Pharmacological modulation of mitochondrial calcium uniporter controls lung inflammation in cystic fibrosis

Alessandro Rimessi1,2,*, Chiara Pozzato1, Lorenzo Carparelli1, Alice Rossi3, Serena Ranucci3, Ida De Fino3, Cristina Cigana3, Anna Talarico4, Mariusz R. Wieckowski5, Carla M. P. Ribeiro6, Claudio Trapella4, Giacomo Rossi7, Giulio Cabrini2,8, Alessandra Bragonzi3, Paolo Pinton1,2*.

Mitochondria physically associate with the endoplasmic reticulum to coordinate interorganelle calcium transfer and regulate fundamental cellular processes, including inflammation. Deregulated endoplasmic reticulum–mitochondria cross-talk can occur in cystic fibrosis, contributing to hyperinflammation and disease progression. We demonstrate that Pseudomonas aeruginosa infection increases endoplasmic reticulum–mitochondria associations in cystic fibrosis bronchial cells by stabilizing VAPB-PTPIP51 (vesicle-associated membrane protein–associated protein B–protein tyrosine phosphatase interacting protein 51) tethers, affecting autophagy. Impaired autophagy induced mitochondrial unfolding protein response and NLRP3 inflammasome activation, contributing to hyper-inflammation. The mechanism by which VAPB-PTPIP51 tethers regulate autophagy in cystic fibrosis involves calcium transfer via mitochondrial calcium uniporter. Mitochondrial calcium uniporter inhibition rectified autophagy and alleviated the inflammatory response in vitro and in vivo, resulting in a valid therapeutic strategy for cystic fibrosis pulmonary disease.

INTRODUCTION

Cystic fibrosis (CF) is a genetic disease caused by mutations of the gene coding for the CF transmembrane conductance regulator (CFTR) protein (1). More than 2000 variants and 300 disease-causing mutations have been identified thus far, with the deletion of phenylalanine at position 508 (Phe508del-CFTR) being the most common, affecting approximately 80% of patients with CF in Europe and the United States (2). Because of protein misfolding, Phe508del-CFTR is prematurely degraded. In the lungs, the primary organ affected in CF, CFTR gene defects impair chloride transport, reducing water content in the airway surface liquid. This defect increases susceptibility to bacterial infections (such as those by Pseudomonas aeruginosa), resulting in hyperinflammation and progressive pulmonary tissue damage, and leading to respiratory insufficiency (3). In addition to the lung tissue damage induced by the chronic inflammatory process itself, P. aeruginosa has been shown to compromise fundamental processes, including the immune response and the expression of rescued Phe508del-CFTR to the apical membrane (4). Thus, the presence of P. aeruginosa infection could exacerbate pulmonary CF pathophysiology and render recent CF therapies less effective. Therefore, alternative approaches aimed to activate early anti-inflammatory pathways to prevent organ damage before patients become symptomatic are needed (5).

Mitochondria have recently gained much attention in the medical field due to their involvement in several inflammatory-associated diseases, including CF. Perturbation of mitochondrial activity is sufficient to activate innate immune responses (6, 7), indicating that cells use mitochondrial stress to potentiate innate immunity programs when specific exogenous or endogenous stress alters mitochondrial homeostasis (8). The maintenance of functional mitochondria in cells, through mitochondrial stress response and quality control pathways, is thus essential to avoid exacerbation of the inflammatory response. A conserved lysosomal degradation pathway, called mitophagy, controls the quality of mitochondria in stressed cells, acting as a mitochondrial stress response together with the mitochondrial unfolded protein response (UPRmt) (8). In relation to CF pathophysiology, a similar mechanism, called xenophagy, plays a role in sequestering and degrading invading pathogens, while macroautophagy controls CFTR intracellular trafficking and function (9, 10). Several protein complexes and signaling pathways, including Ca2+ signaling, are involved in these processes (11–13). However, whether Ca2+ signaling and autophagy are directly related to the CF lung–specific pathogenic cascade remains unclear. Impaired Ca2+ homeostasis and autophagic defects in CF have been reported, suggesting that both could be directly involved (7, 14–19).

We recently demonstrated that the degree and quality of the inflammatory response in CF bronchial cells are supported by P. aeruginosa–dependent mitochondrial perturbation, in which the mitochondrial Ca2+ uniporter (MCU) is involved in NLRP3 (NOD-LRR-, and pyrin domain-containing protein 3) inflammasome activation and inflammatory exacerbation in vitro (7). Consequently, mitochondrial Ca2+ signaling represents a potentially useful but relatively unexploited area for therapeutic innovation and intervention. From this viewpoint, the identified MCU is a strong candidate (20). The activity of MCU induces increases in mitochondrial Ca2+ concentration with defined timing, amplitude, and kinetics that affect mitochondrial activities, including adenosine triphosphate (ATP) production, inflammation, autophagy, and cell death (21, 22). However, mitochondrial Ca2+ homeostasis is primarily conditioned by

---

1Department of Medical Sciences and Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, 44121 Ferrara, Italy. 2Center of research on Innovative Therapies for Cystic Fibrosis, University of Ferrara, 44121 Ferrara, Italy. 3Infections and Cystic Fibrosis Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, 20132 Milano, Italy. 4Department of Chemistry and Pharmaceutical Sciences and Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, 44121 Ferrara, Italy. 5Laboratory of Mitochondrial Biology and Metabolism, Nencki Institute of Experimental Biology, 02-093 Warsaw, Poland. 6Department of Medicine/Pulmonary Division, Marsico Lung Institute and Cystic Fibrosis Center, Chapel Hill, NC 27599-7248, USA. 7School of Biosciences and Veterinary Medicine, University of Camerino, 62024 Macerata, Italy. 8Department of Neurosurgery, Biomedicine and Movement, University of Verona, 37126 Verona, Italy. 9Corresponding author. Email: alessandro.rimessi@unife.it (A.R.); paolo.pinton@unife.it (P.P.)
The increase of VAPB and PTPIP51 expression impairs autophagy in CF bronchial cells during *P. aeruginosa* infection

To gain insight into the role of ER-mitochondria associations in CF during pathogen infection, we first monitored whether infection with *P. aeruginosa* affected the interaction of key ER-mitochondria Ca\(^{2+}\) exchange proteins, such as IP\(_3\)Rs and VDAC, using a proximity ligation assay (PLA). Different human non-CF (S9 and NuLi) and CF (IB3-1 and CuFi) bronchial cell models, grown as monolayer on plastic supports, were exposed to *P. aeruginosa* laboratory strain (PA01) or supernatant from mucopurulent material (SMM) from airways of patients with CF. No changes in IP\(_3\)R3–VDAC interactions were quantified in non-CF bronchial cells challenged with *P. aeruginosa* or SMM (Fig. 1A and fig. S1A). In contrast, in CF bronchial cells, challenge with *P. aeruginosa* or SMM increased the interactions between IP\(_3\)R3 and VDAC (Fig. 1A and fig. S1A). To test whether the increase in ER-mitochondria contacts was due to altered expression of ER-mitochondria tethers, we probed immunoblots of non-CF and CF bronchial cells exposed for different hours to *P. aeruginosa*. No change in the expression of IP\(_3\)R3 and VDAC in both cell lines was detected (Fig. S1B), whereas the expression of ER-mitochondria tethers, VAPB and PTPIP51, was increased in CF bronchial cells during pathogen exposure, suggesting that their increase could justify the augmented interaction of IP\(_3\)R3 and VDAC (Fig. 1B and fig. S1, C and D). Similar effect on ER-mitochondria tethers has been observed also in polarized mucociliary-differentiated CF patient–derived airway epithelial cells reconstituted on Transwell air-liquid interface (fig. S1E). CF primary airway cells showed enhanced VAPB and PTPIP51 expression compared to wild-type (WT) CFTR-expressing human primary cells when exposed to *P. aeruginosa*, which suggests that defective CFTR channel favors ER-mitochondria interaction during pathogen infection. A major ER-mitochondria interaction in CF bronchial cells when exposed to *P. aeruginosa* or SMM is also confirmed by the enhanced percentage of VAPB–PTPIP51 colocalization (fig. S2A).

By manipulating the expression of VAPB or PTPIP51, it is possible to modulate ER-mitochondria contacts affecting interorganelle Ca\(^{2+}\) exchange and autophagy (26). We quantified the level of autophagy present in non-CF and CF bronchial cells exposed to *P. aeruginosa* using the most common marker, microtubule-associated protein 1 light chain 3 (LC3) protein. This protein is processed as LC3-I (~16 kDa) and the membrane-bound form LC3-II (~14 kDa), localized to autophagosomes. By immunoblotting, the level of autophagy was quantified as ratio of LC3-II to LC3-I. *P. aeruginosa* led to significantly down-regulated autophagic response in different CF cellular models compared to non-CF cells under the same experimental conditions, as assessed by a decrease in the LC3-II/LC3-I ratio, which is indicative of defective autophagy (Fig. 1B and fig. S1, D and E). Negative regulation of autophagic machinery in CF cells was also confirmed by microscopy using the green fluorescent protein chimera LC3 (GFP-LC3) to monitor the formation of autophagosomes and by quantifying the number of cells that presented ring-shaped GFP-LC3 structures after pathogen infection. Many cells with ring-shaped GFP-LC3 structures were observed in non-CF cells at different stages of infection (Fig. 1C). A marked reduction in the percentage of GFP-LC3–clustering cells was observed in CF cells, indicating minor processing and translocation of LC3-II from the cytosol to autophagic vacuoles. These data were also confirmed by autophagic flux assay. The levels of LC3-II formation were monitored by immunoblotting in different non-CF and CF bronchial cellular models exposed to *P. aeruginosa* and then treated with saturating concentration of bafilomycin A1. Bafilomycin A1 blocks the fusion of autophagosomes and lysosomes, and by inhibiting LC3-II degradation may modulate the kinetics of autophagosome synthesis. The ratio of LC3-II/LC3-I in the presence of bafilomycin A1 increased in non-CF cells exposed to *P. aeruginosa*, but this increase was restrained in CF cells, suggesting that the pathogen-induced autophagic flux is reduced in CF cells (Fig. 1D and fig. S2, B and C). The reduction in the autophagic response observed in CF cells, during *P. aeruginosa* infection, could be due to the down-regulation of autophagy as a consequence of the enhanced VAPB–PTPIP51 tethers. To address this issue, we monitored autophagy by manipulating the expression of VAPB and PTPIP51 in non-CF bronchial cells. We transfected S9 cells with empty vector (mock), VAPB, and PTPIP51. This overexpression of VAPB or PTPIP51 markedly increased ER-mitochondria contacts enhancing the interactions between IP\(_3\)R3 and VDAC in non-CF cells both under basal conditions and after *P. aeruginosa* exposure (Fig. 1E). Transfection of VAPB or PTPIP51 decreased both the ratio LC3-II/LC3-I (Fig. 1F) and the
Fig. 1. The increase of ER-mitochondria tethering inhibits autophagy in CF bronchial cells during *P. aeruginosa* infection. (A) S9 (non-CF) and IB3-1 cells (CF) were infected with *P. aeruginosa* at an MOI of 100, and after 6 hours, proximity ligation assay (PLA) for IP3R3 and VDAC interactions was performed. Representative images with PLA signals (red) in the different cells are shown. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (blue). The bar chart shows quantification of PLA signals (%), respect to uninfected S9 cells (n = 25 to 30 independent visual field for each condition of three independent experiments). (B) (I) Immunoblots show VAPB and PTPIP51 expression in S9 (non-CF) and IB3-1 (CF) cells during *P. aeruginosa* infection. The cells were uninfected or infected for 3, 6, and 12 hours. The samples were probed using the antibodies indicated, where actin is used as loading control. Protein molecular mass markers are indicated in kilodalton. (II) Bar chart shows the ratio LC3-II/LC3-I following quantification of signals from immunoblots (n = 5). (C) S9 (non-CF) and IB3-1 cells (CF) were transfected with GFP-LC3–encoding plasmid then infected with *P. aeruginosa*, as indicated. Representative images of GFP-LC3–transfected non-CF and CF cells have been reported. The bars depict the percentage of cells showing the accumulation of GFP-LC3 in cluster (n = 10 to 20 independent visual field for each condition of three independent experiments). (D) Cells were infected with PAO1 at an MOI of 100 MOI for 6 hours and then treated with either vehicle or bafilomycin A1 (100 nM) as indicated. (I) Samples were probed on immunoblots for LC3 and β-tubulin as a loading control. (II) Bar chart shows the ratio LC3-II/LC3-I following quantification of signals from immunoblots (n = 10). (E) VAPB or PTPIP51 overexpression increases the IP3R3-VDAC interactions in S9 cells. The bar chart shows quantification of PLA signals (%) respect to uninfected non-CF mock cells (n = 45 independent visual field for each condition of three independent experiments). (F) (I) VAPB or PTPIP51 overexpression was probed using Myc-tag and HA-tag antibodies, respectively, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a loading control. (II) Bar chart shows the ratio LC3-II/LC3-I following quantification of signals from immunoblots (n = 4). (G) LC3-clustering cell count in mock and VAPB- or PTPIP51-overexpressing S9 cells during pathogen infection was performed. The bars depict the percentage of cells showing the accumulation of GFP-LC3 in cluster (n = 40 independent visual field for each condition of three independent experiments). (H) (I) VAPB or PTPIP51 overexpression inhibits autophagic flux in S9 cells during *P. aeruginosa* infection. (II) Bar chart shows the ratio LC3-II/LC3-I following quantification of signals from immunoblots (n = 6). The reported data are means ± SE of at least three independent experiments. Student’s t test was used for indicated comparisons (*P < 0.05 and **P < 0.01).
percentage of GFP-LC3–clustering cells (Fig. 1G) in non-CF cells exposed to pathogen at different time points. Moreover, whereas treatment with bafilomycin A1 increased the levels of LC3-II in mock non-CF cells, the magnitude of this increase was reduced in VAPB- and PTPP51-transfected non-CF cells, confirming that the enhanced expression of VAPB or PTPP51 tethers reduces autophagic response also in non-CF bronchial cells (Fig. 1H).

**Effects of *P. aeruginosa*–induced VAPB and PTPP51 expression on selective autophagic responses**

During pathogen infection, selective autophagic responses (mitophagy and xenophagy) contribute together to protect and maintain cellular homeostasis. To test whether their roles could be compromised in CF, we first checked whether *P. aeruginosa* induced a mitophagic response in non-CF and CF bronchial cells. The cells were plated and infected with *P. aeruginosa*, and after intracellular protein fractionation, the expression level and localization of early [optineurin and nuclear dot protein 52 kDa (NDP52)] and late (LC3) mitophagic markers were immunoblotted (Fig. 2A). Optineurin and NDP52 are cytosolic receptors that are also involved in the xenophagic response and are recruited by PINK1 (phosphatase and tensin homolog-induced kinase 1) in the first steps of mitophagy (27). They are responsible for recognizing damaged mitochondria migrating to organelles and for promoting their sequestration into autophagosomes. In both cell lines, mitophagic receptors accumulated in the mitochondrial fraction after *P. aeruginosa* infection, triggering the mitophagic response (Fig. 2A and fig. S3A). The reduced mitochondrial redistribution of LC3-II in CF cells suggested slower kinetics of autophagosome synthesis and thus of mitochondrial sequestration (Fig. 2B). These data were confirmed by colocalization analysis between GFP-LC3 and mitochondrial-compared with non-CF cells (fig. S3A, III). These data were confirmed by fewer mitochondria colocalization with Parkin cluster (Fig. 2C, II). These results highlight a defect in mitochondrial sequestration during the *P. aeruginosa*–triggered mitophagic response in CF cells, characterized by reduction of the Parkin-mediated amplifying signal.

Although *P. aeruginosa* is primarily considered an extracellular pathogen, reports have demonstrated that, throughout the course of infection, the bacterium acquires the ability to enter and reside within host cells (28). CFTR channel defects could also lead to a reduction in xenophagy during pathogen infection, considering that xenophagy and mitophagy are two different events linked by common factors. We measured the xenophagic clearance activity and the *P. aeruginosa* invasion capacity in CF and non-CF bronchial cells. As expected, reduced xenophagic clearance of invading pathogen was detected in CF compared to non-CF cells (Fig. 2D, I). Similar effect on xenophagy has also been confirmed in other CF bronchial cell model and in polarized CF patient–derived airway cells (fig. S3B). In both cases, the higher number of colony-forming unit (CFU) per milliliter of invading bacteria in CF bronchial cells with respect to non-CF indicates a reduction of xenophagic clearance capacity. Through a bacterial invasion assay, we excluded that the accumulation of intracellular bacteria in CF bronchial cells depended on potentiated bacterial invasion (Fig. 2D, II). Last, colocalization analysis was performed between NDP52 and invading pathogens (Fig. 2E). Representative images show the intracellular redistribution of NDP52 around invading bacteria to facilitate sequestration. A reduced percentage of colocalization between NDP52 and invading bacteria was detected in CF bronchial cells with respect to non-CF cells, confirming a minor xenophagic clearance capacity of CF bronchial cells.

These data are in line with the previously described results of mitophagy, confirming that, in CF bronchial cells, defects of CFTR channel led to down-regulation of xenophagic and mitophagic responses with subsequent accumulation of invading bacteria (Fig. 2D and fig. S3B) and dysfunctional mitochondria, characterized by reduced mitochondrial membrane potential (ΔΨm) (fig. S3C) and increased mitochondrial reactive oxygen species (ROS) (fig. S3D).

The defective mitophagy in CF cells could be a crucial mechanism of the *P. aeruginosa*–dependent inflammatory exacerbation because its failure to remove *P. aeruginosa*–damaged mitochondria during infection leads to mitochondrial ROS production and inflammasome activation, with profound effects on cell physiology and cell viability. Similar effects are ascribable to defective xenophagy because impaired degradation of invading bacteria could result in cell stress and pyroptosis induction.

**Reduced selective autophagic responses potentiate UPRmt and inflammasome activation, favoring a vicious cycle**

Cells sense and respond to mitochondrial dysfunction by activating a protective transcriptional program known as UPRmt, which includes genes that promote mitochondrial protein homeostasis and the recovery of defective organelles. A genetic screen in *Caenorhabditis elegans* identified the transcription factor ATFS-1 (activating transcription factor 4) and ATF5, which have consider-able homology to ATFS-1, are indicated (29). During mitochondrial dysfunction, ATFS-1 traffics from the cytosol to the nucleus, where it induces transcription of mitochondrial chaperones [HSP10 and HSP60 (heat shock 10 kDa protein and heat shock 60 kDa protein)], proteases [CLPP (Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit)], and antibacterial innate immune genes (8). A similar transcriptional response has been described in mammals in which the putative roles of lc ATF4 (activating transcription factor 4) and ATF5, which have consider-able homology to ATFS-1, are indicated (29). During mitochondrial dysfunction, ATF5 fails to be imported into mitochondria and traffics to the nucleus, inducing gene transcription.

The *P. aeruginosa*–dependent triggering of UPRmt was evaluated by ATF5 and ATF4 nuclear translocation in different CF and non-CF bronchial cellular models. Representative images show the nuclear translocation of both transcription factors in CF cells after...
Fig. 2. During *P. aeruginosa* infection the mitophagic and xenophagic response in CF bronchial cells is impaired. (A) Uninfected and *P. aeruginosa*–infected airways cells homogenates (hom) of IB3-1 (CF) and S9 (non-CF) cells were fractionated to obtain pure mitochondria (mit) and cytosol (cyt) fractions. Equal amount of proteins (10 μg) from each fraction were analyzed by Western blot using the indicated antibodies. VDAC and actin are, respectively, mitochondrial and cytosolic markers. (B) (I) Representative images of S9 and IB3-1 cells cotransfected with GFP-LC3 and mitochondrial RFP and then treated with PAO1 at an MOI of 100 for 6 hours. (II) Quantitative analysis of S9 and IB3-1 cells that contain mitochondria-localized LC3 puncta; the values are expressed as mean number of colocalized-puncta counts per cell ($n = 15$ to 20 independent visual field for each condition of three independent experiments). (C) (I) Representative images of mitochondrial redistribution of Parkin in S9 and IB3-1 cells after *P. aeruginosa* infection. Cells were cotransfected with Parkin cherry and mitochondrial GFP and then treated with PAO1 at an MOI of 100 for 6 hours. (II) The bars depict the percentage of cells showing the accumulation of Parkin in cluster. (III) Quantitative analysis of cells that contain mitochondria-localized Parkin cluster, the values are expressed as mean number of colocalized-cluster counts per cell ($n = 20$ to 22 independent visual field for each condition of at least three independent experiments). (D) (I) Infected S9 and IB3-1 cells were lysed after the addition of impermeable antibiotics and streaked on LB agar plates for the determination of intracellular colony-forming units (CFUs) ($n = 8$ of independent experiments). (II) Double bacteria labeling and confocal microscopy to quantify bacterial invasion in whole S9 and IB3-1 cells exposed for 6 hours to GFP-*P. aeruginosa*. The bars show the ratio GFP/RFP signal in non-CF and CF bronchial cells, as described in experimental procedure section. (E) (I) Representative images of intracellular *P. aeruginosa* internalization and recruitment of NDP52 to pathogen in IB3-1 cells. (II) The bar chart shows quantification of colocalization signals (%) between NDP52 and pathogen in S9 and IB3-1 cells ($n = 8$ to 13 independent visual field for each condition of at least three independent experiments). The reported data are means ± SE of at least three independent experiments. Paired data were analyzed by Student's t test (*P < 0.05 and **P < 0.01).
Fig. 3. Abnormal *P. aeruginosa*–dependent UPR\(^{\text{mt}}\) activation lead to worsening of autophagic defect and of inflammatory response in CF bronchial cells. (A) Representative confocal images of nuclear translocation of endogenous ATF5 (I) and ATF4 (II) in S9 (non-CF) and IB3-1 (CF) cells during pathogen infection. The graphs report the nuclear redistribution of ATF5 and ATF4 after PAO1 infection, expressed as percentage increase in fluorescent ratio signals (from cytosol to nucleus) with respect to uninfected condition (n = 6 to 10 independent visual field for each condition of three independent experiments). (B) Immunoblots of IB3-1 and S9 cells uninfected or infected for 3, 6, and 12 hours, as indicated. The samples were probed using the antibodies indicated, where actin is used as loading control. (C) (I) Autophagic flux in IB3-1 cells overexpressing ATF5 during *P. aeruginosa* infection. GFP- and ATFS-GFP-transfected cells were infected with PAO1 at 100 MOI for 6 hours and then treated with bafilomycin A1, as indicated. (II) Bar chart shows the ratio LC3-II/LC3-I following quantification of signals from immunoblots (n = 6). (D) Immunoblots of cleaved caspase-1 and processed IL-1\(\beta\) from lysates (lys) or supernatants (sup) of IB3-1 and ATF5-overexpressed IB3-1 cells infected for 6 hours with *P. aeruginosa*. Actin was used as loading control. The quantification is expressed as the ratio of p–10/actin and cleaved IL-1\(\beta\)/actin. The bars are the means ± SE of four independent immunoblots. (E) IB3-1 and S9 cells were transfected with ATFS-GFP and after 6 hours of PAO1 infection, and the cultured cell supernatant were collected to quantify the levels of proinflammatory cytokines by enzyme-linked immunosorbent assay (ELISA) (n = 5 of independent experiments). The selective inhibitor of caspase-1, 20 \(\mu\)M Ac-YVAD-cmk, was added 30 min before infection. (F) Murine WT and NLRP3-null (NLRP3 KO)–derived embryonic fibroblasts (MEFs) were infected with PAO1 at an MOI of 100 for 6 hours and then treated with either vehicle or bafilomycin A1 as indicated. (I) Samples were probed on immunoblots for LC3 and actin as a loading control. (II) Bar chart shows the ratio LC3-II/LC3-I following quantification of signals from immunoblots (n = 4). The reported data are means ± SE of at least three independent experiments. (G) Infected WT and NLRP3 KO MEFs were lysed after the addition of irreversible antibiotics and streaked on LB agar plates for the determination of intracellular CFUs (n = 3 of independent experiments). (H) WT and NLRP3 KO MEFs were transfected with ATFS-GFP and after 6 hours of PAO1 infection, and the cultured cell supernatant were collected to quantify the levels of proinflammatory cytokines by ELISA (n = 3 of independent experiments). Paired data were analyzed by Student’s t test (*P < 0.05 and **P < 0.01).
P. aeruginosa infection (Fig. 3A and fig. S4A). Major ATF5 and ATF4 nuclear redistribution was measured in CF cells with respect to non-CF cells challenged with P. aeruginosa, suggesting that UPR\textsuperscript{mt} is mainly induced in CF cells (Fig. 3A and fig. S4A). P. aeruginosa–dependent UPR\textsuperscript{mt} activation was also monitored by immunoblotting and quantification of UPR\textsuperscript{mt} reporters, such as HSP10, HSP60, and CLPP (Fig. 3B and fig. S4, B and C). Increased expression levels of UPR\textsuperscript{mt} reporters followed the abnormal UPR\textsuperscript{mt} induction promoted by the stabilization of ATF5 and ATF4 in CF cells during pathogen infection. Similar results were also obtained in polarized CF patient–derived airway cells, where the stabilization and dimerization of ATF5, induced by P. aeruginosa infection, corresponded to an increase of UPR\textsuperscript{mt} reporters (fig. S4D).

Considering the defective mitophagy and consequent accumulation of dysfunctional mitochondria, persistent activation of UPR\textsuperscript{mt} was assessed in CF cells when exposed to pathogen infection. Since ATF5 has been shown to suppress autophagy in human BCR-ABL–transformed cells (30), we questioned whether the stabilization of ATF5 in CF cells, and thus the persistent UPR\textsuperscript{mt} activation, affected P. aeruginosa–triggered autophagy and inflammation. To mimic UPR\textsuperscript{mt} hyperactivation, we transfected the chimera ATF5-GFP into CF bronchial cells. This overexpression favored the stabilization of ATF4 and markedly increased the susceptibility to P. aeruginosa–triggered UPR\textsuperscript{mt} activation, enhancing the expression level of UPR\textsuperscript{mt} reporters in CF cells, both under basal conditions and upon P. aeruginosa infection (Fig. 3C and fig. S5A). Moreover, transfection of different CF bronchial cellular models with ATF5, then exposed to P. aeruginosa, decreased the LC3-II/LC3-I ratio according to the autophagic flux assay, indicating that persistent UPR\textsuperscript{mt} activation in CF cells during pathogen infection further decreases the rate of autophagic response (Fig. 3C and fig. S5B). ATF5 overexpression resulted in enhanced inflammatory sensitivity to pathogen infection in CF bronchial cells, as shown by increased expression level of cleaved caspase-1 and cleaved cytokine interleukin–1β (IL-1β) in cell supernatant (Fig. 3D and fig. S5C) and by higher level of inflammasome-dependent IL-1β and IL-18 release (Fig. 3E and fig. S5D). The forced expression of ATF5 strengthened the inflammasome-dependent IL-1β release after incubation with P. aeruginosa in non-CF bronchial cells (Fig. 3, E to 1, and fig. S5, D to I), the same cells, where the presence of wt CFTR mitigates the P. aeruginosa–dependent mitochondrial effects, avoiding NLRP3 inflammasome activation (7).

However, the exacerbation of the P. aeruginosa–triggered inflammation promoted by ATF5 overexpression was attenuated by the pretreatment with caspase-1 inhibitor Ac-YVAD-cmk (20 μM), which, by affecting the inflammasome, reduced the release of cytokines (Fig. 3E and fig. S5D) and ameliorated the rate of autophagic response in CF bronchial cells (fig. S5E). These results confirm that the synergy between P. aeruginosa–dependent mitochondrial stress and consequent abnormal UPR\textsuperscript{mt} activation leads to worsening of autophagic defects and inflammatory responses in CF cells.

Additional data obtained in NLRP3 KO MEFs (KO) and WT mouse embryonic fibroblasts (MEFs) suggested that the downregulation of autophagy during P. aeruginosa infection is also strictly related to NLRP3 inflammasome activation (Fig. 3F). By autophagic flux assay, the LC3-II conversion in the presence of bafilomycin A1 increased in WT fibroblasts, but this increase was augmented in NLRP3 KO fibroblasts, both under basal condition and after P. aeruginosa infection, indicating that loss of NLRP3 stimulates autophagic flux. NLRP3 deficiency augmented bacterial clearance capacity in NLRP3 KO MEFs with respect to WT MEFs (Fig. 3G), while the NLRP3 activator nigericin (10 μM) treatment reduced the bacterial clearance capacity in CF bronchial cells, enhancing further the number of CFU per milliliter of invading pathogen (fig. S5F). Last, the deletion of NLRP3 in MEFs prevented the inflammasome-dependent IL-1β and IL-18 release in ATF5-overexpressing NLRP3 KO MEFs during P. aeruginosa infection (Fig. 3H). Collectively, these findings show that the worsening of defective autophagy in CF bronchial cells, started by P. aeruginosa–induced VAPB and PTPIP51 expression, is also sustained by abnormal UPR\textsuperscript{mt} and NLRP3 inflammasome activation, contributing to persistent accumulation of damaged mitochondria and invading bacteria.

**Regulation of ER-mitochondria tethering controls Ca\textsuperscript{2+} exchange and abrogates the effects of tethering on autophagy**

The expression of VABP or PTPIP51 both led to an increase in IP\textsubscript{3}R3–VDAC interactions (fig. S6A). We monitored whether overexpression of VABP or PTPIP51 affected the uptake of Ca\textsuperscript{2+} by mitochondria following IP\textsubscript{3}R-mediated release from the ER store. For these experiments, we cotransfected, in different CF bronchial cellular models, empty vector (pcDNA3), VABP, or PTPIP51 and mitochondrial–targeted aqueorin Ca\textsuperscript{2+} probe. We triggered physiological IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release by stimulation with 100 μM histamine. As expected, transfection of both VABP and PTPIP51 induced a significant increase in mitochondrial Ca\textsuperscript{2+} response (Fig. 4A and fig. S6B) and had no effects on cytosolic Ca\textsuperscript{2+} levels (fig. S6C). We therefore investigated whether the reduction in autophagy induced by VABP or PTPIP51 overexpression is caused by a higher Ca\textsuperscript{2+} transfer to mitochondrial matrix. To verify this hypothesis, we first quantified the changes in IP\textsubscript{3}R3–VDAC interactions by PLA in MCU-silenced CF bronchial cells (shMCU-CF cells) transfected with pcDNA3, VABP, or PTPIP51 during pathogen infection or SMM treatment (Fig. 4B and fig. S6D). The silencing of MCU induced a reduction in the protein expression (fig. S6E) and in histamine–dependent mitochondrial Ca\textsuperscript{2+} uptake (fig. S6F) without affecting the ΔΨ\textsubscript{m} (fig. S6G). The reduction in mitochondrial Ca\textsuperscript{2+} uptake in shMCU-CF cells protected the mitochondria from P. aeruginosa–dependent ΔΨ\textsubscript{m} loss, decreasing mitochondrial superoxide production and NLRP3 activation (7). The maintenance of mitochondrial physiology in shMCU-CF cells during P. aeruginosa infection is also confirmed by lacked P. aeruginosa–triggering nuclear translocation of transcription factors ATF5 and ATF4, avoiding the UPR\textsuperscript{mt} activation (fig. S6H).

Under basal conditions, the overexpression of VABP or PTPIP51 increased the association between the two ER-mitochondria Ca\textsuperscript{2+} exchange proteins, IP\textsubscript{3}R3–VDAC in shMCU-CF cells. In contrast, no significant differences emerged between empty vector–transfected shMCU-CF cells (pcDNA3) and nonsilenced CF cells (Mock). In CF cells, P. aeruginosa exposure led to a marked enhancement of IP\textsubscript{3}R3–VDAC PLA signaling (Figs. 1A and 4B). This increase was abrogated both in pcDNA3-transfected shMCU-CF cells and in VABP- or PTPIP51-overexpressing shMCU-CF cells, indicating that the changes in IP\textsubscript{3}R3–VDAC interactions during pathogen infection are strictly dependent on MCU (Fig. 4B). The gain of IP\textsubscript{3}R3–VDAC interaction in CF bronchial cells exposed to P. aeruginosa or SMM could be the consequence of compensatory responses that promote the increase of VABP and PTPIP51 expression, at front of pathogen–dependent mitochondrial perturbations, which impairs ΔΨ\textsubscript{m} and thus Ca\textsuperscript{2+} uptake into the mitochondrial matrix.
Fig. 4. MCU targeting abrogates the effects of VAPB and PTPIP51 on autophagy, restoring the mitochondrial physiology in CF bronchial cells exposed to pathogen. (A) Mitochondrial Ca\textsuperscript{2+} response in IB3-1 cells (CF cells) overexpressing VAPB or PTPIP51; representative traces are shown. Cells were cotransfected with mitochondrial-targeted aequorin with control empty vector (pcDNA3), VAPB, or PTPIP51 and stimulated with 100 \textmu M histamine. (B) Stable MCU-silenced IB3-1 clone (IB3-1 shMCU) was transfected with pcDNA3, VAPB, or PTPIP51 and then infected with PAO1 at an MOI of 100 for 6 hours. PLA for IP3R3 and VDAC interactions was performed. The bar chart shows quantification of PLA signals (%), with respect to uninfected IB3-1 cells (mock) (n = 15 independent visual field for each condition of three independent experiments). (C) LC3-clustering cell count in pcDNA3 and VAPB- or PTPIP51-overexpressing IB3-1 shMCU cells during pathogen infection was performed. The bars depict the percentage of cells showing the accumulation of GFP-LC3 in cluster (n = 25 independent visual field for each condition of at least three independent experiments). (D) Effects of VAPB and PTPIP51 on autophagic flux in IB3-1 shMCU cells during pathogen infection. (I) Samples were probed on immunoblots for LC3 and GAPDH as a loading control. (II) Bar chart shows the quantification of the area under the curve. a.u., arbitrary unit. Student’s t test used for indicated comparisons (*P < 0.05 and **P < 0.01).
We also monitored how MCU silencing affected autophagy in VAPB- or PTPIP51-overexpressing CF cells. MCU silencing in CF cells, challenged with *P. aeruginosa*, induced a significant increase in the percentage of GFP–LC3–clustering cells with respect to naive CF cells (Fig. 4C). However, the inhibitory effects of VAPB and PTPIP51 overexpression on the percentage of GFP–LC3–clustering cells were abrogated in shMCU-CF cells exposed to *P. aeruginosa* (Fig. 4C). Moreover, treatment with bafilomycin A1 not only confirmed that the silencing of MCU increased the LC3-II/LC3-I ratio, demonstrating an induction of autophagy, but also showed that this increase was unaffected by overexpression of VAPB or PTPIP51 (Fig. 4D). Thus, the inhibitory effect of VAPB and PTPIP51 on autophagy in CF bronchial cells is lost by blocking mitochondrial Ca\(^{2+}\) uptake. In this case, it is also possible to prevent the increase in IP\(_3\)R3–VDAC interactions during bacterial infection or SMM damage that could further affect the mitochondrial Ca\(^{2+}\) uptake capacity of CF bronchial cells (Fig. 4H).

We also demonstrated that, when preventing *P. aeruginosa*–dependent mitochondrial dysfunction by KB-R7943, the increases in IP\(_3\)R3–VDAC interactions during bacterial infection or SMM were lost (Fig. 4I). We suppose that the exacerbation of the *P. aeruginosa*–triggered inflammatory response is mitigated by MCU inhibition, which avoids a mitochondrial Ca\(^{2+}\) overload due to compensatory increased ER-mitochondria interaction, which, in turn, favors a higher ER-mitochondria Ca\(^{2+}\) transfer in CF bronchial cells during pathogen infection. To demonstrate this, we performed mitochondrial Ca\(^{2+}\) measurement using the ultrasensitive mitochondrial GFP-based Ca\(^{2+}\) probe, mtGCaMP6, useful to measure fast and small mitochondrial Ca\(^{2+}\) changes (Fig. 4J and fig. S7F). Acute exposure of CF bronchial cells to *P. aeruginosa* or SMM induced a sustained increase of mitochondrial Ca\(^{2+}\) concentration (Fig. 4J and fig. S7F). Conversely, by inhibiting MCU with KB-R7943, the mitochondrial Ca\(^{2+}\) uptake in CF bronchial cells exposed to pathogen or SMM was abrogated. No perturbation in mitochondrial Ca\(^{2+}\) signaling was detected in non-CF bronchial cells when exposed to *P. aeruginosa* or SMM (Fig. 4J and fig. S7F), confirming a higher ER-mitochondria Ca\(^{2+}\) transfer in CF bronchial cells. The concept of mitochondrial Ca\(^{2+}\) overload, however, does not necessarily refer solely to a very large increase in mitochondrial Ca\(^{2+}\) concentration, rather much smaller, but prolonged, increases of Ca\(^{2+}\) may activate the organelle dysfunctional machinery, leading to ΔΨ\(_{m}\) dissipation, reduced ATP production, increased release of ROS, protein tyrosine phosphatase opening, and mitochondria swelling (20).

Then, we monitored whether KB-R7943 affected autophagy in CF bronchial cells when exposed to *P. aeruginosa*. The higher LC3-II/LC3-I ratio (Fig. 5A and fig. S8A) and percentage of GFP–LC3–clustering cells (Fig. 5B) in KB-R7943–treated CF cells than in untreated CF cells indicated recovery of autophagy during infection. Treatment with bafilomycin A1 confirmed that KB-R7943 pretreatment, by preventing mitochondrial Ca\(^{2+}\) overload, increased the LC3-II/LC3-I ratio in CF cells upon *P. aeruginosa* challenge and thus autophagy (Fig. 5C). However, the increase in LC3-II/LC3-I ratio (Fig. 5D) and percentage of GFP–LC3–clustering cells (Fig. 5E) in KB-R7943–treated CF cells exposed to the pathogen were unaffected by overexpression of tethers, VAPB or PTPIP51. The recovery of autophagy in KB-R7943–treated CF cells, by MCU inhibition, potentiated the bacterial clearance activity in different CF bronchial cell models when exposed to bacteria (Fig. 5F and fig. S8B), ameliorating cell viability (Fig. 5G and fig. S8C). To exclude the possibility that KB-R7943 has toxic effects on bacterial viability and proliferation, we evaluated *P. aeruginosa* growth under different concentrations of KB-R7943. No changes in the bacterial growth curve emerged (fig. S8D). In line with the data of MCU silencing, the inhibition of mitochondrial Ca\(^{2+}\) uptake abrogates the inhibitory effects of VAPB and PTPIP51 on autophagy in CF bronchial cells, promoting cell resistance to pathogen infection.
KB-R7943 mitigates the P. aeruginosa–triggered inflammatory response in vivo

Last, we analyzed the efficacy of KB-R7943 in counteracting pathogenic CF lung inflammation in vivo. C57Bl/6 Cfttm1 UNC TgN(FABPCFTR)#Jaw (CFTR-KO) mice and their WT (non-CF) littermates were infected with P. aeruginosa AA43 clinical isolate embedded in agar beads and treated with KB-R7943 (300 μg/kg) or vehicle (ctrl) via aerosol administration by the Penn-Century MicroSprayer Aerosolizer. The schedule of treatment was 1 hour before infection and then every 12 hours. Two days after infection and 1 hour after the last treatment, murine lungs and bronchialalveolar lavage fluid (BALF) were collected and processed. First, we evaluated the KB-R7943 distribution in tissues of treated mice by mass spectrometry. The drug was detected both in the lung homogenates [677.1 ± 305.6 pg/ml (WT) versus
Fig. 6. KB-R7943 limits the *P. aeruginosa*–triggered inflammatory response in CF mice. C57BL/6 Chtm1/Junc5N(FA8PCFTR)#Jaw mice (CFTR-KO) (*n* = 20 for each experimental condition) and their WT littermates (*n* = 20 for each experimental condition) were inoculated with 1.5 × 10^6 CFU of *P. aeruginosa* AA43 isolate embedded in agar beads. Mice were treated with KB-R7943 (300 μg/kg) or vehicle via aerosol administration by the Penn-Century MicroSprayer Aerosolizer every 12 hours starting 1 hour before infection. Every 12 hours, mice were monitored for the health status. Two days after infection, 1 hour after the last treatment, murine lungs and BALFs were collected and processed. (A) Kaplan-Meier survival curve for untreated and KB-R7943–treated CFTR-KO mice. The data were analyzed by log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test (*P* = 0.0378; GraphPad Prism, USA) (*n* = 20 mice for each condition of three independent experiments). (B) Clearance and infection were determined on surviving mice. The data were analyzed by Fisher’s test (confidence intervals, 95%; *P* < 0.05; GraphPad Prism, USA) (*n* = 20 mice for each condition of three independent experiments). (C) Bacterial burden in the lungs of KB-R7943–treated and untreated mice after 2 days from *P. aeruginosa* challenge is shown. Bar chart represent means ± SE of lung CFU in mice. Data were analyzed by two-way ANOVA and Tukey’s post hoc test (*P* < 0.05, *n* = 15 mice analyzed of three independent experiments). (D) The images exemplify the lungs of WT and CFTR-KO mice stained with hematoxylin and eosin (H&E), pretreated 1 hour before infection with KB-R7943 and vehicle. Scale bars, 250 μm (I, IV, VII, and X) and 500 μm (II, III, V, VI, VIII, IX, XI, and XII). (E) Graphs summarize histological scoring of global inflammation grading based on H&E staining (*n* = 15 of independent experiments). Data were analyzed by Student’s *t* test used for indicated comparisons and by two-way ANOVA and Tukey’s post hoc test (**P* < 0.01). (F) Immunoblots show NLRP3 and LC3 expression in murine WT and CFTR-KO lung homogenates after *P. aeruginosa* infection. The samples were probed on immunoblots for NLRP3, LC3, and actin as a loading control. Bar chart shows the ratio NLRP3/actin (II) and ratio LC3-II/LC3-I (III) following quantification of signals from immunoblots (*n* = 9 to 12). Student’s *t* test was used for indicated comparisons (*P* < 0.05 and **P* < 0.01). (G) The levels of released IL-1β (I) and IL-18 (II) were measured in BALF of WT and CFTR-KO mice exposed to *P. aeruginosa* infection, treated with KB-R7943 (300 μg/kg) and vehicle. The data were analyzed by two-way ANOVA and Tukey’s post hoc test (*P* < 0.05 and **P* < 0.01; *n* = 25 of independent experiments).
We investigated the effect of KB-R7943 in CFTR-KO and WT mice, measured the survival, bacterial burden, and inflammatory response. Survival of CFTR-KO mice after *P. aeruginosa* infection was significantly improved by KB-R7943 treatment (80% [vehicle] versus 100% [KB-R7943]) (Fig. 6A). In addition, KB-R7943 treatment of CFTR-KO mice led to significantly increased clearance of *P. aeruginosa* infection both in the lung and BALF (Fig. 6B), with significant decrease of bacterial burden (Fig. 6C and fig. 9B) compared to vehicle. Differently, both KB-R7943 and vehicle-treated WT mice survived to infection and did not show differences in bacterial clearance (Fig. 6B) or bacterial burden (Fig. 6C and fig. 9B). These results show that KB-R7943 impacts on a better health status and *P. aeruginosa* infection in CFTR-KO mice.

Lung histopathology and the degree of inflammation were investigated by scoring global inflammatory response, extension, and type in tissue sections of murine lungs stained with hematoxylin and eosin (H&E) (Fig. 6, D and E, and fig. S8, C and D). In CFTR-KO and, in part, in WT mice, *P. aeruginosa* infection favored the diffusion of a massive exudate in lungs (Fig. 6D, I and VII), with a large involvement of alveolar spaces and broncho-bronchiolar lumina that markedly reduced the aerate areas (Fig. 6D, II, III, VIII, and IX). KB-R7943 treatment reduced the inflammatory status in CFTR-KO lungs. A marked reduction of the inflammatory cells in alveoli and deep airways was documented (Fig. 6D, IV, V, VI, X, XI, and XII) in KB-R7943–treated mice compared to vehicle. Moreover, *P. aeruginosa* infection significantly increased histological inflammation scores, which were diminished when the animals were pretreated with KB-R7943 (Fig. 6E and fig. S9, C and D).

In vivo, the pretreatment with KB-R7943 in CFTR-KO mice reduced NLRP3 inflammasome expression in the lung homogenates after infection to levels detected in ctrl WT mice, thus preventing the *P. aeruginosa*–triggered NLRP3 inflammasome priming observed in vehicle-treated CFTR-KO mice (Fig. 6F). In line with the data in vitro, the higher LC3-II/LC3-I ratio in KB-R7943–treated CFTR-KO mice than in vehicle-treated CFTR-KO mice confirmed the recovery of autophagy in vivo (Fig. 6F).

Last, the efficacy of KB-R7943 on the exacerbated *P. aeruginosa*–triggered inflammation in CF was confirmed by the reduced IL-1β and IL-18 levels detected in BALF of treated CFTR-KO mice (Fig. 6G). Conversely, no significant differences in inflammatory cells and tumor necrosis factor–α (TNFα) levels between KB-R7943 and vehicle-treated mice were detected (fig. S9, E and F), suggesting that the “first proinflammatory signal,” or Toll-like receptor signaling, in both lungs is unchanged with the drug treatment. The reduction of released cytokines in BALF is due to rectified autophagy and escaped *P. aeruginosa*–dependent mitochondrial dysfunction, avoiding thus abnormal UPRmt and NLRP3 inflammasome activation in KB-R7943–treated CFTR-KO mice.

**DISCUSSION**

The excessive inflammatory response due to chronic airway infection by *P. aeruginosa* plays a critical role in the CF lung pathology and disease progression. Within the innovative CF therapy pipeline, including CFTR "correctors" and "potentiators," effective and alternative anti-inflammatory drugs are lacking. CFTR correctors and potentiators have garnered much attention in the CF community, although their impact on downstream consequences, such as inflammation, has been debated. Evidence shows that *P. aeruginosa* burden decreased in the first 6 months of modulator therapy but rebounded thereafter, increasing the inflammatory response (36). A number of bacterial pathogens perturb mitochondrial function to promote proliferation and infection, such as *P. aeruginosa*. Conversely, mitochondria present different roles in resistance against bacterial infection, including mitochondrial ROS production and inflammasome activation (37, 38), and protective roles, such as mitophagy and UPRmt (8, 39).

Previous data showed that CFTR-defective bronchial cells have increased intracellular [Ca2+] associated to increased susceptibility to pathogen-dependent mitochondrial dysfunction (7). The perturbed Ca2+ homeostasis in patients with CF underlies the compromising mechanisms of mitochondrial stress response and quality control pathways. The prolonged and abnormal UPRmt activation is the negative consequence of the down-regulation of selective autophagic response in CF, which leads to an increase in pathogen survival, resulting in enhanced innate immune and mitochondrial-protective gene expression (40) and, ultimately, worsened pulmonary inflammation (Fig. 3).

During infection, the interactions between ER and mitochondria change to sustain a range of physiological processes. This structural perturbation is proposed to represent a physiological response of the cell to altered mitochondrial bioenergetics during infection because mitochondria require Ca2+ for efficient production of ATP (41). ER-mitochondria interactions arise as a result of ER-resident VAPB closely associated with mitochondrial protein PTPIP51, enabling ER membrane recruitment to mitochondria. Here, we reported that *P. aeruginosa*–induced VAPB–PTPIP51 tether regulates autophagy in CF bronchial cells. The increase of VAPB and PTPIP51 expression triggered by bacterial infection in CF bronchial cells induces tightening of the tether and concomitant impairment of autophagy (Figs. 1 and 2). We also demonstrated that blocking the ER-mitochondria Ca2+ exchange via MCU-targeted short hairpin RNA (shRNA) or pharmacological inhibition, by KB-R7943, abrogates the effects of VAPB and PTPIP51 on autophagy in CF cells during bacterial infection (Figs. 4 and 5). Our findings are in line with a large number of studies that show that perturbation of Ca2+ delivery to mitochondria stimulates autophagy and mitophagy (11, 42, 43).

Our discoveries about the regulation of mitophagy and UPRmt in CF during *P. aeruginosa* infection have suggested a therapeutic strategy to promote the overall health of the mitochondrial network to limit the CF lung disease progression and bacterial infection. Consistent with the ability of KB-R7943 to reduce CF pulmonary inflammation, the promotion of autophagy appeared to contribute to the protective activity of KB-R7943.

Our findings qualify KB-R7943 as a mitochondrial Ca2+ regulator that rectifies unbalanced autophagic activity controlling UPRmt and NLRP3 inflammasome activation, known to contribute significantly to pathologic airway inflammation in CF (7). The cross-regulation between autophagy and inflammasomes may also explain the ability of KB-R7943 to limit *P. aeruginosa*–triggered NLRP3 inflammasome priming in the lungs of CFTR-KO mice in vivo (Fig. 6) (44, 45).

According to our study, mitochondrial Ca2+ regulators have emerged as a new option for CF therapy in controlling inflammation and bacterial infection. Currently, there are two specific MCU inhibitors, ruthenium red and Ru360, which are known to be
impermeable to the PM (20). In 2017, a high-throughput assay that screened for human MCU-specific small-molecule modulators identified mitoanxtrone as selective inhibitor of MCU among more than 600 clinically approved drugs. Unfortunately, mitoanxtrone was toxic after 48 hours of treatment, independent of its action on MCU (46). Despite mechanistic limitations and the availability of newer, more selective MCU inhibitors, KB-R7943 is the first available MCU inhibitor, which is freely permeable through the PM, with protective activity (31). KB-R7943, which was developed to inhibit the reverse mode of NCX in intact cells (47), has been shown to protect against myocardial ischemia–reperfusion injury and neuronal damage (34, 48, 49). The effect of KB-R7943 is about threefold more selective for NCX3 than for NCX1 and NCX2 (50), and NCX3 is the only isoform that is also located on the outer mitochondrial membrane involved in the regulation of mitochondrial Ca$^{2+}$ homeostasis (51).

The mechanism through which KB-R7943 confers protection remains controversial and debated. If used at concentrations up to 1 μM, KB-R7943 may inhibit a variety of ion channels (Na$^+$, K$^+$, and Ca$^{2+}$), neurotransmitters receptors (nicotinic and N-methyl-D-aspartate), and store-operated Ca$^{2+}$ entry (52). In the kidney, KB-R7943 increases renal vascular resistance, causing vasoconstriction that may be harmful (53), and is toxic to cancer cells at high concentrations (30 μM and more) by inducing apoptosis and affecting autophagy (54). The nonspecificity of the molecule and its toxicity at high concentrations have long limited the clinical usefulness of KB-R7943. Nevertheless, it remains an important pharmacological tool in the laboratory setting to define and develop new therapeutic approaches.

Here, we show that KB-R7943 is endowed with unique activity to correct CF pulmonary physiopathology through the regulation of autophagy and inflammation. Although KB-R7943 appears to play roles in multiple activities through which it may rectify P. aeruginosa–triggered autophagy and preserve mitochondrial homeostasis, inhibition of mitochondrial Ca$^{2+}$ signaling appears to be a key to these mechanisms, as well as considering that the CF bronchial cells used for in vitro experiments lack any PM NCX activity (32).

Through its multitasking activity, KB-R7943 treatment could represent viable strategy to rectify the multifunctional defect in individuals with CF. Airway epithelia absorb Na$^+$ through the epithelial Na$^+$ channel (ENaC), which is negatively regulated by CFTR. In patients with CF, the absence of CFTR results in ENaC hyperactivity and increased Na$^+$ absorption (55). This increase results in membrane depolarization that activates the NCX reverse mode, promoting a further influx of Ca$^{2+}$ in CF cells (56). Intracellular Ca$^{2+}$ overload provides a positive feedback loop for autophagy and chloride secretion inhibition and mitochondrial dysfunction (13, 57–60). This indicates that KB-R7943 could be exploited in CF for pharmacologic correction of outcomes related to (i) intracellular Ca$^{2+}$ overload through inhibition of PM NCX reverse mode and (ii) mitochondrial Ca$^{2+}$ overload through the simultaneous inhibition of MCU and NCX3 reverse mode. In this view, KB-R7943 represents an excellent example of a multitasking drug with anti-inflammatory effects that can also favor rescuing of chloride secretion and CFTR functioning in patient with CF, ameliorating airways disease.

**METHODS**

**Antibodies and other reagents**

For immunoblot and immunostaining, the following antibodies were used:

- anti-actin (A3853), anti-LC3 (L7543), and anti–β-tubulin (T5201; all from Sigma-Aldrich); anti-MCU (ab121499), anti-myc (ab9106), anti-ATF5 (ab60126), anti-pseudomonas (ab68538), anti-VAPB (ab196487), anti-PTPIP51 (ab224081), and anti-VDAC (ab15895; all from Abcam); anti-GFP (sc9996), anti–hemagglutinin (HA) (sc7392), anti–HSP60 (sc13115), anti-mito-photopsin 2 (MFN2) (sc50331), and anti–HSP10 (sc20958; all from Santa Cruz Biotechnology); anti-CLPP (14181), anti-ATF4 (11815), anti–glyceraldehyde-3-phosphate dehydrogenase (2118), anti–IL-18 (12242), and anti–NDP52 (9036; all from Cell Signaling Technology); anti–optineurin (10837-1-AP, ProteinTech); anti–caspase-1 (NB100-56565, Novus Biologicals); anti-NLRP3 (ag20boo14, AdipoGen); and anti–IP3 R3 (BD Biosciences). All the reagents were from Sigma-Aldrich, unless otherwise indicated.

KB-R7943 (sc202681) was dissolved in dimethyl sulfoxide and was from Santa Cruz Biotechnology.

**Cell culture, transfection, and infection**

IB3-1 cells (CF cells) are human bronchial epithelial cells derived from patient with CF with a mutant ΔF508/W1282X genotype. The CF phenotype of the IB3-1 cells was corrected in the S9 cell line (non-CF cells; non-CF) by transfection with WT adenov-associated virus expressing CFTR. Both cells were grown on plastic support under liquid-liquid conditions in LHC-8 basal medium (Life Technologies) supplemented with 5% fetal bovine serum.

CuFi-1 (CF cells) and NuLi cells (non-CF) were a gift from J. Zabner (University of Iowa). The CuFi-1 cells were derived from human bronchial epithelia from a patient with CF (CFTR mutant genotype ΔF508/ΔF508), and the NuLi cells were derived from the normal lung of a 36-year-old patient. These cells were grown under liquid-liquid conditions on human placental collagen-coated flasks (Sigma-Aldrich) with bronchial epithelial growth medium (Lonza).

The CF patient–derived cells are represented by polarized mucociliary-differentiated human airway epithelium that was reconstituted in vitro using cells isolated from patient (MucilAir Epithelix CF-202; a 24-year-old female with homozygous ΔF508/ΔF508) and grown on Transwell air–liquid interface. The wt CFTR human primary cells are represented by polarized mucociliary-differentiated human airway epithelium that was reconstituted in vitro with cells isolated from donor (MucilAir Epithelix; MD-801, a 59-year-old female donor with no pathology reported) and grown on Transwell air–liquid interface.

Stable MCU-silencing IB3-1 cell clone was obtained using shRNA targeting MCU (TRCN0000133861) purchased from Sigma-Aldrich. IB3-1 cells were cultured in tissue dish to 75% of confluence and infected with lentiviral-driven shRNA targeting MCU for 48 hours. The infected cells were selected by the addition of 5 μM puromycin to the culture medium, fed every 2 to 3 days with selection medium, and checked for cell death after 3 to 7 days. Resistant cells were normally observed after about 2 weeks of selection. Individual cells were selected randomly and placed in separate wells within a 24-well plate. Clones were grown to confluence and expanded in a maintenance puromycin media (0.5 μM). Expanded clones were restaged for MCU expression before any further studies. Primary Nlrp3$^{−/−}$ and Nlrp3$^{−/−}$ MEFs were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin-streptomycin-glutamine (100×) liquid.

For the transient overexpression experiments, pNEO plasmid encoding a myc-tagged VAPB and a HA-tagged PTPIP51 and ATF5...
cloned in pEGFP-N1 were transfected with Lipofectamine LTX (Life Technologies) according to the manufacturer’s recommendations. After 36 hours, the transfected cells were infected with the well-characterized, motile, nonmucoid \textit{P. aeruginosa} laboratory strains, named PAO1, donated by A. Prince (Columbia University). Bacterial colonies from overnight cultures on trypticase soy agar (Difco) plates were grown in 20 ml of trypticase soy broth (Difco) at 37°C with shaking until an optical density (OD$_{600nm}$) corresponding to $1 \times 10^7$ CFU/ml was reached. The bacteria were washed twice with Krebs-Ringer buffer (KRB), and diluted in each specific serum-free medium before infection. PAO1 was added to cells as indicated in the captions for Figs. 1 through 5.

Mucopurulent material was harvested from the lumens of excised human CF lungs infected with \textit{P. aeruginosa} at the UNC Adult Cystic Fibrosis Center. SMM from several patients was pooled to study disease progression. SMM from several patients was pooled to study disease progression.

Proximity ligation assay
PLAs to quantify IP$_{3}$R3–VDAC interactions were performed in according to the manufacturer’s protocol of Duolink reagents (Sigma-Aldrich). Cells were fixed in 4% paraformaldehyde in KRB and probed with mouse IP$_{3}$R3 (BD Biosciences) and rabbit anti-VDAC1 (Abcam) antibodies. Signals were developed using a Duolink In Situ Detection Kit (Abcam). The images were acquired using a computer algorithm based on the Ca$^{2+}$ response curve of WT and mutant aquorins.

Immunoblotting
Briefly, to obtain whole-cell extracts, cells were washed, harvested, and lysed in radioimmunoprecipitation assay buffer supplemented with 2 mM Na$_3$VO$_4$, 2 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Roche Diagnostics Corp.).

Thereafter, protein extracts were separated on precast 4 to 12% SDS–polyacrylamide gel electrophoresis gels (Life Technologies), electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad), and probed with the specific antibodies. Last, the membranes were incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies (SouthernBiotech), followed by chemiluminescence detection using West Pico reagent (Thermo Scientific Pierce). The immunoreactive bands were acquired using the ImageQuant LAS-4000 system (GE Healthcare) and quantified using ImageJ. The immunoblots shown are representative of at least three independent experiments.

Immunofluorescence
After infection, the cells were washed with KBR and fixed in 4% paraformaldehyde for 10 min. Then, cells were permeabilized for 10 min with 0.1% Triton X-100 in KBR and blocked in KBR containing 2% bovine serum albumin and 0.05% Triton X-100 for 1 hour. Cells were then incubated with primary antibody overnight at 4°C and washed three times with KBR. The appropriate isotype-matched, Alexa Fluor–conjugated secondary antibodies (Life Technologies) were used. Images were taken with a Nikon Swept Field confocal equipped with CFI Plan Apo VC60XH objective [numerical aperture (NA), 1.4] (Nikon Instruments) and an Andor DU885 electron multiplexing charge-coupled device camera (Andor Technology Ltd.).

Ca$^{2+}$ measurements
For cytosolic aquorin and mitochondrial aquorin measurements, cells growing on coverslips were incubated with 5 μM coelenterazine for 2 hours in KRB supplemented with 1% fetal calf serum and then transferred to the perfusion chamber. To reconstitute the aquorin chimeras targeted to the ER, the luminal [Ca$^{2+}$] of this compartment had to be first reduced by incubating the cells for 1 hour at 4°C in KRB supplemented with 5 μM ionomycin and 600 μM EGTA in the presence of 5 μM coelenterazine, followed by extensive washing of the cells with KRB supplemented with 2% BSA and 1 mM EGTA. All aquorin measurements were performed in KRB supplemented with 1 mM CaCl$_2$, and agonists and other drugs were added to the same medium, as specified in the figure legends. Experiments terminated by lysing the cells with 100 μM digitonin in a hypotonic, Ca$^{2+}$-rich solution to discharge the remaining aquorin pool. The output of the discriminator was captured by a Thorn EMI photon counting board and stored in an IBM-compatible computer for further analyses. The aquorin luminescence data were calibrated offline into [Ca$^{2+}$] values using a computer algorithm based on the Ca$^{2+}$ response curve of WT and mutant aquorins.

Microscopy analysis
For autophagic and mitophagic count, the cells were seeded and then transfected with LC3 in pEGFP-C1 and Parkin cherry, respectively. After 36 hours, images were taken on a Zeiss Axiovert 200 fluorescence microscope (63×, 40×, or 20× objectives). For each condition, the number of GFP-LC3 or Parkin cherry–clustering cells was counted in at least 10 to 20 independent visual fields and expressed as percentage. For colocalization analysis, the cells were seeded and then cotransfected with mtRFP and GFP-LC3 or mtGFP and Parkin cherry using Lipofectamine LTX. After 36 hours, coverslips were placed in an incubated chamber with controlled temperature, CO$_2$, and humidity, and z stacks at 21 planes and a distance of 0.2 μm were taken to allow acquisition of the entire cell. The images were restored with the AutoQuant three-dimensional blind deconvolution module and loaded into Imaris 4.0 (Bitplane AG). Obtained puncta images were merged to compare the GFP signals with RFP or cherry signals using ImageJ software. For each condition, the colocalization of these two signals was quantified in at least 20 independent visual fields, expressing the data as number of LC3 or Parkin dots localized to mitochondria/cell.

Subcellular fractionation
After infection, the cells (10$^9$) were harvested, washed two times with KBR, resuspended in homogenization buffer [225 mM mannitol, 75 mM sucrose, 30 mM tris-HCl (pH 7.4), 0.1 mM EGTA, and phenylmethylsulfonyl fluoride], and gently disrupted by Dounce homogenization. The homogenate was centrifuged twice at 600g for 5 min to remove nuclei and unbroken cells. The supernatant was then centrifuged at 10,300g for 10 min to pellet crude mitochondria. The crude mitochondrial fraction was resuspended in isolation buffer [250 mM mannitol, 5 mM Hepes (pH 7.4), and 0.5 mM EGTA] and then subjected to Percoll gradient centrifugation. For Percoll medium: 225 mM mannitol, 25 mM Hepes (pH 7.4), 1 mM EGTA, and 30% (v/v) Percoll. After centrifugation at 95,000g for 30 min (SW40 rotor), a dense band containing purified mitochondria was collected, washed by centrifugation at 6300g for 10 min to remove the Percoll, and lastly, resuspended in isolation medium.
Bacterial clearance

Bacterial killing was performed infecting human airway epithelial cell with GFP-expressing *P. aeruginosa* strains (ampicillin resistant strains) at different multiplicities of infection (MOIs). Briefly, the cells were infected in serum-free LHC-8 media with ampicillin-resistant *P. aeruginosa* strain at an MOI of 1:10 and 1:100 for 6 hours. Extracellular bacteria were killed with cell-impermeable antibiotics [penicillin-streptomycine (50 U/ml each; Life Technologies) and gentamycine (200 μg/ml; Life Technologies)]. Cells were then washed in KBR and lysed in KBR containing 0.2% Triton X-100 (Sigma-Aldrich), and a serial dilution of lysate was streaked in duplicate on ampicillin-selected LB (lysogeny broth) agar plates and incubated overnight at 37°C. The day after, CFUs were counted. Samples containing no cells were used to identify background CFUs, which were subtracted from samples.

Bacterial invasion

Double bacteria labeling, confocal microscopy, and image analysis allow identification and quantification of the number of internalized bacteria. To identify intracellular and extracellular bacteria, images from whole cells were analyzed in three steps. First, a binary mask of the *P. aeruginosa* antibody channel (594 nm) highlighting extracellular bacteria was generated. Then, the mask was subtracted from the bacterial channel (GFP-expressing *P. aeruginosa* strains; 488 nm), giving intracellular bacteria as a result. Last, the logical AND operation was performed between the mask and the bacterial channel, giving extracellular bacteria as a result. Images of pathological and non-CF cells infected with GFP-expressing *P. aeruginosa* and stained with anti–*P. aeruginosa* antibody were acquired. All data are expressed as ratio between the 488- and 594-nm signal.

ROS production measurements

Mitochondrial ROS production was measured by staining cells in KBR for 15 min at room temperature with 5 μM MitoSox Red (Life Technologies) and successively analyzed on a Tali image-based cytometer (Life Technologies).

Mitochondrial membrane potential measurements

Cells were loaded with 10 nM tetramethylrhodamine methyl ester (TMRM) (Life Technologies) at 37°C for 20 min, placed in a humidified (95% humidity) incubator at 37°C, and successively analyzed on a Tali image-based cytometer (Life Technologies). The annexin V–positive cells were recognized as dead cells by the cytometer software and expressed as percentage of cellular death.

Cell viability assay

Cells were plated in 60-mm plates and pretreated with 1 μM KB-R7943 for 1 hour before PAO1 infection. Cells were harvested 72 hours after treatment and stained using annexin V Alexa Fluor 488/propidium iodide, as described by a Tali apoptosis kit (Life Technologies). Cell viability was evaluated using the Tali image-based cytometer (Life Technologies). The annexin V–positive cells were recognized as dead cells by the cytometer software and expressed as percentage of cellular death.

Subjects and housing

Animals used in all procedures were 10- to 19-week-old gut-corrected CFTR-deficient male C57BL/6 Cfrtm1UNCtgN(FABPCFTR) #1 mouse (CFTR-KO) and their WT littermates. All mice were maintained under specific pathogen–free conditions in sterile cages, which were put into a ventilated isolator. Fluorescent lights were cycled 12 hours on and 12 hours off, and ambient temperature (23°C ± 1°C) and relative humidity (40 to 60%) were regulated. Mice were fed with irradiated 5K-52 rodent chow (Safe, France) and autoclaved tap water.

Mouse model of chronic infection and lung histological analysis

For the infection procedure, *P. aeruginosa* AA43 strain isolated from a patient with CF before death was embedded in agar beads. Mice were anesthetized to implant into the lung an inoculum of 50 μl of agar bead suspension, embedding 1.5 × 10^6 CFU of AA43 strain.

Mice were treated with KB-R7943 (300 μg/kg) or vehicle via aerosol administration by Penn-Century MicroSprayer Aerosolizer every 12 hours starting 1 hour before infection. Two days after infection (1 hour after last treatment), mice were sacrificed to collect BALF and lungs for histopathological analysis. A detailed description of all the procedures used is reported in Supplementary Materials and Methods section.

Mass spectrometry

All the quantitative analysis of KB-R 7943 were performed using a Xevo TQD mass spectrometry instruments (Waters, USA) equipped with a Acquity ultraperformance liquid chromatography system with a BEH (ethylene bridged hybrid) C18 column using a linear gradient of water and acetonitrile with 0.1% of formic acid.

Cytokines assay

Airway epithelial cells were plated in six-well plates and left uninfected, pretreated with the selective inhibitor of caspase-1 [20 μM; 30 min before infection or KB-R7943 (1 μM) 1 hour before infection], and primed with bacteria (MOI of 100) for 6 hours at 37°C. IL-1β, TNFα, and IL-18 released in the cell culture supernatants and/or in 1 mM CaCl_2), and imaging was performed on an Olympus Xcelflour widefield system equipped with a 40x/1.3 NA Oil Plan Fluor objective. Excitation was performed at 490 and 400 nm, and one field was collected per coverslips. Images were captured each 500 ms, and after the first 50 frames, PAO-1 (MOI of 100) or SMM (MOI of 1:100) was added to cells as indicated in the figure. Analysis was performed with ImageJ where both images were background and corrected frame by frame. The data are presented as the means ± SE of the ratio of all time points of all the experiments, using Prism (GraphPad Prism, USA), and we have calculated the area under curve for each experiments.
SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/19/eaax9093/DC1

SCIENCE ADVANCES | RESEARCH ARTICLE

View request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES

1. J. S. Elborn, Cystic fibrosis. Lancet 388, 2519–2531 (2016).
2. L.-C. Tsui, R. Dorfman, The cystic fibrosis gene: A molecular genetic perspective. Cold Spring Harb. Perspect. Med. 3, a009472 (2013).
3. E. A. Rose, D. P. Nicholas, J. F. Chmiel, Inflammation in cystic fibrosis: An update. Pediatr. Pulmonol. 50, 118–126 (2015).
4. R. Rubino, V. Bezzerri, M. Favia, M. Facchini, M. Tebon, A. K. Singh, B. Riederer, U. Seidler, M. Lazarou, D. A. Sliter, L. A. Kane, S. A. Sarraf, C. Wang, J. L. Burman, E. F. McKone, P. K. Singh, M. K. Brittain, T. R. Cummins, V. Pinelis, N. Brustovetsky, C. G. Gallagher, J. E. Bruce, D. A. Stoltz, M. J. Welsh, L. R. Hoffman, E. F. McKone, P. K. Singh, M. R. Knowles, P. Pinton, P. Gasparini, G. Brenton, G. Cablini, Phosphopilase C-J3 is a key modulator of IL-8 expression in cystic fibrosis bronchial epithelial cells. J. Immunol. 186, 4946–4958 (2011).
5. B. M. Wiczer, R. Marcu, B. J. Hawkins, KB-R7943, a plasma membrane Na+/Ca2+ exchanger inhibitor, blocks opening of the mitochondrial permeability transition pore. Cell Death Differ., 2037–2043 (2016).
6. C. Giorgi, S. Marchi, P. Pinton, The machineries, regulation and cellular functions of mitochondrial Ca2+. Nat. Rev. Mol. Cell Biol. 19, 713–730 (2018).
7. C. Giorgi, A. Danese, S. Missirol, S. Paternagni, P. Pinton, Calcium dynamics as a machine for decoding signals. Trends Cell Biol. 28, 258–273 (2018).
8. A. Rimessi, C. Giorgi, P. Pinton, R. Rizzuto, The versatility of mitochondrial calcium signals: From stimulation of cell metabolism to induction of cell death. Biochim. Biophys. Acta 1777, 808–816 (2008).
9. S. Marchi, S. Paternagni, S. Missirol, G. Morciano, A. Rimessi, M. R. Wieckowski, C. Giorgi, P. Pinton, Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. Cell Calcium 62, 62–78 (2017).
bacteria and inflammation in people with cystic fibrosis and chronic lung infections. Am. J. Respir. Crit. Care Med. 195, 1617–1628 (2017).

37. M. A. Puertollano, E. Puertollano, G. A. de Cienfuegos, M. A. de Pablo, Dietary antioxidants: Immune and host defense. Curr. Top. Med. Chem. 11, 1752–1766 (2011).

38. R. Zhou, A. S. Yazdi, P. Menu, J. Tschopp, A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225 (2011).

39. N. V. Kirienko, F. M. Ausubel, G. Ruwkul, Mitophagy confers resistance to siderophore-mediated killing by Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U.S.A. 112, 1821–1826 (2015).

40. M. A. Qureshi, C. M. Haynes, M. W. Pellegrino, The mitochondrial unfolded protein response: Signaling from the powerhouse. J. Biol. Chem. 292, 13500–13506 (2017).

41. M. Bonora, S. Patergnani, A. Rimessi, E. De Marchi, J. M. Suski, A. Bononi, C. Giorgi, S. Marchi, S. Missiroli, F. Poletti, M. R. Wieckowski, P. Pinton, ATP synthesis and storage. Purinergic Signal 8, 343–357 (2012).

42. S. Böckler, B. Westermann, Mitochondrial ER contacts are crucial for mitophagy in yeast. Dev. Cell 28, 450–458 (2014).

43. K. Malliankaraman, C. Cárdenas, P. J. Doonan, H. C. Chandramoorthy, K. M. Irininki, T. Golenar, G. Córdas, P. Madireddi, J. Yang, M. Müller, R. Miller, J. E. Kolesar, J. Molgó, B. Kaufman, G. Hajnóczky, J. K. Foskett, M. Madesh, MCU1 is an essential component of mitochondrial Ca^{2+} uptake that regulates cellular metabolism. Nat. Cell Biol. 14, 1336–1343 (2012).

44. M. A. Rodgers, J. W. Bowman, Q. Liang, J. U. Jung, Regulation where autophagy intersects the inflammasome. Antioxid. Redox Signal. 20, 495–506 (2018).

45. M. Takahama, S. Akira, T. Saijo, Autophagy limits activation of the inflammasomes. Immunol. Rev. 281, 62–73 (2018).

46. D. M. Arduini, J. Wettmarshausen, J. H. Cheng, A. Leimpek, D. M. Arduino, J. Wettmarshausen, H. Vais, P. Navas-Navarro, Y. Cheng, A. Leimpek, A. Feliciello, L. Annunziato, NCX3 regulates mitochondrial Ca^{2+} handling through domains influencing sensitivity to isothiourea derivative inhibitor KB-R7943 in cardiac myocytes. Mol. Pharmacol. 81, 1132–1140 (2005).

47. H. Hagihara, Y. Yoshikawa, Y. Ohga, C. Takenaka, K.-Y. Murata, S. Taniguchi, M. Takaki, Na^{+}/Ca^{2+} exchanger. Ca^{2+} overload. Curr. Top. Med. Chem. 17, 15–30 (2017).

48. Z. Ma, A. Delrio-Lorenzo, A. Giordano, C. Garcia-Perez, G. Médard, B. Kuster, B. Liu, S. E. Peel, J. Fox, I. P. Hall, Reverse mode Na^{+}/Ca^{2+} exchange mediated by STIM1 inhibitor. A. Facchini, I. De Fino, C. Cigana, A. Talarico, A. Rimessi, C. Pozzato, L. Carparelli, A. Rossi, S. Ranucci, I. De Fino, C. M. Haynes (University of Massachusetts) for ATF5 in pEGFP plasmid; and B. Tümmler (Medizinische Hochschule Hannover) for supplying the P. aeruginosa AA43 clinical isolate. Funding: This study was supported initially by the Italian Cystic Fibrosis Research Foundation (grant FFC no. 19/2014 to P.F., FFC no. 20/2015 to A.R., and CFAcore to A.B.). Moreover, the Signal Transduction Laboratory is supported by the following: the local funds from University of Ferrara, FIR-2017, the Italian Ministry of Health (GR-2016-02364602), and the Italian Ministry of Education, University and Research and PRIN grant 2017AX5JSN) to A.R. and the Italian Association for Cancer Research (AIRC, IG-23670), Telethon (GGP111398), local funds from the University of Ferrara, and the Italian Ministry of Education, University and Research (PRIN grant 2017E5LSP3) to P.P. M.R.W. was supported by the Polish National Science Centre (grant UMO-2014/15/NZ1/00490). P.P. is grateful to C. d. Scrovegni for continuous support. Author contributions: A.Ri. and P.P. designed the experiments. A.Ri., C.P., and L.C. performed the experiments in vivo, while the histopathological analysis was performed by G.R. C.T. and A.T. performed the drug distribution analysis. A.B., A.Ro., S.R., I.D.F., and C.C. carried out most of the experiments and data analysis. A.B., A.Ro., S.R., I.D.F., and C.C. performed the experiments in vivo, while the histopathological analysis was performed by G.R. C.T. and A.T. performed the drug distribution analysis. A.Ri., A.B., C.M.P.R., M.R.W., G.C., and P.P. contributed to the interpretation and discussion of results. A.Ri. and P.P. wrote the manuscript with input from all authors. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or in the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 6 May 2019
Accepted 20 February 2020
Published 6 May 2020
10.1126/sciadv.aax9093

Citation: A. Rimessi, C. Pozzato, I. Carparelli, A. Rossi, S. Ranucci, I. De Fino, C. Cigana, A. Talarico, M. R. Wieckowski, C. M. P. Ribeiro, C. Trapella, G. Rossi, G. Cabrini, A. Bronaghi, P. Pinton, Pharmacological modulation of mitochondrial calcium uniporter controls lung inflammation in cystic fibrosis. Sci. Adv. 6, eaax9093 (2020).