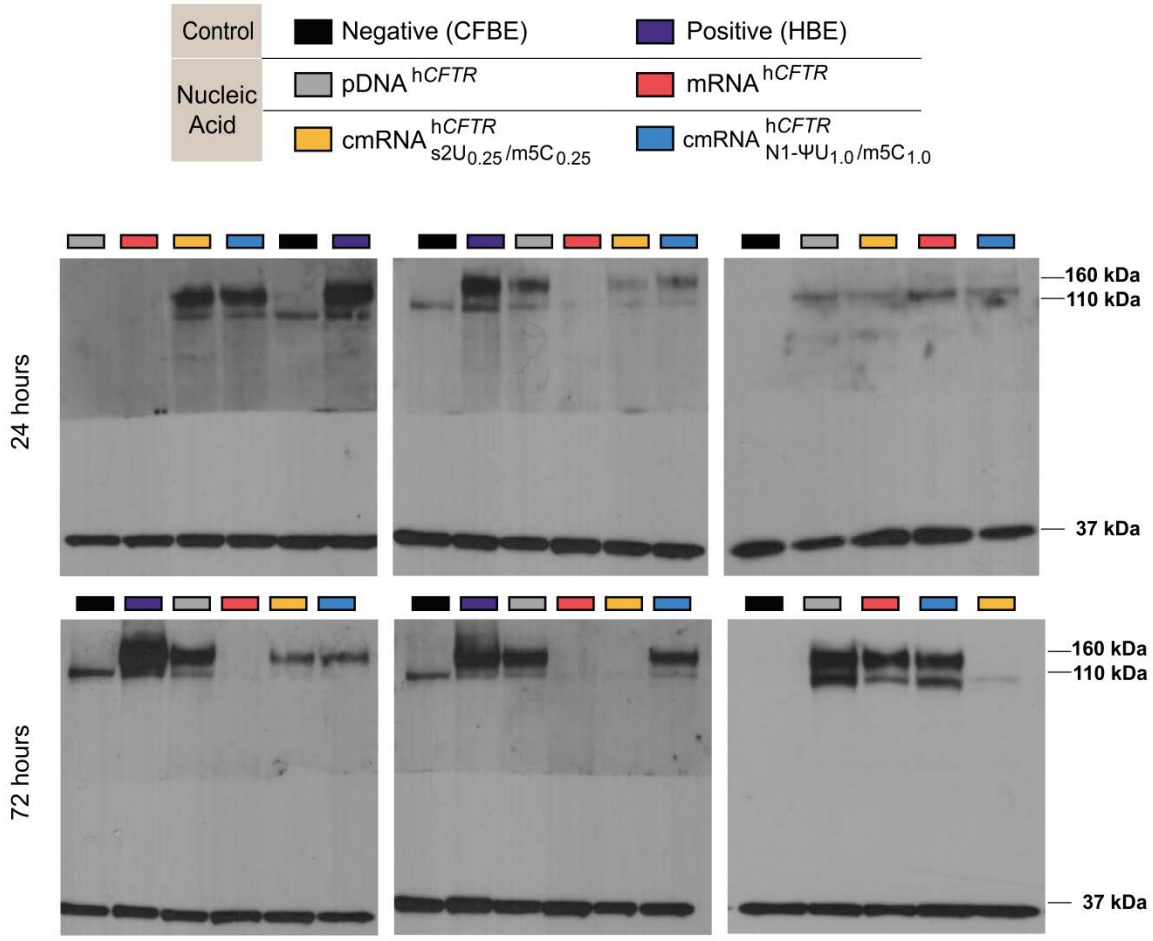




24h	Treatment	Sample #	% pos cells	MFI	Total expression
	pDNA h*CFTR*	1	14,5	15,0	217,5
		2	15,6	15,5	241,8
		3	17,2	16,6	285,5
	mRNA h*CFTR*	1	25,4	22,8	579,1
		2	21,0	17,3	363,3
		3	24,6	19,9	489,5
	cmRNA h*CFTR*,s2U$_{0.25}$/m5C$_{0.25}$	1	32,1	32,4	1040,0
		2	33,0	33,0	1089,0
		3	35,7	35,3	1260,2
	cmRNA h*CFTR*,N1Ψ$_{1.0}$/m5C$_{1.0}$	1	50,9	57,2	2911,5
		2	48,0	55,1	2644,8
		3	50,0	56,1	2805,0
72h	Treatment	Sample #	% pos cells	MFI	Total expression
	pDNA h*CFTR*	1	80,6	14,2	1140,5
		2	72,9	13,8	1007,5
		3	75,3	13,4	1007,5
	mRNA h*CFTR*	1	52,7	9,3	488,0
		2	55,8	10,0	555,2
		3	54,2	9,4	507,3
	cmRNA h*CFTR*,s2U$_{0.25}$/m5C$_{0.25}$	1	45,8	7,8	355,9
		2	42,6	8,0	372,3
		3	47,1	8,8	416,4
	cmRNA h*CFTR*,N1Ψ$_{1.0}$/m5C$_{1.0}$	1	48,6	7,9	385,4
		2	45,3	8,8	434,3
		3	44,8	6,4	305,6

Fig. S1: Expression analyses of hCFTR protein by flow cytometry; n=3

907 **Figure S2**



908

909 **Fig. S2:** Western blot analyses of hCFTR protein in CFBE and HBE cells; n=3.

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Source data:

Figure 1A: Total expression, **n=3**, 24hour- **Source 1**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

				P values (95% CI)
h*CFTR* pDNA	210.50	241.80	285.52	**N/A**
mRNA h*CFTR*	519.12	363.30	489.54	**0.05**
cmRNA h*CFTR* S2U$_{25}$ m5C$_{25}$	1040.04	1089.40	1260.21	**0.05**
cmRNA h*CFTR* m1U$_{25}$ m5C$_{25}$	2911.48	2644.80	2805.31	**0.05**

Figure 1A: Total expression, **n=3**, 72hour- **Source 2**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

				P values (95% CI)
h*CFTR* pDNA	1140.49	1007.47	1007.51	**N/A**
mRNA h*CFTR*	488.02	555.21	507.31	**0.05**
cmRNA h*CFTR* S2U$_{25}$ m5C$_{25}$	355.86	372.33	416.36	**0.05**
cmRNA h*CFTR* m1U$_{25}$ m5C$_{25}$	385.39	434.33	305.59	**0.05**

Figure 1A : Median Fluorescence Intensity, **n=3**, 24hour- **Source 3**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

				P values (95% CI)
h*CFTR* pDNA	15	15.5	16.6	**N/A**
mRNA h*CFTR*	22.8	17.3	19.9	**0.05**
cmRNA h*CFTR* S2U$_{25}$ m5C$_{25}$	32.4	33	35.3	**0.05**
cmRNA h*CFTR* m1U$_{25}$ m5C$_{25}$	57.2	55.1	56.1	**0.05**

Figure 1A: hCFTR Positive cell(%) **n=3**, 24hour- **Source 4**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

				P values (95% CI)
h*CFTR* pDNA	14.5	15.6	17.2	**N/A**
mRNA h*CFTR*	25.4	21	24.6	**0.05**
cmRNA h*CFTR* S2U$_{25}$ m5C$_{25}$	32.1	33	35.7	**0.05**
cmRNA h*CFTR* m1U$_{25}$ m5C$_{25}$	50.9	48	50	**0.05**

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Figure 1A: Median Fluorescence Intensity, **n=3**, 72 hours- **Source 5**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

				P values (95% CI)
h*CFTR* pDNA	14.15	13.82	13.38	**N/A**
mRNA h*CFTR*	9.26	9.95	9.36	**0.05**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$	7.77	7.99	8.84	**0.05**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$	7.93	8.81	6.42	**0.05**

Figure 1A: hCFTR Positive cells (%), **n=3**, 72 hours- **Source 6**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

				P values (95% CI)
h*CFTR* pDNA	80.6	72.9	75.3	**N/A**
mRNA h*CFTR*	52.7	55.8	54.2	**0.05**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$	45.8	42.6	47.1	**0.05**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$	48.6	45.3	44.8	**0.05**

Figure 1B: hCFTR expression by Western blot, n=3- Source 7
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

	24hours			**P value (95%CI)**	72hours			**P value (95%CI)**
h*CFTR* pDNA	0	8.31	60.2	**0.2**	136	70	76	**0.05**
mRNA h*CFTR*	16.37	55.55	56.14	**0.05**	65	16	23	**0.05**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$	73.6	32.93	54.19	**0.05**	11	29	48	**0.05**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$	72.28	33.05	64.59	**0.05**	85	38	67	**0.05**

Figure 1C: YFP assay for CFTR function, **n=4** for each time point. **Source 8**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

	24 hours				**P value (95%CI)**	48 hours				**P value (95%CI)**	72 hours				**P value (95%CI)**
h*CFTR* pDNA	0.86	1.48	1.18	0.64	**0.99**	1.97	1.67	2.59	1.98	**0.028**	1.61	2.04	1.76	1.97	**0.028**
mRNA h*CFTR*	0.58	0.89	0.29	0.74	**NA**	1.47	1.74	1.36	1.35	**0.028**	0.93	0.95	1.08	0.68	**0.3134**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$	0.94	1.30	0.68	0.95	**0.3134**	2.13	1.52	1.88	1.27	**0.028**	0.78	1.22	0.68	1.38	**0.99**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$	1.79	1.55	0.83	1.30	**0.3134**	1.53	2.09	1.88	2.32	**0.028**	0.38	0.74	0.93	0.73	**0.028**

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Figure 2A: Expression fold change by qPCR. **Source 9**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

						P value (95%CI)
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$ 40μgi.v.	19.51	27.61	24.01		**n=3**	**0.028**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$ 40μgi.v.	1.25	12.38	5.076		**n=3**	**0.028**
h*CFTR* pDNA i.v 40ug	10.03	3.39	0.43		**n=3**	**0.028**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$ 40μgi.t.	1	1	1		**n=3**	**N/A**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$ 80μgi.t.	1.40	1.70	1.80	2.03	**n=4**	**0.028**
h*CFTR* pDNA i.t 80 ug	2.41	1.75	2.08	1.94	**n=4**	**0.028**

Figure 2B: hCFTR Elisa. **Source 10**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

								P value (95%CI)
Cftr	1.58	1.71	2.11	1.79			**n=4**	**N/A**
cmRNA DsRed S2U$_{0.25}$ m5C$_{0.25}$ 40μgi.t.	1.75	1.52	1.87	2.49			**n=4**	**0.88**
Nanoparticles (NPs) i.v.	1.85	1.79	1.06	1.69			**n=4**	**0.88**
Nanoparticles (NPs) i.t.	1.81	1.94	1.21	1.78			**n=4**	**0.88**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$ 40μgi.v.	2.79	2.44	4.91	3.19	3.34	2.32	**n=5**	**0.0061**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$ 40μgi.v.	2.29	2.54	1.50	2.50			**n=4**	**0.3429**
pDNA h*CFTR* 40μg i.v.	2.25	2.02	1.54	1.81			**n=4**	**0.6857**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$ 40μgi.t.	1.46	2.25	2.39	2.87			**n=4**	**0.3429**
cmRNA h*CFTR* S2U$_{0.25}$ m5C$_{0.25}$ 80μgi.t.	2.65	4.35	3.67	2.35			**n=4**	**0.0286**
pDNA h*CFTR* 80μg i.t.	3.75	2.93	2.19	3.97			**n=4**	**0.0286**

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Figure 2C: Immune Assay INF-Alpha *in vivo*, n=4 for each time point. Source 11
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant
No Significant P value can be measured

	6 hours				24 hours				72 hours			
Cftr	0	0	0	0	0	0	0	0	0	0	0	0
Nanoparticles (NPs) i.v.	0	0	0	0	0	0	0	0	0	0	0	0
E.coli	61.05	63.91	69.62	59.48	45.97	49.25	0	0	0	0	0	0
pDNA h*CFTR* 40μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0
mRNA h*CFTR* 40μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0
cmRNA h*CFTR* $S2U_{0.25}$ $m5C_{0.25}$ 40μgi.v	0	0	0	0	0	0	0	0	0	0	0	0
cmRNA h*CFTR* $m1U_{1.0}$ $m5C_{1.0}$ 40 μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0
cmRNA h*CFTR* $S2U_{0.25}$ $m5C_{0.25}$ 40μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0

Figure 2C: Immune Assay TNF-Alpha *in vivo*, n=4 for each time point. Source 12
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant
No Significant P value can be measured

	6 hours				24 hours				72 hours			
Cftr	0	0	0	0	0	0	0	0	0	0	0	0
Nanoparticles (NPs) i.v.	0	0	0	0	0	0	0	0	0	0	0	0
E.coli	17.21	15.70	17.68	16.98	0	0	0	0	0	0	0	0
pDNA h*CFTR* 40μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0
mRNA h*CFTR* 40μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0
cmRNA h*CFTR* $S2U_{0.25}$ $m5C_{0.25}$ 40μgi.v	0	0	0	0	0	0	0	0	0	0	0	0
cmRNA h*CFTR* $m1U_{1.0}$ $m5C_{1.0}$ 40 μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0
cmRNA h*CFTR* $S2U_{0.25}$ $m5C_{0.25}$ 40μgi.v.	0	0	0	0	0	0	0	0	0	0	0	0

Figure 2D : Whole blood assay INF-Alpha (*ex vivo*) **Donor (n=3) Source 13**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

Donor 1	6 hours		**P value (95%CI)**	24 hours		**P value (95%CI)**
Blood	0	0	**N/A**	0	0	**N/A**
NP	0	0	**N/A**	0	0	**N/A**
R848	125.63	119.24	**0.05**	103.69	96.63	**0.05**
pDNA h*CFTR*	7.80	16.56	**0.05**	23.58	21.69	**0.05**
mRNA h*CFTR*	35.64	32.63	**0.05**	12.25	13.03	**0.05**

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cmRNA h*CFTR* S2U$_{xx}$ m5C$_{xx}$	0	0	**N/A**	0	0	**N/A**
cmRNA h*CFTR* m1U$_{xx}$ m5C$_{xx}$	0	0	**N/A**	0	0	**N/A**

Donor 2	6 hours		**P value (95%CI)**	24 hours		**P value (95%CI)**
Blood	0	0	**N/A**	0	0	**N/A**
NP	0	0	**N/A**	0	0	**N/A**
R848	188.43	134.90	**0.05**	151.87	165.95	**0.05**
pDNA h*CFTR*	21.74	19.05	**0.05**	21.37	19.49	**0.05**
mRNA h*CFTR*	29.77	27.63	**0.05**	15.84	21.71	**0.05**
cmRNA h*CFTR* S2U$_{xx}$ m5C$_{xx}$	0	0	**N/A**	0	0	**N/A**
cmRNA h*CFTR* m1U$_{xx}$ m5C$_{xx}$	0	0	**N/A**	0	0	**N/A**

Donor 3	6 hours		**P value (95%CI)**	24 hours		**P value (95%CI)**
Blood	0	0	**N/A**	0	0	**N/A**
NP	0	0	**N/A**	0	0	**N/A**
R848	95.20	74.16	**0.05**	90.74	73.63	**0.05**
pDNA h*CFTR*	19.70	18.63	**0.05**	17.89	26.93	**0.05**
mRNA h*CFTR*	26.89	31.32	**0.05**	15.62	21.32	**0.05**
cmRNA h*CFTR* S2U$_{xx}$ m5C$_{xx}$	0	0	**N/A**	0	0	**N/A**
cmRNA h*CFTR* m1U$_{xx}$ m5C$_{xx}$	0	0	**N/A**	0	0	**N/A**

Figure 2D : Whole blood assay TNF-Alpha (*ex vivo*) **Donor (n=3) Source 14**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

Donor 1	6 hours		**P value (95%CI)**	24 hours		**P value (95%CI)**
Blood	143.96	141.98	**N/A**	186.11	187.60	**N/A**
NP	174.47	179.94	**0.99**	159.82	162.08	**0.99**
R848	1120.90	1112.60	**0.05**	1007.07	1021.46	**0.05**
pDNA h*CFTR*	459.40	531.17	**0.05**	200.94	207.50	**0.70**
mRNA h*CFTR*	116.81	130.23	**0.99**	153.08	147.37	**0.99**
cmRNA h*CFTR* S2U$_{xx}$ m5C$_{xx}$	121.79	132.26	**0.99**	152.85	158.69	**0.99**
cmRNA h*CFTR* m1U$_{xx}$ m5C$_{xx}$	125.89	140.19	**0.99**	154.47	162.27	**0.99**

Donor 2	6 hours		**P value (95%CI)**	24 hours		**P value (95%CI)**
Blood	193.33	193.47	**N/A**	231.80	235.07	**N/A**
NP	146.65	147.56	**0.99**	211.78	221.70	**0.99**
R848	1126.01	1124.93	**0.05**	1095.66	1100.5	**0.05**
pDNA h*CFTR*	983.81	982.88	**0.05**	259.15	260.60	**0.99**
mRNA h*CFTR*	148.08	153.05	**0.99**	223.90	208.53	**0.99**

^

cmRNA h*CFTR* S2U$_{0.25}$m5C$_{0.25}$	142.29	143.81	**0.99**	167.02	164.55	**0.99**
cmRNA h*CFTR* m1U$_{0.25}$m5C$_{0.25}$	153.86	155.00	**0.99**	164.18	153.43	**0.99**

Donor 3	6 hours		**P value (95%CI)**	24 hours		**P value (95%CI)**
Blood	346.41	344.34	**N/A**	459.53	450.27	**N/A**
NP	366.34	380.61	**0.99**	403.56	460.78	**0.99**
R848	1079.81	1088.11	**0.05**	1111.01	1084.73	**0.05**
pDNA h*CFTR*	867.98	887.78	**0.05**	536.78	552.76	**0.99**
mRNA h*CFTR*	401.89	404.76	**0.99**	484.33	544.94	**0.99**
cmRNA h*CFTR* S2U$_{0.25}$m5C$_{0.25}$	413.58	462.21	**0.99**	537.38	537.27	**0.99**
cmRNA h*CFTR* m1U$_{0.25}$m5C$_{0.25}$	372.28	407.73	**0.99**	582.94	569.24	**0.99**

Figure 3B: Salivary Chloride assay, **n=4**: **Source 15**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

					P value (95%CI)
Cftr$^{-/-}$	4263.55	3876.60	4331.90	3905.40	**N/A**
Cftr$^{+/+}$	797.33	699.35	819.34	608.47	**0.028**
cmRNA DsRed S2U$_{0.25}$m5C$_{0.25}$ 40 µgi.v	4103.80	3912.10	4792.50	3834.76	**0.99**
Nanoparticles (NPs) i.v.	4615.20	3976.31	4047.64	3621.93	**0.99**
Nanoparticles (NPs) i.t.	3656.50	4934.50	5467.91	3550.96	**0.99**
cmRNA h*CFTR* S2U$_{0.25}$m5C$_{0.25}$ 40 µg i.v.	1764.35	2016.40	1952.50	2218.75	**0.028**
cmRNA hCFTR S2U$_{0.25}$m5C$_{0.25}$ 40 µg i.t.	3187.90	2931.59	3479.70	3461.25	**0.028**
cmRNA hCFTR S2U$_{0.25}$m5C$_{0.25}$ 80 µg i.t.	2252.97	2553.87	2862.01	2698.51	**0.028**

Figure 3C: Lung Compliance. **Source Data 16**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

									P value (95%CI)
Cftr$^{-/-}$	0.01951	0.02338	0.02288	0.02380	0.02690			**n=5**	**N/A**

^

Cftr$^{-/-}$	0.02857	0.02767	0.02693	0.02780				**n=4**	**0.0159 /0.159**
cmRNA DsRed S2U$_{0.25}$m5C$_{0.25}$ 40 μgi.v	0.01803	0.01891	0.02122	0.01692	0.01267			**n=5**	**0.0159**
Nanoparticles (NPs) i.v.	0.01910	0.02403	0.02376	0.02787				**n=4**	**0.7302**
Nanoparticles (NPs) i.t.	0.02833	0.02183	0.02258	0.02518				**n=4**	**0.9048**
cmRNA h*CFTR* S2U$_{0.25}$m5C$_{0.25}$ 40 μg i.v.	0.03629	0.02453	0.03325	0.03424	0.03809	0.02841	0.02700	**n=7**	**0.0051**
cmRNA hCFTR S2U$_{0.25}$m5C$_{0.25}$ 40 μg i.t.	0.01874	0.01854	0.02621	0.02136				**n=4**	**0.4127**
cmRNA hCFTR S2U$_{0.25}$m5C$_{0.25}$ 80 μg i.t.	0.04120	0.04109	0.02912	0.03129				**n=4**	**0.0159**

Figure 3D:Lung Resistance. **Source 17**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

									P value (95%CI)
Cftr$^{-/-}$	1.65944	1.34315	1.46782	1.27120	1.16761			**n=5**	**N/A**
Cftr$^{+/+}$	0.73260	0.72022	0.74576	0.74889				**n=4**	**0.0159 /0.0159**
cmRNA DsRed S2U$_{0.25}$m5C$_{0.25}$ 40 μgi.v	1.52008	0.95717	1.29285	1.22658	1.25748			**n=5**	**0.4206**
Nanoparticles (NPs) i.v.	1.17250	1.05098	1.15327	1.29601				**n=4**	**0.111**
Nanoparticles (NPs) i.t.	1.10923	1.18400	1.13412	1.29250				**n=4**	**0.111**
cmRNA h*CFTR* S2U$_{0.25}$m5C$_{0.25}$ 40 μg i.v.	0.87531	0.78846	0.89476	0.77062	0.75756	0.82743	0.83438	**n=7**	**0.0025**
cmRNA hCFTR S2U$_{0.25}$m5C$_{0.25}$ 40 μg i.t.	0.81922	1.19989	1.19767	0.87647				**n=4**	**0.0635**
cmRNA hCFTR S2U$_{0.25}$m5C$_{0.25}$ 80 μg i.t.	0.67500	0.76931	1.08940	0.90550				**n=4**	**0.0159**

Figure 3E: Tissue Elastance. **Source Data 18**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

									P value (95%CI)
Cftr$^{-/-}$	64.12	41.06	44.07	55.52	63.26			**n=5**	**N/A**
Cftr$^{+/+}$	28.36	33.40	33.67	30.68				**n=4**	**0.0159 /0.0159**
cmRNA DsRed S2U$_{0.25}$m5C$_{0.25}$	48.93	50.57	35.04	46.23	40.47			**n=5**	**0.222**

^

40 μgi.v									
Nanoparticles (NPs) i.v.	53.65	61.42	44.21	54.56				**n=4**	**0.7302**
Nanoparticles (NPs) i.t.	57.40	62.43	49.50	51.20				**n=4**	**0.999**
cmRNA h*CFTR* $S2U_{0.25}m5C_{0.25}$ 40 μg i.v.	30.03	31.58	34.95	33.39	35.88	30.85	23.25	**n=7**	**0.0025**
cmRNA hCFTR $S2U_{0.25}m5C_{0.25}$ 40 μg i.t.	32.94	34.33	47.41	41.88				**n=4**	**0.111**
cmRNA hCFTR $S2U_{0.25}m5C_{0.25}$ 80 μg i.t.	21.30	23.05	27.86	29.61				**n=4**	**0.0159**

Figure 3F: $FEV_{0.1}$. **Source Data 19**
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

							P value (95%CI)
Cftr$^{-/-}$	0.598	0.678	0.712	0.638		**n=5**	**N/A**
Cftr$^{-/-}$	0.910	0.975	1.190	1.071	0.980	**n=4**	**0.0079 /0.0079**
cmRNA DsRed $S2U_{0.25}m5C_{0.25}$ 40 μgi.v	0.515	0.532	0.665	0.523		**n=5**	**0.0571**
Nanoparticles (NPs) i.v.	0.686	0.703	0.638	0.650		**n=4**	**0.3858**
Nanoparticles (NPs) i.t.	0.610	0.542	0.703	0.721		**n=4**	**0.500**
cmRNA h*CFTR* $S2U_{0.25}m5C_{0.25}$ 40 μg i.v.	0.873	0.825	0.849	0.886	0.904	**n=5**	**0.0079**
cmRNA hCFTR $S2U_{0.25}m5C_{0.25}$ 40 μg i.t.	0.655	0.672	0.689	0.834		**n=4**	**0.2429**
cmRNA hCFTR $S2U_{0.25}m5C_{0.25}$ 80 μg i.t.	0.830	0.895	0.851	0.810		**n=4**	**0.0143**

Figure 4B. Salivary Assay, n=4: Source Data 20
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

					P value (95%CI)
Cftr$^{-/-}$	4263.55	3876.60	4331.20	3905.00	**N/A**
Cftr$^{-/-}$	797.33	699.35	819.34	608.47	**0.028**
cmRNA DsRed $S2U_{0.25}m5C_{0.25}$ 40 μgi.v	4103.80	3912.10	4792.50	3834.70	**0.99**
Nanoparticles (NPs) i.v.	4615.31	3976.77	4047.40	3621.51	**0.99**

^

Nanoparticles (NPs) i.t.	3656.50	4934.50	5467.30	3550.51	**0.99**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$ 40 µgi.v.	3383.15	3059.39	2999.75	3345.875	**0.0286**
pDNA hCFTR 40 µgi.v.	3088.50	3479.99	3159.52	3053.40	**0.0286**
pDNA hCFTR 80 µgi.t.	2875.56	2804.50	2840.38	2449.50	**0.0286**

Figure 4C : Lung Compliance. Source Data 21

Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

							P value (95%CI)
Cftr$^{-/-}$	0.01951	0.02338	0.02288	0.02380	0.02670	**n=5**	**N/A**
Cftr$^{-/-}$	0.02857	0.02767	0.02693	0.02780		**n=4**	**0.0159 /0.159**
cmRNA DsRed S2U$_{0.25}$ m5C$_{0.25}$ 40 µgi.v	0.01803	0.01891	0.02122	0.01690	0.01268	**n=5**	**0.0159**
Nanoparticles (NPs) i.v.	0.01910	0.02403	0.02376	0.02787		**n=4**	**0.7302**
Nanoparticles (NPs) i.t.	0.02833	0.02183	0.02258	0.02510		**n=4**	**0.9048**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$ 40 µgi.v.	0.02027	0.02748	0.01823	0.02745		**n=4**	**0.9048**
pDNA hCFTR 40 µgi.v.	0.03159	0.02526	0.02450	0.02777		**n=4**	**0.0635**
pDNA hCFTR 80 µgi.t.	0.03650	0.03159	0.02468	0.03628		**n=4**	**0.0317**

Figure 4D: Lung Resistance. Source Data 21

Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

							P value (95%CI)
Cftr$^{-/-}$	1.659	1.343	1.467	1.271	1.160	**n=5**	**N/A**
Cftr$^{-/-}$	0.732	0.720	0.745	0.748		**n=4**	**0.0159 /0.0159**
cmRNA DsRed S2U$_{0.25}$ m5C$_{0.25}$ 40 µgi.v	1.520	0.957	1.292	1.226	1.257	**n=5**	**0.4206**
Nanoparticles (NPs) i.v.	1.172	1.059	1.153	1.296		**n=4**	**0.111**
Nanoparticles (NPs) i.t.	1.109	1.184	1.130	1.2925		**n=4**	**0.111**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$ 40 µgi.v.	1.036	0.891	1.027	0.874		**n=4**	**0.0159**
pDNA hCFTR 40 µgi.v.	1.283	1.119	1.033	1.070		**n=4**	**0.0635**
pDNA hCFTR 80 µgi.t.	0.710	0.817	1.001	0.917		**n=4**	**0.0159**

^

Figure 4E: Tissue Elastance. Source Data 22

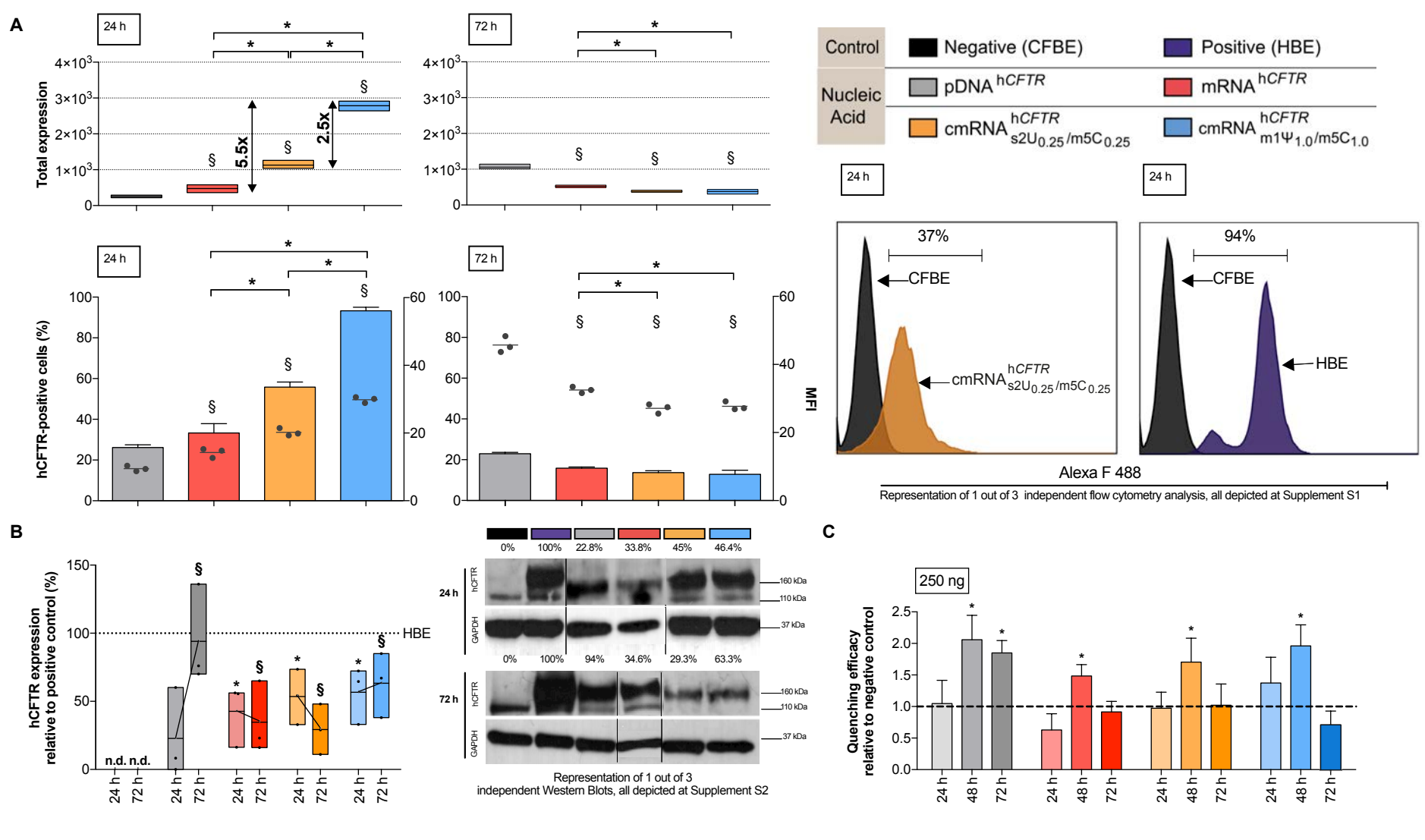
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ (two-sided) was considered statistically significant

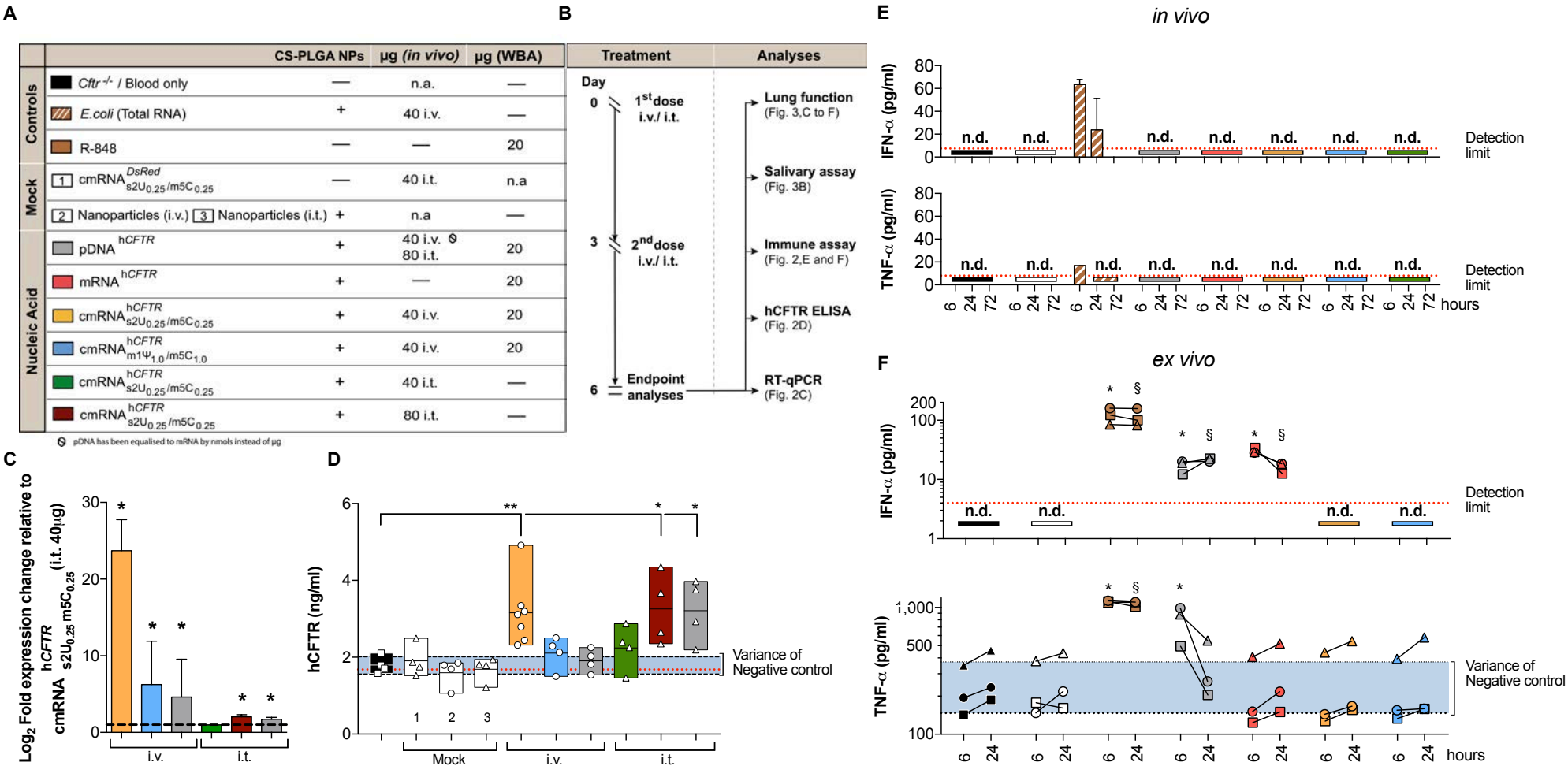
							P value (95%CI)
Cftr$^{-/-}$	64.11	41.06	44.07	55.52	63.27	**n=5**	**N/A**
Cftr$^{+/+}$	28.36	33.41	33.67	30.69		**n=4**	**0.0159 /0.0159**
cmRNA DsRed S2U$_{0.25}$ m5C$_{0.25}$ 40 µgi.v	48.93	50.57	35.04	46.23	40.48	**n=5**	**0.222**
Nanoparticles (NPs) i.v.	53.60	61.30	44.70	54.32		**n=4**	**0.7302**
Nanoparticles (NPs) i.t.	57.60	62.08	49.85	51.70		**n=4**	**0.999**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$ 40 µgi.v.	37.31	31.49	40.52	30.76		**n=4**	**0.0159**
pDNA hCFTR 40 µgi.v.	37.52	41.61	34.88	22.43		**n=4**	**0.0159**
pDNA hCFTR 80 µgi.t.	31.39	36.65	35.08	38.98		**n=4**	**0.0159**

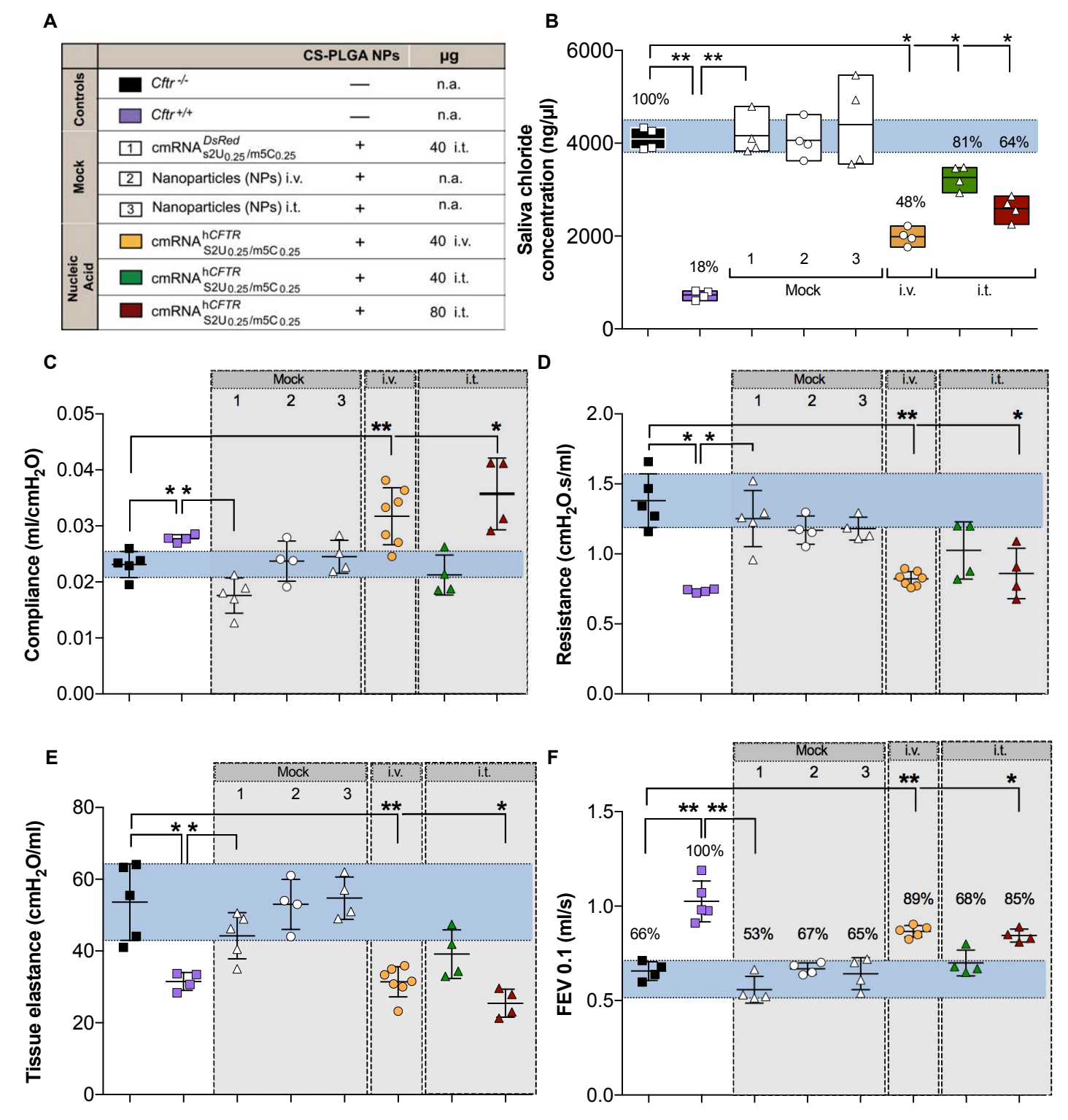
Figure 4E. FEV$_{0.1}$ Source Data 23.

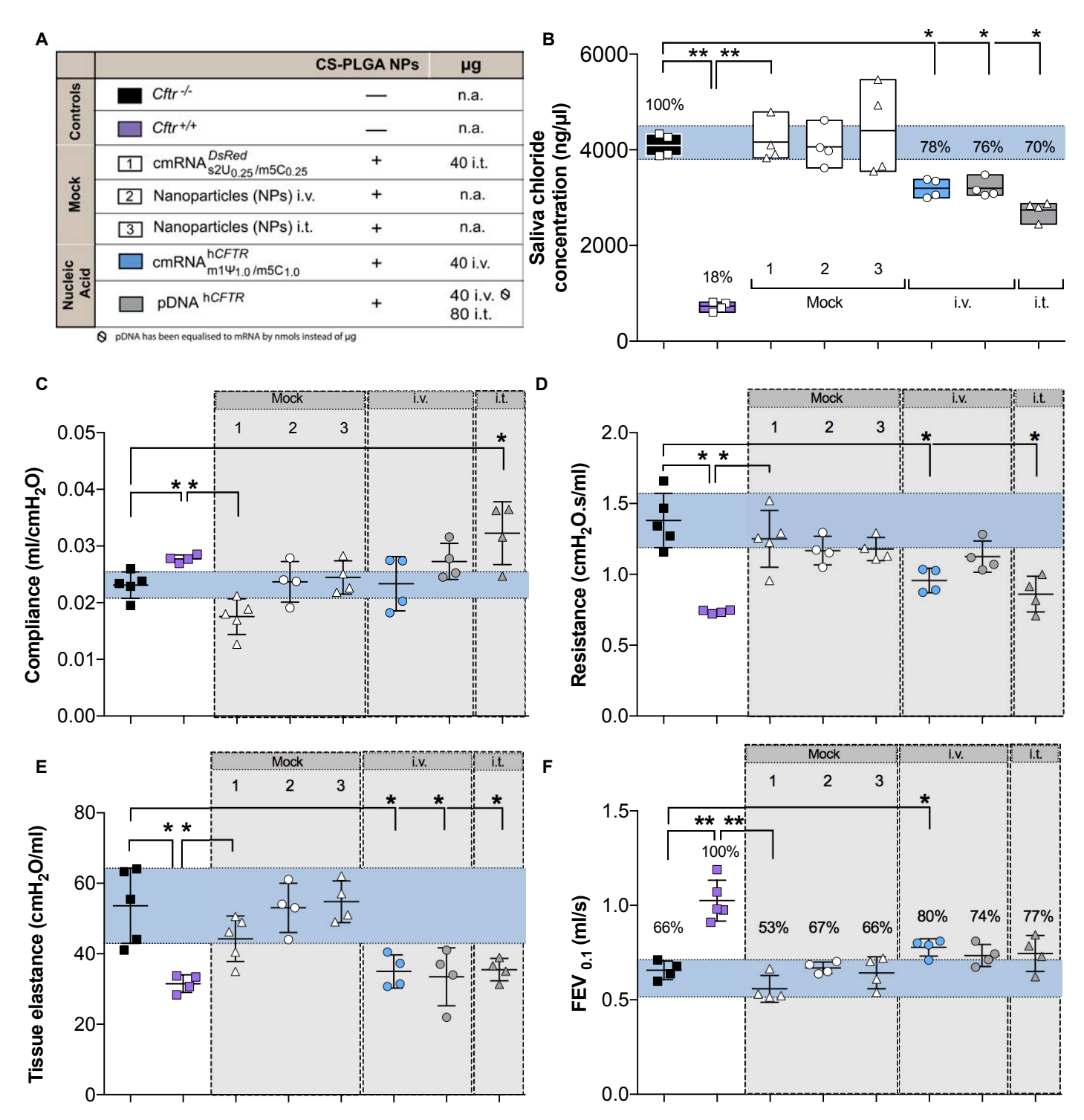
Statistic: Wilcoxon-Mann-Whitney test and $P \leq 0.05$ was considered statistically significant

							P value (95%CI)
Cftr$^{-/-}$	0.60	0.68	0.71	0.64		**n=4**	**N/A**
Cftr$^{+/+}$	0.91	0.98	1.19	1.07	0.98	**n=5**	**0.0079 /0.0079**
cmRNA DsRed S2U$_{0.25}$ m5C$_{0.25}$ 40 µgi.v	0.52	0.53	0.67	0.52		**n=4**	**0.0571**
Nanoparticles (NPs) i.v.	0.69	0.70	0.64	0.65		**n=4**	**0.3858**
Nanoparticles (NPs) i.t.	0.61	0.54	0.70	0.72		**n=4**	**0.500**
cmRNA h*CFTR* m1U$_{1.0}$ m5C$_{1.0}$ 40 µgi.v.	0.71	0.81	0.79	0.80		**n=4**	**0.0286**
pDNA hCFTR 40 µgi.v.	0.71	0.74	0.81	0.68		**n=4**	**0.0571**
pDNA hCFTR 80 µgi.t.	0.84	0.73	0.79	0.62		**n=4**	**0.100**


A
24 h
72 h
Total expression
5.5x
2.5x
hCFTR-positive cells (%)
MFI
Control
Nucleic Acid
Negative (CFBE)
Positive (HBE)
pDNA hCFTR
mRNA hCFTR
cmRNA hCFTR s2U0.25/m5C0.25
cmRNA hCFTR m1Ψ1.0/m5C1.0
37%
94%
CFBE
HBE
Alexa F 488
Representation of 1 out of 3 independent flow cytometry analysis, all depicted at Supplement S1
B
hCFTR expression relative to positive control (%)
n.d. n.d.
HBE
0%
100%
22.8%
33.8%
45%
46.4%
hCFTR
GAPDH
160 kDa
110 kDa
37 kDa
0%
100%
94%
34.6%
29.3%
63.3%
Representation of 1 out of 3 independent Western Blots, all depicted at Supplement S2
C
250 ng
Quenching efficacy relative to negative control


A
CS-PLGA NPs
µg (in vivo)
µg (WBA)
Controls
Cftr -/- / Blood only — n.a. —
E.coli (Total RNA) + 40 i.v. —
R-848 — — 20
Mock
1 cmRNA DsRed s2U0.25/m5C0.25 — 40 i.t. n.a
2 Nanoparticles (i.v.) 3 Nanoparticles (i.t.) + n.a —
Nucleic Acid
pDNA hCFTR + 40 i.v. 80 i.t. 20
mRNA hCFTR + — 20
cmRNA hCFTR s2U0.25/m5C0.25 + 40 i.v. 20
cmRNA hCFTR m1Ψ1.0/m5C1.0 + 40 i.v. 20
cmRNA hCFTR s2U0.25/m5C0.25 + 40 i.t. —
cmRNA hCFTR s2U0.25/m5C0.25 + 80 i.t. —
pDNA has been equalised to mRNA by nmols instead of µg
B
Treatment
Analyses
Day
0 1st dose i.v./ i.t.
3 2nd dose i.v./ i.t.
6 Endpoint analyses
Lung function (Fig. 3,C to F)
Salivary assay (Fig. 3B)
Immune assay (Fig. 2,E and F)
hCFTR ELISA (Fig. 2D)
RT-qPCR (Fig. 2C)
C
Log2 Fold expression change relative to hCFTR cmRNA s2U0.25 m5C0.25 (i.t. 40µg)
i.v.
i.t.
D
hCFTR (ng/ml)
Variance of Negative control
Mock
i.v.
i.t.
E
in vivo
IFN-α (pg/ml)
TNF-α (pg/ml)
n.d.
Detection limit
hours
F
ex vivo
IFN-α (pg/ml)
TNF-α (pg/ml)
Detection limit
Variance of Negative control
hours


A
CS-PLGA NPs
µg
Controls
Cftr -/-
—
n.a.
Cftr +/+
—
n.a.
Mock
1 cmRNA DsRed s2U0.25/m5C0.25
+
40 i.t.
2 Nanoparticles (NPs) i.v.
+
n.a.
3 Nanoparticles (NPs) i.t.
+
n.a.
Nucleic Acid
cmRNA hCFTR S2U0.25/m5C0.25
+
40 i.v.
cmRNA hCFTR S2U0.25/m5C0.25
+
40 i.t.
cmRNA hCFTR S2U0.25/m5C0.25
+
80 i.t.
B
Saliva chloride concentration (ng/µl)
6000
4000
2000
0
100%
81%
64%
48%
18%
1
2
3
Mock
i.v.
i.t.
C
Compliance (ml/cmH2O)
0.05
0.04
0.03
0.02
0.01
0.00
Mock
i.v.
i.t.
D
Resistance (cmH2O.s/ml)
2.0
1.5
1.0
0.5
0.0
E
Tissue elastance (cmH2O/ml)
80
60
40
20
0
F
FEV 0.1 (ml/s)
1.5
1.0
0.5
0.0
100%
66%
53%
67%
65%
89%
68%
85%


A
CS-PLGA NPs
µg
Controls
Cftr -/-
—
n.a.
Cftr +/+
—
n.a.
Mock
1 cmRNA DsRed s2U0.25/m5C0.25
+
40 i.t.
2 Nanoparticles (NPs) i.v.
+
n.a.
3 Nanoparticles (NPs) i.t.
+
n.a.
Nucleic Acid
cmRNA hCFTR m1Ψ1.0/m5C1.0
+
40 i.v.
pDNA hCFTR
+
40 i.v. ⊘ 80 i.t.
⊘ pDNA has been equalised to mRNA by nmols instead of µg
B
Saliva chloride concentration (ng/µl)
6000
4000
2000
0
100%
18%
78%
76%
70%
1
2
3
Mock
i.v.
i.t.
C
Compliance (ml/cmH2O)
0.05
0.04
0.03
0.02
0.01
0.00
Mock
i.v.
i.t.
D
Resistance (cmH2O.s/ml)
2.0
1.5
1.0
0.5
0.0
E
Tissue elastance (cmH2O/ml)
80
60
40
20
0
F
FEV0.1 (ml/s)
1.5
1.0
0.5
0.0
100%
66%
53%
67%
66%
80%
74%
77%