Thymosin α1 represents a potential potent single-molecule-based therapy for cystic fibrosis

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Cystic fibrosis (CF) is caused by mutations in the gene encoding the CFTR protein (see URLs)¹. Presently, various pharmacological agents that target specific classes of CFTR mutations to address dysfunctional CFTR activities in CF improve CFTR function and alter the disease process². These agents generally come in two classes: potentiators and correctors. The former promote an increase in the residual activity of mutant CFTR proteins, whereas the latter promote proper folding or trafficking of mutant CFTR to increase its activity. The p.Phe508del mutation in the first nucleotide-binding domain, which is the most common mutation among individuals with CF, results in the production of a misfolded protein with residual activity that is degraded by the ubiquitin–proteasome system during biogenesis²,³. Corrector drugs rescue trafficking of p.Phe508del-CFTR to the plasma membrane by directly targeting the mutant protein²,⁴. However, in spite of their high efficacy in vitro, CFTR correctors show modest clinical benefits in individuals with CF who harbor the p.Phe508del mutation, even when these correctors are combined with the CFTR potentiator ivacaftor⁵,⁶. Given the complex molecular and cellular defects that occur in CF, a multidrug approach is desirable to both rescue and stabilize p.Phe508del-CFTR in vivo and, hence, prevent the progressive decline of lung function in most individuals with CF⁷. Recently, general strategies aimed at improving the proteostasis network by means of proteostasis regulators have emerged as an alternative approach to promote p.Phe508del-CFTR plasma membrane targeting and stability⁸,⁹. Instead of directly targeting the mutant protein, proteostasis regulators favor p.Phe508del-CFTR rescue and stability by altering signaling pathways and checkpoints in cellular proteostasis⁸,¹⁰.

Optimal CF treatments should not only rescue CFTR localization and functionality but also alleviate the associated hyperinflammatory pathology²,⁸. Rescuing chloride channel activity might be sufficient per se to reverse the inflammatory pathology in chronic CF lung disease¹¹,¹². However, the complexity of the p.Phe508del-CFTR molecular defect makes this task challenging. Irrespective of whether the inflammatory response is due to multiple, additive effects—including chronic infection—or is a primary outcome of CFTR dysfunction, a hyperinflammatory state in individuals with CF is associated, as a rule, with early and nonresolving activation of innate immunity, which impairs microbial clearance and promotes a self-sustaining condition of progressive lung damage¹³. The remarkable persistence of chronic lung infections in the face of intensive antibiotic therapy¹⁴ and, likewise, the major side-effects of both corticosteroids¹⁵ and non-steroidal anti-inflammatory drugs (NSAIDs), including inhibition of the CFTR chloride channel¹⁶, have revealed the need for therapeutic approaches aimed at activating early anti-inflammatory pathways to

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In vivo to C57BL/6 mice homozygous for a

Concomitantly, contributes to chronic lung disease25. Mice received T

often breaking the vicious circle that perpetuates chronic lung inflam-

Inflammation plays a critical role in lung disease development and pro-

α1 is endowed

α1 restores IDO1 expression both basally and after infection (Fig. 1f)

α1 treatment in knockouts (Supplementary Fig. 2a–d). This further suggested that Tα1, by influencing CF inflammation and microbiology, favorably alters the natural history of the disease.

Tα1 improves the localization and stability of mutant CFTR

Infection and inflammation may produce secondary alterations in 

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**Figure 1** Tα1 limits the inflammatory response in cystic fibrosis via IDO1. (a) Representative images (n = 5 images per treatment) of TLR9 colocalization with transferrin receptor and LAMP-1 on endosomes in HEK293 cells transfected with human TLR9–GFP and stimulated with a suboptimal CpG ODN dose with or without 100 ng/ml Tα1. Scale bars, 100 µm. Shown are merged images of cells (single-channel FITC or TRITC images are on the right). See Supplementary Figure 10 for colocalization coefficients. (b) CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR were treated with Tα1 or 100 U/ml interferon (IFN)-γ as a positive control for 24 h at 37 °C. Shown are representative immunoblots (n = 3) of IDO1 protein. (c) CFBE41o- cells were transfected with p.Phe508del-CFTR and exposed to MALP-2 in the presence of Tα1 for 2 h. Representative immunoblots (n = 3) of NF-κB/p65 (p-p65) and phosphorylated NF-κB/p65 (p-p65) are shown. (d) CFBE41o- cells were transfected with an NF-xB luciferase reporter plasmid and p.Phe508del-CFTR with MALP-2 exposure and Tα1 treatment. NF-xB relative luciferase units (RLU) are shown from n = 3 biological replicates. (e) CFBE41o- cells were transfected with p.Phe508del-CFTR and exposed to CpG ODN in the presence of Tα1 for 2 h. Representative immunoblots (n = 3) of IRF3 and phosphorylated IRF3 (p-IRF3) (top) and IL10 gene expression (n = 3) (bottom) are shown. (f–h) Control C57BL/6 and C57BL/6 F508del mice were infected intranasally with live *A. fumigatus* conidia and treated with 200 µg per kg bodyweight Tα1 intraperitoneally for 6 d. Lungs were assessed for IDO1 protein (f) and caspase-1 cleavage (g) by immunoblotting and examined for histology (periodic acid–Schiff (PAS) staining) and immunofluorescence staining with NLRP3 antibody (n = 5 images per mouse) (h). Scale bars, 100 µm. (i) Immunoblotting and lung sections are representative of three independent experiments with n = 6 mice per group. (j) Fungal growth (log_{10} colony-forming units (CFU)). (k) Number of polymorphonuclear neutrophils (PMNs) in the bronchoalveolar lavage (BAL) and myeloperoxidase (MPO) (l) as well as cytokine (k) production in lung homogenates. Assays were performed 7 d after infection. Data (means ± s.d.) are presented as box-and-whisker plots; bars represent maximal and minimal values, and the center of the box represents the mean. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, two-way ANOVA with Tukey’s post test. None, treatment with scrambled peptide; −, untreated cells.
the early endosomes, prevents movement to the late endosomes and/or lysosomes and favors recycling from the endosomes to the plasma membrane. Thus, Tα1 facilitates proper folding and trafficking of p.Phe508del-CFTR and also stabilizes the rescued CFTR mutant protein at the plasma membrane.

Tα1 rescues CFTR protein through USP36 deubiquitination and autophagy

Next, we measured the half-life of p.Phe508del-CFTR in CFBE41o- cells treated with Tα1 for 24 h at 37 °C. Tα1 did not increase steady-state CFTR mRNA expression (data not shown) and marginally affected the half-life of wild-type (WT) CFTR, but it significantly increased the half-life of p.Phe508del-CFTR, which was shorter (1 h) than that of WT CFTR (4 h) (Fig. 3a,b). The effect of Tα1 could not be traced to inhibition of the proteasomal and lysosomal degradation pathways, as Tα1 did not inhibit degradation of reporter substrates (Supplementary Fig. 4a,b). However, Tα1 was detected in immunoprecipitated p.Phe508del-CFTR from CFBE41o- cells that overexpressed Tα1 or were treated with Tα1 for 2 h at 37 °C. While present in bronchial epithelial cells expressing WT CFTR, Tα1 was not detected in immunoprecipitated p.Phe508del-CFTR from CFBE41o- cells that overexpressed Tα1.

Figure 2 Tα1 increases cell surface expression and stability of p.Phe508del-CFTR. (a) Representative CFTR immunoblots (n = 3) from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR and treated with 100 ng/ml Tα1 or vehicle at various time points. Arrowheads indicate the B and C (mature) forms of CFTR. (b) Percentage of band C to total (bands B + C) CFTR as quantified by densitometry for the cells in a. (c–e) CFTR immunoblots from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR and treated with 100 ng/ml Tα1 or vehicle at various time points. Arrowheads indicate the B and C (mature) forms of CFTR. (f) Relative percentage of band C to total CFTR (expressed as the percentage of cells expressing WT CFTR at 37 °C (control)), as quantified by densitometry for the cells in e (100 ng/ml Tα1). (g) Representative immunoblots (n = 3) of CFTR and FLOT-1 (an integral membrane protein) for purified plasma membranes from CFBE41o- cells incubated with Tα1 for 37 °C. (h) Ratio of plasma membrane CFTR/total CFTR as quantified by densitometry of cells exposed as in g. (i) Immunofluorescence staining of CFTR (n = 5 images per treatment) in CFBE41o- cells transfected with mutant CFTR and treated with Tα1 for 24 h. (j) Immunoblots of CFTR in CFBE41o- cells transfected with mutant CFTR and treated with Tα1 for 24 h at 37 °C and exposed to trypsin. (k) Immunostaining of CFTR with Rab5, Rab7, or Rab9 (n = 5 images per treatment) in CFBE41o- cells transfected with mutant CFTR and treated with Tα1 at 37 °C for 2 h. Shown are merged images of cells. See Supplementary Figure 10 for colocalization coefficients. In i and k, scale bars, 100 μm. Data are presented as means ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, two-way (b) or one-way (g,h) ANOVA with Tukey's post test.
WT CFTR (Supplementary Fig. 5a,b). Overexpression of Tα1 increased CFTR maturation (Supplementary Fig. 5c) and IL10 expression and decreased IL6 expression (Supplementary Fig. 5d), suggesting functional Tα1 activity. In accordance with the ER localization of Tα1 that is different from that of its precursor, prothymosin37, these results indicate that Tα1 associates with p.Phe508del-CFTR.

Figure 3 Tα1 rescues p.Phe508del-CFTR by promoting USP36 deubiquitination. (a,b) Representative immunoblots (n = 3) (a) and corresponding densitometric analysis (b) of CFTR in lysates from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR and treated with 100 ng/ml Tα1 for 24 h at 37 °C and then with cycloheximide (CHX) for up to 6 h as compared to untreated cells. (c) Representative immunoblots (IB) of ubiquitin (Ub) in total lysates and lysates in which CFTR was immunoprecipitated (IP) from CFBE41o- cells transfected with p.Phe508del-CFTR and treated with Tα1 for 2 h (n = 3). (d) Representative immunoblots of HA and USP proteins in lysates from CFBE41o- cells transfected with p.Phe508del-CFTR and expressing HA-Ub-VME in which the HA probe was immunoprecipitated (n = 3). (e,f) Representative immunoblots of USP36 in total lysates (e) or lysates in which CFTR was immunoprecipitated (f) from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR and treated with Tα1 for 30 min or 24 h (n = 3). (g) Colocalization of USP36 with either Rab5 or Rab9 (n = 5 images per treatment) in CFBE41o- cells transfected with p.Phe508del-CFTR and treated with Tα1 for 2 h. Scale bars, 100 µm. See Supplementary Figure 10 for colocalization coefficients. (h) Representative immunoblots of CFTR and USP36 (n = 3) in CFBE41o- cells treated with siRNA specific to USP36 or control siRNA (scrambled version of the siRNA target sequence) or transfected with plasmid encoding USP36. (i) Representative immunoblots of USP36 (n = 3) in lysates in which IDO1 was immunoprecipitated from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR and treated with Tα1 at 37 °C for 2 h. Data are presented as means ± s.d. *P < 0.05, **P < 0.001, ****P < 0.0001, two-way ANOVA with Bonferroni post test.
suggesting a chaperone activity of Tø1 that may affect the protein’s ER quality control and degradation. Tø1 perturbed the physical interaction of p.Phe508del-CFTR with the ER chaperon Hsp70 but not with Hsp90 or calnexin, which are known to assist protein folding or degradation18 (Supplementary Fig. 5e.f).

Poorly folded plasma membrane–bound CFTR is subjected to proteolysis by the ER-associated degradation mechanism via the cytosolic ubiquitin (Ub)–26S proteasome system. Ubiquitination was reduced in immunoprecipitated p.Phe508del-CFTR from CFBE41o- cells treated with Tø1 (Fig. 3c). Most of the ubiquitinated p.Phe508del-CFTR was ~20 kDa in molecular weight. Because the CFTR detected by western blotting was in an ~180 kDa complex and a single ubiquitin has a molecular mass of 8 kDa, it appears that Tø1 reduced mult ubiquitination, a signal that targets proteins in early endosomes for lysosomal degradation.

The removal of ubiquitin by deubiquitinating enzymes (DUBs) regulates sorting of ubiquitinated plasma membrane–associated proteins39. Using a hemagglutinin-tagged ubiquitin probe engineered to regulate sorting of ubiquitinated plasma membrane–associated proteins in early endosomes for lysosomal degradation.

~200 kDa in molecular weight. Because the CFTR detected by western immunoprecipitation p.Phe508del-CFTR from CFBE41o- cells treated with Tø1 (Fig. 3d). Parallelly, the promotion of mature CFTR (Fig. 2), the induction of USP36 occurred at 30 min after initial exposure and was still present at 24 h of exposure (Fig. 3e). USP36 associated with immunoprecipitated p.Phe508del-CFTR (Fig. 3f) and deubiquitinated CFTR in Rab5+ endosomes and even more so in Rab9+ endosomes in Tø1-treated cells (Fig. 3g). This is in line with the finding that Rab9 enters the endosomal pathway at the transition stage between early Rab5+ and late Rab7+ endosomes and colocalizes to the trans-Golgi network42. Depletion of USP36 by RNA interference abrogated the rescuing effect of Tø1 on mature p.Phe508del-CFTR, whereas its overexpression further increased the capacity of Tø1 to rescue mutated CFTR (Fig. 3h). Thus, the corrector activity of Tø1 correlated with USP36-mediated deubiquitination of CFTR in endosomes, thereby promoting CFTR trafficking to the post-endocytic compartment and more efficient ER export to the cell surface. USP36 was not involved in promotion of IDO1 activity by Tø1 (Fig. 3i), a finding consistent with the importance of the postendosomal degradation pathway for IDO1 activity21 and, likewise, arguing for USP36 selectivity in CFTR correction.

The ubiquitin system is deeply influenced by autophagy. The increase in CF autoimmunity in CF leads to an increased pool of ubiquitinated proteins, including the ubiquitin-binding protein SQSTM1/p62, which is pivotal in the aggresome sequestration of ubiquitinated p.Phe508del-CFTR as well as in p.Phe508del-CFTR disposal from the plasma membrane after rescue32. Enhanced expression of SQSTM1/p62 lacking the UBA domain stabilizes p.Phe508del-CFTR at the plasma membrane and promotes its recycling10. In accordance with the ability of autophagy to rescue CFTR function9, Tø1 was able to promote IDO1–dependent autophagy (Supplementary Fig. 6a–d), to reduce SQSTM1/p62 levels (Supplementary Fig. 6e) and, notably, to correct CFTR in an autophagy-dependent manner, as indicated by the decreased rescue activity in mice bearing CfrF508del in a Beclin1−/− haploinsufficient background, which confirms a major autophagy defect9 (Supplementary Fig. 6f).

Tø1 increases p.Phe508del-CFTR and CLCA1 function

The corrector and stabilizing capacity of Tø1 would anticipate increased chloride ion channel function of the rescued p.Phe508del-CFTR.

We treated p.Phe508del-CFTR-transfected CFBE41o- cells with Tø1 for either 2 or 24 h at 37 °C before determining the channel open probability (Po) of p.Phe508del-CFTR. Studies have estimated that the extent of correction in p.Phe508del airway epithelial cells must approximate 20–30% of WT CFTR function to provide therapeutic benefit44. Treatment with Tø1 for 2 h restored channel gating of rescued p.Phe508del-CFTR (Fig. 4a) with a fourfold increase in Po, namely, from 0.08 ± 0.02 of untreated CFTR to 0.35 ± 0.07 after treatment (Fig. 4b), with the latter Po values being similar to those for WT CFTR45. A twofold increase in chloride current density was still observed at 24 h after treatment as assessed by whole-cell patch-clamp recordings of cells expressing p.Phe508del-CFTR (Fig. 4c,d), and the rate of iodide efflux would then approximate 70% of the control value in WT cells, as assessed by the halide-sensitive fluorescent probe 6-methoxy-N-(sulfopropyl)quinolinium after stimulation with forskolin (Fig. 4e). This was confirmed by the results obtained in human bronchial epithelial (HBE) cells from a subject with two distinct mutations, p.Gly1244Glu and p.Phe508del, whereby a gating defect46 occurred in association with the p.Phe508del defect. The effect of Tø1 was qualitatively similar, although of a lesser extent, to that obtained with potentiator ivacaftor (Fig. 4f), which is known to restore channel activity in CFTR gating (class III) mutations, including the p.Gly1244Glu mutation47. This is in agreement with recent evidence indicating that proteostasis regulators rescue functional CFTR expression in human and mouse cells bearing only one copy of the p.Phe508del allele9.

Bypassing CFTR by targeting additional ion channels is an alternative strategy to circumvent the CFTR defect48. On the basis of data gathered from DNA microarray analysis of Tø1-treated mice (Supplementary Fig. 7a,b), we assessed whether Tø1 would increase mRNA expression of the calcium-activated chloride channel regulator 1 (CLCA1), a member of the CLCA protein family capable of paracrine modulation of the activity of TMEM16A49, an alternative chloride channel that could obviate the primary defect in CF48. Tø1 potentiated calcium-activated chloride currents (Fig. 4g,h) and persistently increased the expression of CLCA1 (Fig. 4i) in CFBE41o- cells. Depleting CLCA1 with specific small interfering RNAs (siRNAs) (Supplementary Fig. 7) greatly reduced the ion channel activity promoted by Tø1 (Fig. 4e). These findings suggested that Tø1 is endowed with effects that can further ameliorate chloride channel activity in CF.

Tø1 rescues p.Phe508del-CFTR in mice and HBE cells

To determine whether Tø1 corrects CFTR in vivo, we assessed CFTR expression and activity in CfrF508del C57BL/6 mice aged 4 weeks treated daily for 6 d with 200 µg of Tø1 per kg of body weight. Tø1 restored CFTR expression in the lung (Fig. 5a,b) and small intestine (Fig. 5c,d), as assessed by immunoblotting of cell lysates (Fig. 5a,c), and it promoted localization of mature CFTR to the plasma membrane (Fig. 5b,d), as revealed by immunohistochemistry. Functionally, Tø1 treatment restored channel gating in lung epithelial cells with a twofold increase in Po from 0.23 ± 0.02 before stimulation to 0.45 ± 0.04 after forskolin and genisten stimulation (Fig. 5e,f). In intestines from CfrF508del mice mounted in Ussing chambers, Tø1 significantly increased CFTR-dependent chloride ion conductance in response to forskolin, much like the proteostasis regulator cysteamine9 (Fig. 5g,h). Rescue of CFTR activity by Tø1 was also observed in CfrF508del mice on an FVB/129 background (data not shown). Of interest, Tø1 also increased CLCA1 expression in both the lung (Fig. 5i,j) and the gut (Fig. 5k,l) of CfrF508del mice.
Taken together, these data indicated that administration of Tα1 ameliorated chloride ion fluxes in the intestinal and respiratory tracts of Cft5408del mice.

To test the activity of Tα1 in a more relevant clinical setting, HBE cells from five subjects homozygous for the p.Phe508del-CFTR mutation were assessed for CFTR, USP36 protein levels and CLCA1 gene expression as determined by RT–PCR in CFBE41o- cells from five subjects homozygous for the p.Phe508del-CFTR mutation.

Supplementary Figure 10

Figure 4 Tα1 rescues p.Phe508del-CFTR functional activity. (a) CFTR single-channel currents recorded at +100 mV from p.Phe508del-CFTR-transfected CFBE41o- cells treated with 100 ng/ml Tα1 or vehicle for 2 h (dotted lines correspond to the channel’s closed state). (b) Popen calculated at +100 mV (n = 4; ***P < 0.001, Student’s t-test) in cells treated as in a. (c) Time course of whole-cell CFTR current densities induced by forskolin (Fsk) + genistein (Gen) at +50 mV in p.Phe508del-CFTR-transfected CFBE41o- cells treated with 100 ng/ml Tα1 or vehicle for 24 h followed by blockade with CFTR inhibitor 172 (CFTR inh-172). The horizontal bars indicate the time period of drug application (left). Current–voltage (I–V) relationships were elicited by ramps from −100 mV to +50 mV (holding potential, −40 mV) and are constructed for the time points corresponding to the colored dots (right). CFTR inhibitor 172–sensitive chloride current densities were calculated by subtracting the residual current density recorded after the application of CFTR inhibitor 172 from the current density induced by forskolin and genistein (insets). pA/pF, current density. (d) Average CFTR current density induced by forskolin and genistein (n = 8; **P < 0.01, Student’s t-test) in cells treated as in c. (e) Iodide efflux assessed by a fluorescence assay (SPQ) upon forskolin stimulation of CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR and treated with Tα1 at 37 °C (n = 3). Treatment with siRNA targeting CLCA1 or control siRNA was performed 24 h before Tα1 addition for 5 min only. (f) Iodide efflux in cells from a subject with the p.Gly1244Glu gene mutation that were treated with Tα1 or with ivacaftor alone or in combination with lumacaftor for 24 h at 37 °C (n = 3). Results are representative of three experiments. (g) Time course of ionomycin-induced calcium-activated chloride current densities at +50 mV in CFBE41o- cells treated and depicted as in c except that the blockade was with 100 µM niflumic acid (NFA) instead of CFTR inhibitor 172 (left). Current–voltage relationships were elicited and are depicted as in c (right). NFA-sensitive chloride current densities were calculated by subtracting the residual current density recorded after the application of NFA from the ionomycin-induced current density (insets). (h) Average current density (n = 8; ****P < 0.0001, Student’s t-test) in cells treated as in g. (i) CLCA1 expression as determined by RT–PCR in CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR and treated with Tα1 at 37 °C (n = 5). *P < 0.05, **P < 0.01, two-way ANOVA with Bonferroni post test. Data are presented as means ± s.d. For statistical analysis, see Supplementary Figure 10.
Figure 5 Tα1 rescues p.Phe508del-CFTR activity in CfrF508del mice. (a–d) Control C57BL/6 and CfrF508del mice were treated with 200 µg per kg bodyweight Tα1 intraperitoneally for 6 d before the assessment of CFTR protein expression in the lung (a,b) and small intestine (c,d) by immunoblotting of lysates (n = 3) (a,c) and immunohistochemistry (n = 5 images per mouse) (b,d). Scale bars, 25 µm in b, 200 µm in d, and 100 µm in the inset. (e) CFTR single-channel currents recorded at +100 mV from ex vivo purified epithelial cells, obtained from mutant mice, in response to forskolin and genistein (n = 4 experiments) (dotted lines represent the zero-current baseline when the channel is in its closed state). (f) P0 of channels calculated at +100 mV (n = 4) in cells treated as in e. (g,h) Ex vivo ileum-mounted cells from C57BL/6 and CfrF508del mice were placed in Ussing chambers in the presence of CFTR inhibition (CFTR inhibitor 172) and amiloride. Cysteamine was used as a positive control. (g) CFTR-dependent chloride secretion measured by means of the forskolin-induced increase in the chloride current (Isc). (h) Summary of the results in g (n = 4 experiments). (i–l) Control C57BL/6 and CfrF508del mice were treated with 200 µg per kg bodyweight Tα1 intraperitoneally for six consecutive days, and CLCA1 protein expression in lung (i,j) and small intestine (k,l) was assessed by immunoblotting of lysates (i,k) and immunohistochemistry (n = 5 images per mouse) (j,l). Scale bars, 25 µm in j, 200 µm in l, and 100 µm in the insets. Immunoblotting and immunohistochemical sections are representative of three independent experiments with n = 6 mice per group. Data are presented as means ± s.d. **P < 0.01, ***P < 0.001, ****P < 0.0001, two-way ANOVA with Bonferroni post test.
Figure 6 Tα1 rescues p.Phe508del-CFTR activity in CF cells and human bronchial epithelial cells from subjects with CF. (a-d) Representative CFTR (a) and USP36 (b) protein expression (n = 3) and CLCA1 gene expression (n = 3) (c), and iodide efflux as assessed by SPQ assay upon stimulation with forskolin (d) in HBE cells from five subjects (subj.) with the p.Phe508del-CFTR mutation and one representative control treated with 100 ng/ml Tα1 for 24 h at 37 °C. (e, f) Tα1 production in sputa from controls and subjects with CF (n = 3; *P < 0.05, Student’s t-test) (e) and lysates or supernatants from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR (n = 3; **P < 0.01, two-way ANOVA with Bonferroni post test) (f) and lysates or supernatants from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR (n = 3; **P < 0.01, two-way ANOVA with Bonferroni post test). (g) Representative immunoblots (n = 3) of CFTR and C band pixel density in cells transfected with siRNA targeting PTMA (prothymosin) or plasmid encoding PTMA and treated with Tα1 at 37 °C for 2 h. (h–j) CFTR-dependent chloride secretion measured by means of forskolin-induced increase in the Isc in HBE cells from two subjects with CF (h) and one representative control (i) treated with Tα1, ivacaftor or lumacaftor for 24 h at 37 °C and mounted in Ussing chambers in the presence of CFTR inhibition (CFTR inh-172) and amiloride. (j) Summary of the results in h and i (n = 4). Data are shown in box-and-whisker plots: the box center line represents the mean, and whiskers show the minimum to maximum. Otherwise, data are presented as means ± s.d. ***P < 0.01, **P < 0.001, two-way ANOVA with Bonferroni post test.

expression after exposure to Tα1 for 24 h at 37 °C. Tα1 increased expression of the mature p.Phe508del-CFTR protein (Fig. 6a), USP36 (Fig. 6b) and CLCA1 (Fig. 6c) in three of the five subjects with CF and concomitantly increased ion channel activity (Fig. 6d). To assess the clinical relevance of these findings, we measured the concentrations of Tα1 in sputa from subjects with the p.Phe508del-CFTR mutation and control subjects, taking into consideration that Tα1 is highly expressed not only in human thymic epithelium but also in peripheral tissues. We found lower concentrations of Tα1 in subjects with CF than in controls (Fig. 6e), which is consistent with the lower levels observed in supernatants and lysates from p.Phe508del-CFTR-transfected CFBE41o- cells relative to cells transfected with WT CFTR (Fig. 6f). Because signaling via TLRs influences Tα1 production by lung epithelial cells (Supplementary Fig. 8), the deregulated TLR activity observed in subjects with CF may affect local Tα1 production and, ultimately, CFTR activity. This likely occurs, as endogenous Tα1 affected functional expression of CFTR. We assessed the expression of mature CFTR in HBE cells from subjects with the p.Phe508del-CFTR...
mutation and controls after prothymosin depletion (by specific siRNA) or overexpression (via transfection) because T\(\alpha\)1 is produced by cleavage of prothymosin by the lysosomal asparaginyl endopeptidase legumain\(^{37}\). We found that mature p.Phe508del-CFTR expression was negated by prothymosin inhibition and increased by prothymosin overexpression (Fig. 6g). Of interest, prothymosin overexpression also increased mature CFTR expression in cells from controls (Fig. 6g), which points to a role for the endogenous prothymosin/T\(\alpha\)1 system in CFTR physiology.

We finally comparatively assessed the ion channel activity of T\(\alpha\)1 with that of lumacaftor or ivacaftor, either alone or in combination, in primary HBE cells from controls or subjects homozygous for the p.Phe508del-CFTR mutation. Using chamber tracings revealed that T\(\alpha\)1 promoted the forskolin-induced increase in the chloride current (Isc) in HBE cells from subjects with CF (Fig. 6h) but not controls (Fig. 6i), an effect that was sensitive to CFTR inhibition and was similar to that observed with lumacaftor (Fig. 6j). With the only exception of subject 8 (Supplementary Fig. 9), the activity of T\(\alpha\)1 was not additive to that of ivacaftor, which is unable to rescue p.Phe508del-CFTR at the plasma membrane\(^{47}\). Similar results were obtained by means of a halide-sensitive fluorescent probe (Supplementary Fig. 9). In spite of the reported interindividual variability, even in patients bearing the same genotype, these results suggest that T\(\alpha\)1 alone or in combination can be used for treating subjects with the p.Phe508del-CFTR mutation.

DISCUSSION

While regulation of lung homeostasis and inflammation by CFTR is an established notion\(^{51}\), whether regulation of inflammation will affect CFTR functioning is less clear. Here we show that T\(\alpha\)1 is endowed with the unique ability to correct CFTR defects through the regulation of inflammation. Although T\(\alpha\)1 appears to have a multitasking chaperon activity through which it may affect the balance of protein folding versus degradation (for example, by associating with p.Phe508del-CFTR, perturbing the interaction of mutated CFTR with the ER chaperons and activating USP36-mediated deubiquitination of misfolded CFTR), the induction of IDO1 appears to be key to this mechanism. IDO1 is known to play a major role in preventing excessive pathology in immune-mediated tissue injury through multiple effector mechanisms, including the induction of autophagy\(^{31}\). In accordance with the ability of autophagy to rescue CFTR function\(^{9}\), the promotion of autophagy appeared to contribute to the corrector activity of T\(\alpha\)1 and qualified T\(\alpha\)1 as a proteostasis regulator that rectifies an unbalanced degradasome activity in CF cells. Proteostasis regulators, such as cysteamine, have emerged as a new option for CFTR repair by avoiding unwanted protein–protein interactions, favoring the trafficking and stability of mutant CFTR and hence controlling inflammation\(^{9,10,32}\). Unlike cysteamine, the anti-inflammatory effects of T\(\alpha\)1 are not independent on an ability to rescue functional p.Phe508del-CFTR, as they also occur in CFTR-knockout mice. Therefore, T\(\alpha\)1 is an excellent example of a drug with primary anti-inflammatory effects that can also favor rescue of p.Phe508del-CFTR.

The cross-regulation between autophagy and inflammasomes\(^{52}\) may also explain the ability of T\(\alpha\)1 to inhibit NLRP3-based inflamma- some activity, which is known to contribute to respiratory infections and pathologic airway inflammation in CF\(^{28}\). Moreover, as immune tolerance induced by IDO1 includes tissue repair and remodeling activity\(^{33}\), this may explain the remarkable effect of T\(\alpha\)1 in restoring host tissue architecture in the lung and the gastrointestinal tract of Cftr\(^{F508del}\) mice. Similarly to subjects with CF\(^{30}\), an altered pulmonary histopathophysiology is present in those mice in the absence of infection\(^{54}\). Altogether, these findings may point to T\(\alpha\)1 as a drug candidate capable of preventing CF progression at very early stages of the disease.

Through its multitasking activity, T\(\alpha\)1 may represent a proper means of rectifying the multifunctional defect in individuals with CF, including the increase in CLCA1 activity. Both clinical and animal model studies have suggested a compensatory role for CLCA1 in CF\(^{55,56}\). Even though calcium-activated chloride channels are abundant in mouse but not in human airways\(^{48,57}\), mutations in CLCA1 are found in a subset of people with CF marked by aggravated intestinal disease\(^{55}\), a finding consistent with the observation that calcium-activated chloride channels are defective in the intestine of individuals with CF\(^{57}\). This may explain the remarkable activity of T\(\alpha\)1 in the gastrointestinal tract of Cftr\(^{F508del}\) mice, in which chloride channel activity was rectified. This indicates that T\(\alpha\)1 could be clinically exploited for pharmacologic correction of defective CFTR not only in the lung but also in the gut, implying that gastrointestinal outcome measures could be promising clinical endpoints of T\(\alpha\)1 therapeutics.

Only a combination of molecules with different mechanisms of action is expected to induce a considerable degree of p.Phe508del-CFTR correction, as shown by the combination of ivacaftor with lumacaftor\(^{58}\) or VX-661 (refs. 59,60). Although the corrector activity of T\(\alpha\)1 has yet to be verified relative to the agents moving into development in the clinic, the excellent safety profile and cost effectiveness of Zadaxin in adults and children\(^{20}\) suggest that T\(\alpha\)1 could be tested in clinical trials for possible pulmonary and extrapulmonary benefits in individuals with CF.

URLs. List of CF-associated mutations, http://www.genet.sickkids.on.ca/cftr/StatisticsPage.html.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

V.O., R.G.I. and M. Pariano performed most immunoblotting and immunofluorescence experiments; R.G.I., M.B., S.M. and E.F. performed murine in vivo experiments; M.C.D.A., L.S. and M. Pesia performed electrophysiology experiments; F.F. and M.T.P. performed TLR9 colocalization experiments; M.M.B. and G.S. performed transfection experiments; V.R.V. performed Using chamber experiments; A.L.G., L.M., G.K., M. Pessia, P.P., E.G. and L.R. designed the experiments, analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

General experimental approaches. Sample size was chosen empirically based on our previous experiences in the calculation of experimental variability; no statistical method was used to predetermine sample size and no samples, mice or data points were excluded from the reported analyses. Experimental groups were balanced in terms of animal age, sex and weight. No blinding was applied upon harvesting samples after the treatments.

Mice. Mouse experiments were performed according to Italian Approved Animal Welfare Authorization 360/2015–PR and Legislative Decree 26/2014 regarding the animal license obtained by the Italian Ministry of Health lasting for 5 years (2015–2020). Infections were performed under isoflurane anesthesia, and all efforts were made to minimize suffering. CF mice homozygous for the Phe508del-CFTR allele, which had been backcrossed for 12 generations to the C57BL/6 strain or on the FVB/129 outbred background (Cftr+/−/Tm1BR), were obtained from B. Scholte (Erasmus Medical Center) or female mice were backcrossed to C57BL/6-background Becn1+/− male mice to obtain Becn1−/− homozygous mice (abbreviated Cftr+/−/Becn1−/−), as described18. These mice were provided with a special food consisting of an equal mixture of SRM-A (Arie Blok, Woerden, The Netherlands) and Teklad 2019 (Harlan Laboratories, San Diego, CA, USA) and water acidified to pH 2.0 with HCl and containing 60 g/l PEG 3350, 1.46 g/l NaCl, 0.745 g/l KCl, 1.68 g/l NaHCO3 and 5.68 g/l Na2SO4. Newborn mice were genotyped by cutting a small piece of tail 12 d after birth. C57BL/6 mice aged 4–6 weeks purchased from Charles River (Calco, Italy) and genetically engineered homozygous knockout Cftr mice (B6.129P2-KO Cftr+/-;N ENC, abbreviated Cfr−/− and Cfr+/−, gut corrected, on a C57BL/6 background) were bred under specific-pathogen-free conditions at the Animal Facility of San Raffaele Hospital, Milan, Italy. Male and female mice were used in all studies.

Infections and treatments. Mice were anesthetized in a plastic cage by inhalation of 3% isoflurane (Forane, Abbott) in oxygen before intranasal instillation of 2 × 104 A. fumigatus (AF293) resting conidia per 20 µl of saline. For P. aeruginosa infection, appropriate dilutions with sterile PBS were made to prepare the inoculum before intranasal instillation of 3 × 107 CFU per mouse22. Quantification of microbial growth was done as described22. For histology, paraffin-embedded sections were stained with periodic acid–Schiff (PAS) and H&E. Mice were treated either intraperitoneally (i.p.) or intranasally (i.n.) with T1 to the scrambled polypeptide reconstituted in sterile water (200 µg per kg bodyweight in 20 µl of saline (i.n.) or 100 µl of saline (i.p.)) given daily for 4 consecutive days in infected mice either beginning the day of the infection or starting 7 d after the infection. Mice were weighed on the first and last days of the treatment. Mice were gavaged with cysteamine (60 µg per kg bodyweight in 100 µl of saline per day) for 6 d as reported25. For BAL fluid collection, lungs were filled thoroughly with 1 ml aliquots of pyrogen-free saline through a 22-gauge bead-tipped feeding needle introduced into the trachea. BAL fluid was collected in a plastic tube on ice and centrifuged at 400g at 4°C for 5 min. For differential BAL fluid cell counts, cytokin preparation were made and stained with May–Grünwald Giemsa reagents (Sigma–Aldrich). At least 200 cells per field per 10 fields were counted, and the absolute number of neutrophils was calculated. For epithelial cell purification, lung epithelial cells, of which 99% expressed cytokeratin on pan-cytokeratin antibody staining of cyt centrifuge preparations and >90% were viable on trypan blue exclusion assay, were isolated as described63. Cells were cultured with MALP-2, poly(I:C), ultrapure lipopolysaccharide from Salmonella minnesota Re 595 (all from Sigma–Aldrich) and CpG ODNs (all at 10 µg/ml) for 1 h (NF-kB and IRF3 protein expression) or 4 h (IL10 gene expression). Uncropped immunoblots are shown in the Supplementary Data.

Cells. HBE cells, homoygous for the p.Phe508del mutation, and isogenic wild-type cells were obtained from lung transplants (individuals with CF) or lung resection controls) (kindly provided by L.J. Galiotta, Italian Cystic Fibrosis Foundation). Cells were maintained at 37°C in a humidified incubator in an atmosphere containing 5% CO2, and the experiments were done 5 d after plating64. Stable lentiviral-based transductions of the parental CFBF410- cells, homoygous for the p.Phe508del-CFTR mutation24, with either WT CFTR or p.Phe508del-CFTR, were provided by L.J. Galiotta. The transduced CFBF410-cells were maintained in minimum Eagle’s medium supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM l-glutamine, 10% FBS and 1 µg/ml blasticidin (WT CFTR) or 2 µg/ml puromycin (p.Phe508del-CFTR) in a 5% CO2 and 95% air incubator at 37°C. To establish polarized monolayers, CFBF410-cells were seeded on 24-mm-diameter Transwell permeable supports (0.4-mm pore size; Corning Corp., Corning, NY) at 2 × 105 cells/well and grown in an air–liquid interface culture at 37°C for 6–9 d and then at 27°C for 36 h. Cells were incubated with different concentrations of T1 (CIRBI Biotechnology), 3 µM VX-809 (Lumacator, Aurogene), 1 µM VX-770 (Ivactar, Aurogene) alone or in combination for up to 24 h before the assessment of CFTR protein expression and function at either 37°C or 26°C. For washout experiments, after the treatment period, the medium was replaced with complete medium for the indicated time. DMSO vehicle alone (0.1%) was used as a control. T1 and the scrambled polypeptide were supplied as purified (the endotoxin levels were <0.03 µg/ml by a standard limulus lysate assay), sterile, lyophilized, acetylated polypeptide. The sequences were as follows: Ac-SDAAVDTSSSEITTLDEKEKVVEEAEEHN (Tet1) (SEQ ID 1) and Ac-AKSDKVKAETSEIDTDLEKEVFVKANE-OH (scrambled peptide) (SEQ ID 2). For cell cultures, CFBF410-cells were stimulated with CpG ODN (10 µg/ml) or MALP-2 (100 ng/ml) with or without 100 ng/ml of T1 for 2 h and then lysed for immunoblotting or assessed for cytokine analysis by RT–PCR. Recombinant IFN-γ, from Santa Cruz Biotechnology, was used at the concentration of 10 ng/ml. HEK293 cells were maintained in DMEM supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM l-glutamine and 10% FBS in a 5% CO2, 95% air incubator at 37°C. Regular testing for mycoplasma contamination was carried out by PCR. Human studies approval was obtained from institutional review boards at each site, and written informed consent was obtained from the participants or, in case of minors, from parents or guardian. All cell lines have been correctly identified to ensure their specificity.

Immunofluorescence and immunohistochemistry. CFBF410-cells were treated with 100 ng/ml T1 at 37°C for 24 h, fixed in 2% formaldehyde for 15 min at room temperature and permeabilized in blocking buffer containing 5% FBS, 3% BSA and 0.5% Triton X-100 in PBS. The cells were then incubated at 4°C with the primary antibody anti-CFTR (clone CF3, Abcam). After extensive washing with PBS, the slides were then incubated at room temperature for 60 min with goat anti-mouse antibody to CFTR followed by Alexa Fluor 555 (Clone Poly4053, BioLegend) and Alexa Fluor 488 anti-phallolidin (A12379, Thermo Fisher) for F-actin labeling. For intracellular routing, cells were plated in complete medium into chambered coverslips (Lab-Tek/Nunc; Thermo Scientific) in a temperature-regulated environmental chamber and exposed to 100 ng/ml T1 at 37°C for 2 h in serum-free RPMI-1640 medium. After washout, cells were fixed, permeabilized and incubated at 4°C with anti-Rab5, anti-Rab7, anti-Rab9 (all from Sigma) and anti-α1 (clone 7G3, Abcam) primary antibodies. After extensive washing with PBS, the cells were incubated at room temperature for 60 min with a 1:400 dilution of FITC-conjugated anti-rabbit IgG secondary antibody (Sigma–Aldrich). For TL9b colocalization, HEK293 cells that were transfected with human TL9b tagged at its C terminus with enhanced GFP (TL9b–EGFP) were obtained from G. Tetti (University of Messina). Cells were seeded onto a sterilized coverslip, placed in a six-well plate and stimulated with 1 µg/ml CpG ODN in the presence of 100 ng/ml T1 for 30 min. Cells were then fixed with 4% paraformaldehyde, permeabilized by 0.1% Triton X-100 and stained with biotin-conjugated anti-human CD107a antibody (BioLegend) for the LAMP-1 or with biotin-conjugated anti-transferin receptor antibody (DFI153, Abcam) and visualized with streptavidin, Alexa Fluor 568 conjugate (Thermo Fisher). The tissues were removed and fixed in 10% phosphate-buffered formalin, embedded in paraffin and sectioned at 5µm. Sections were then rehydrated, and after antigen retrieval in citrate buffer (10mM, pH 6.0), sections were fixed in 2% formaldehyde for 40 min at room temperature and permeabilized in blocking buffer containing 5% FBS, 3% BSA and 0.5% Triton X-100 in PBS. The sections were incubated at 4°C overnight with anti-NLFR3 (ab4207, Abcam) primary antibody and then incubated at room temperature for 60 min with Alexa Fluor 555–conjugated goat anti-mouse secondary antibody (Poly4053, BioLegend). For immunohistochemistry, sections were incubated overnight with anti-CFTR (CF3, Novus) or anti-CLCA1 (Abcam) antibody followed by biotinylated secondary antibodies. For each primary antibody, validation
contained the cytoplasmic and plasma membrane fractions were centrifuged (X-100-RS, Sigma) and centrifuged 1 h at 60,000 g. PMSF, 1 mg/ml protease inhibitor cocktail (P8340, Sigma), 0.1% Triton X-100.

Membrane fractionation. Blots of cell lysates were incubated with antibodies against the following proteins: CFTR (C3, Abcam; C3, Novus; 2269, Cell Signaling), CLCA1 (Abcam), USP36 (Proteintech), murine IDO1 (monoclonal; cv152R, human IDO1 (10.1, Millipore), phospho-NF-xB/p65 and NF-xB/p65 (Cell Signaling), phospho-IRF3 (Cell Signaling) and IRF3 (Santa Cruz). For immunoprecipitation, cells were lysed in immunoprecipitation buffer containing 150 mM sodium chloride, 50 mM Tris (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, Complete protease inhibitor cocktail (Roche) and PMSF (Roche). CFTR and IDO1 were immunoprecipitated by incubation with 1 µg of the specific antibody. The reaction was performed overnight, and either Protein A (for CFTR) or Protein G (IDO1) Sepharose 4 Fast Flow beads (GE Healthcare) were added and incubated for an additional 2 h. Beads were washed and resuspended in Laemmlli buffer. Immunoprecipitated proteins were separated by SDS–PAGE, and immunoblots were probed with anti-USP36, anti-Hsp70, anti-Hsp90 and anti-calnexin antibodies and with anti-ubiquitin antibody that recognizes mono-, oligo- or polyubiquitinated additions (all from Abcam). For physical association of Tα1 to CFTR, immunoprecipitated proteins were separated by tricine–SDS–PAGE, and immunoblots were probed with anti-Tα1 antibody (ab76557, Abcam). Normalization was performed probing the membrane with β-actin or β-tubulin antibody (AC-15 or TUB 2.1, Sigma). Alternatively, lysates from HEK293 cells transfected with FLAG-Tα1 were immunoprecipitated using an affinity resin with anti-FLAG M2 (clone M2, Sigma) and the resulting FLAG-Tα1 was incubated in the presence of lysates from CFBE41o- cells transfected with WT CFTR or p.Phe508del-CFTR. Samples were resolved by immunoblotting and probed with anti-CFTR antibody. Lysates from p.Phe508del-CFTR CFBE41o- cells were transfected with FLAG-Tα1 and immunoprecipitated using the affinity resin with anti-FLAG M2. In order to avoid nonspecific binding, after elution with FLAG peptide (Sigma), the remaining proteins bound to the resin were loaded as a negative control. Samples were resolved by immunoblotting and probed with anti-CFTR antibody.

Chemiluminescence detection was performed with LightAblePlus chemiluminescence substrate (Euroclone), using the ChemiDoc XRS + Imaging system (Bio-Rad), and quantification was obtained by densitometry image analysis using Image Lab 5.1 software (Bio-Rad). Uncropped immunoblots are shown in the Supplementary Data.

Luciferase assays. CFBE41o- cells were seeded in 24-well plates at a density of 7.5 × 104 cells per well. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Each transfection contained 100 ng of a luciferase reporter plasmid (pLV-SV–NF-xB–RE Luc) and 50 ng of a β-galactosidase internal-control reporter plasmid (pGL3-lacZ) with or without MALP-2 (100 ng/ml). The total amount of DNA applied per well was adjusted to 600 ng by adding pcDNA3 empty vector. Cell extracts were subjected to a luminometry-based luciferase assay, and luciferase activity was normalized to a β-galactosidase internal-control reporter (pGL3-lacZ) plasmid (Thermo Fisher Scientific) to construct the recombinant pcDNA3.1/myc-His A plasmid (Thermo Fisher Scientific) to construct the recombinant pcDNA3.1/myc-His A plasmid. The typical activity, the Lysosome/Cytotoxicity Dual-Staining kit (Abcam) was used per the manufacturer’s instructions. Identification of active deubiquitinas. CFBE41o- cells were lysed in immunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 10 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and 0.1 µg of the HA-UB–VME probe (Enzo) was added to 20 µg of protein extract. HA-UB–VME probe forms an irreversible covalent bond with active DUBs. Identification of DUBs covalently linked to the HA-UB–VME probe was achieved by immunoprecipitation of HA-UB–VME–DUB complexes using an anti-HA antibody (A190-108A, Bethyl Laboratories) followed by SDS–PAGE and western blot analysis using anti-DUB antibodies specific to USP19 (Bethyl Laboratories), USP10 (Bethyl Laboratories) and USP36 (Proteintech). The specificity of the HA–UB–VME probe for active DUBs was confirmed with the addition of N-ethylmaleimide (10 µM) to inhibit cysteine protease DUBs during the labeling reaction. Uncropped immunoblots are shown in Supplementary Data.

Plasmins and transfection. The FLAG-HA-USP36 plasmid (22579) was from Addgene and was used for transfection experiments. For generation of the plasmid encoding pcDNA3.1/myc-His with the prothrombin α (PTMA) fragment, the coding sequence of PTMA cDNA was amplified by PCR with specific primers containing EcoRI and XhoI anchores. The fragment was then subcloned into the EcoRI and XhoI cloning sites of pcDNA3.1/myc-His A plasmid (Thermo Fisher Scientific) to construct the recombinant pcDNA3.1/myc-His PTMA expression vector. To isolate the sequence encoding the Tα1 active peptide, the cDNA sequence corresponding to Tα1 was amplified by PCR with specific primers containing EcoRI and XhoI anchores, using PTMA pCDNA3.1/myc-His A as a template, and was cloned into the pcMV-Tag 2B expression vector (Agilent Technologies). Transient transfections were performed with a PCMV vector expressing FLAG-Tα1 using TransIT Transfection Reagent (Mirus), according to the manufacturer’s instructions, in HEK293 cells and HBE Phe508del cells incubated for 24 h at 37 °C in 5% CO2. The empty vector was used as a negative control.

RNA interference. Pooled duplexes of predesigned siRNA (USP36, MMC.RNAI.N007887.12.1; CLCA1, MMC.RNAI.N001285.12.1) were purchased from IDT (TEGA Ricerca). Specific siRNA for prothrombin α was purchased from Sigma. For siRNA delivery, cells were incubated for 24 h (as indicated by preliminary experiments performed at 12, 24 or 48 h) at 37 °C in 5% CO2 with specific siRNA using TransIT-TKO Transfection Reagent (Mirus) following the manufacturer’s instructions. The effectiveness of the silencing of specific targets was verified by evaluating target mRNA levels using RT–PCR (Supplementary Fig. 8d), and protein expression was evaluated by western blotting (Figs. 3g and 6g).
in the absence of a quenching anion, $C_{Q}$ is the concentration of the quenching anion and $K$ is the Stern-Volmer quench constant.

**Preparation of RNA and microarray hybridization.** Total-lung RNA was extracted using TRIzol reagent (Life Technologies). The integrity of total RNA was evaluated using the Agilent Bioanalyzer 2100 system (Agilent Technologies) and was within RNA integrity number 7–9 and thus considered suitable for further processing. 100 ng of total RNA was processed to produce fragmented biotin-labeled cDNA using the GeneChip WT PLUS Reagent kit according to the manufacturer's instructions. Samples were hybridized to Affymetrix GeneChip Mouse Gene 2.0 ST arrays and quantified. Images were processed and cell intensity files (CEL files) were generated in GeneChip Command Console Software (Affymetrix). CEL files were processed using Expression Console v1.4.1.46 to yield RNA-summarized log2-transformed expression values for probe sets (CHP files). Normalized expression data (CHP files) were analyzed using Transcriptome Analysis Console Software (Affymetrix) and ANOVA in R using the Bioconductor R package.

**Using chamber.** Chambers for mounting tissue biopsy were obtained from Physiologic Instruments (model P2300). Chamber solution was buffered with bubbling with a mixture of 95% O2 and 5% CO2. Tissues were short-circuited using Ag/AgCl agar electrodes. Short-circuit current and resistance were acquired or calculated using the VCC-600 transpithelial clamp from Physiologic Instruments and Acquire & Analyze 2.3 software for data acquisition (Physiologic Instruments), as previously described. A basolateral-to-apical chloride gradient was established by replacing NaCl with sodium gluconate in the apical (luminal) compartment to create a driving force for CFTR-dependent Cl− secretion. CFTR channels present at the apical surface of the epithelium (lumen side of the tissue) were activated. Stimulations with forskolin, CFTR inhibitor 172 and amiloride were performed as described.

**ELISA and real-time PCR.** The levels of cytokines were determined by specific ELISAs (R&D Systems). For Tc1 production (ELISA kit from Immunodiagnostik), either cell culture supernatants or cell lysates were used. RT–PCR was performed using the Bio-Rad CFX96 System and SYBR Green chemistry (Bio-Rad). The PCR primers were as follows: IL10 Forward, 5′-GCCATACGTTGAGATCT-3′ and Reverse, 5′-TGATGCTTGTTGTCGTTCT-3′; IL6 Forward, 5′-CCACCTACCTTCTGAGAACT-3′ and Reverse, 5′-AGGCACCTTCTGTTCAAGATT-3′; and Reverse, 5′-CTGCAATACCTCCTGCTTTG-3′, and Reverse, 5′-CTGCAATACCTCCTGCTTTG-3′. Each data point was examined for integrity by analysis of the amplification plot. The mRNA-normalized data were expressed as relative gene mRNA in treated as compared to untreated experimental groups or cells.

**Statistical analysis.** GraphPad Prism software 6.01 (GraphPad Software) was used for analysis. Data are expressed as means ± s.d. Horizontal bars indicate the means. Plots of in vivo data are presented as box-and-whiskers plots; bars represent maximal and minimal values. Statistical significance was calculated by one- or two-way ANOVA (Tukey's or Bonferroni's post hoc test) for multiple comparisons and by a two-tailed Student's t-test for single comparisons. The distribution of levels tested by the Kolmogorov–Smirnov normality test turned out to be non-significant. The variance was similar in the groups being compared. We considered all P values 0.05 to be significant. The in vivo groups consisted of six mice/group. The data reported are either representative of at least three experiments (histology, immunofluorescence and western blotting) or pooled otherwise.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.
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