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RESEARCH ARTICLE

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Resolvin D1 improves airway inflammation and exercise capacity in cystic fibrosis lung disease

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Abstract

Mucus plugging and non-resolving inflammation are inherent features of cystic fibrosis (CF) that may lead to progressive lung disease and exercise intolerance, which are the main causes of morbidity and mortality for people with CF. Therefore, understanding the influence of mucus on basic mechanisms underlying the inflammatory response and identifying strategies to resolve mucus-driven airway inflammation and consequent morbidity in CF are of wide interest. Here, we investigated the effects of the proresolving lipid mediator resolvin (Rv) D1 on mucus-related inflammation as a proof-of-concept to alleviate the burden of lung disease and restore exercise intolerance in CF. We tested the effects of RvD1 on inflammatory responses of human organotypic airways and leukocytes to CF mucus and of humanized mice expressing the epithelial Na+channel (βENaC-Tg) having CF-like mucus obstruction, lung disease, and physical exercise intolerance. RvD1 reduced pathogenic phenotypes of CF-airway supernatant (ASN)-stimulated human neutrophils, including loss of L-selectin shedding and CD16. RNASeq analysis identified select transcripts and pathways regulated by RvD1 in ASN-stimulated CF bronchial epithelial cells that are involved in sugar metabolism, NF-κB activation and inflammation, and response to stress. In in vivo inflammation using βENaC TG mice, RvD1 reduced total leukocytes, PMN, and interstitial Siglec-MΦ when given at 6–8 weeks of age, and in older mice at 10–12 weeks of age, along with the decrease of pro-inflammatory chemokines and increase of anti-inflammatory IL-10. Furthermore, RvD1 treatment promoted the resolution of pulmonary exacerbation caused by *Pseudomonas aeruginosa* infection and significantly enhanced physical activity and energy expenditure

Abbreviations: AcOH, acetic acid; ASN, airway supernatant; BAL, bronchoalveolar lavage; FEV1%, forced expiratory volume in the first second percent of predicted; G-CSF, granulocyte-colony stimulating factor; HPLC, high performance liquid chromatography; I.P., intra peritoneal; I.T., intratracheal; KC, keratinocyte chemoattractant; K_3EDTA, tripotassium ethylenediaminetetraacetic acid; MeOH, methanol; q.a.d, every other day; RvD1, 7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13Z,15E,19Z-docosahexaenoic acid; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor; VCO_2, volume of carbon dioxide production; VO_2, volume of oxygen consumption.

associated with mucus obstruction, which was impaired in βENaC-Tg mice compared with wild-type. These results demonstrate that RvD1 can rectify features of CF and offer proof-of-concept for its therapeutic application in this and other muco-obstructive lung diseases.

KEYWORDS

airway-on-chip, macrophages, neutrophils, obstructive/restrictive respiratory disease, specialized proresolving lipid mediators

1 | INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease due to mutations in the cystic fibrosis transmembrane regulator (CFTR) gene that result in insufficient protein amounts or function. By occurring in 1:3000 1:30 000 live births and affecting ~100 000 people of all races and ethnicities, CF is the most common life-threatening inherited disease worldwide. Most of the morbidity and mortality of CF arise from a progressive, severe, and non-resolving inflammatory lung disease, characterized by the accumulation of dense and sticky mucus.[1–3] Lung disease in people with CF evolves from small-airway mucus obstruction evident at birth into diffuse inflammation, bronchiectasis, and airway damage that compromise respiratory function and reduce physical exercise capacity.[4–9] Improvements in medical care and therapeutic options offered by CFTR modulators to people with some mutations have increased life expectancy and symptoms. Nonetheless, people with CF still suffer from mucus-related lung disease and physical intolerance, which indicates that additional therapies would be beneficial even if CFTR protein and functions are restored.

According to the classical pathophysiology paradigm, defective CFTR protein levels or function lead to impaired ion transport, hydration, and mucus clearance on the apical membranes of airway epithelial cells.[10] However, accruing evidence indicates that the epithelial Na channel (ENaC) is hyperactive in patients with CF, causing excessive cellular reabsorption of sodium and water from the extracellular environment, thus contributing to the dehydration and increased viscosity of the bronchial mucus.[11,12] This notion is well corroborated by transgenic (Tg) mice with airway epithelial cell-specific overexpression of the beta subunit of the *Scnn* gene that encodes for the beta subunit of the epithelial sodium channel (βENaC).[11,13] βENaC-Tg mice exhibit loss of water from airway surfaces, mucostasis, and lung inflammation characterized by a relentless influx of neutrophils (PMN) and macrophages (MΦ) that are pathophysiologic mechanisms of CF. Moreover, airway leukocytes in βENaC-Tg mice undergo the same phenotypic changes occurring in people with CF that impair their ability to clear infections, resolve inflammation, and restore homeostasis.[14,15] Hence, they allow us to test new strategies to alleviate the burden of mucus-driven lung inflammation and disease that are paramount in CF.

It is now clear that resolution of acute inflammation is an active process where specialized pro-resolving lipid mediators (SPM) play key roles in protecting the host organism.[16,17] SPM are biosynthesized from polyunsaturated fatty acids through enzymatic reactions and include lipoxins (LX), resolvins (Rv), maresins (MaR), and protectins that have protective roles on the airway, for instance, in bacterial pneumonia,[18] chronic obstructive pulmonary disease (COPD),[19] and viral infections.[20] SPM tune leukocyte responses to pathogenic stimuli, blunting activation markers and cytokine storm while activating clearance of infections,[21] and may underly beneficial effects of omega-3, for instance, COVID-19.[22] RvD1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid) is a member of the SPM superfamily biosynthesized from the essential omega-3 DHA through enzymatic reactions occurring within leukocyte and resident cells in the bloodstream and inflammatory exudates,[23,24] including CF airway secretions.[25,26] RvD1 binds two specific G-protein coupled receptors, ALX/FPR and DRV1/GPR32, that are expressed in airway leukocytes and resident cells[26,27] and conveys its anti-inflammatory effects, such as reduction of inflammatory cytokines, neutrophil recruitment, and MΦ activation that contribute to tissue damage.[19] Moreover, RvD1 ameliorates in vivo lung diseases driven by chronic bacterial infections that constitute the clinical phenotype of people with CF.[26,28]

Since roles and functions of RvD1 in persistent CF lung inflammation are of wide therapeutic interest, in the present study, we investigated whether RvD1 reduced lung disease and inflammation associated with mucus obstruction ex vivo using airway mucus and cells from patients with CF as well as in vivo with βENaC-Tg mice.

2 | MATERIALS AND METHODS

2.1 | Study Participants, Sample Collection, and Analyses

K_3EDTA-anticoagulated blood and airway sputum were collected from adult (>18 years of age, $n = 5$) volunteers

with a confirmed diagnosis of CF and mild lung disease (FEV1% 70%–90%). A consent form was signed by participants according to the approved clinical protocol (Prot. RECCHI19 approved on 09/24/2020) conducted according to the Declaration of Helsinki principles.

2.2 | Primary CF Cell Culture and Neutrophil Isolation

Organotypic airway chips were obtained with CF bronchial epithelial cells (CFBEC) grown on Alvetex scaffolds submerged in Ham's F12, 2% Ultroser G (Pall Corp., New York) medium for 7–10 days followed by culture in air-liquid interface up to 2 weeks as in Ref.[26] CFBEC in airway chips were exposed to medium with 3.3% CF ASN (mix of sputum from 3 different volunteers) on the apical side. RvD1 (10 nM) or ethanol (0.01%) as a vehicle control was added in the apical and basolateral compartment. RvD1 was obtained from Cayman Chemical (Ann Arbor, MI, Cat. # 10012554) and stored protected from the light at- -80 °C prior to the experiments. Concentration of each stock solution was determined under UV (in MeOH) using the Lambert-Beer law (molar extinction coefficient ε= 50,000 $M^{-1}cm^{-1}$).

2.3 | RNA sequencing

RNA from CFBEC was extracted and then processed according to the Nanopore protocol (Nanopore SQKDCS 109 with EXP-NBD 104 and EXP-NBD 114). Barcoded cDNA libraries were loaded in a MinION flow cell (Nanopore). Raw data are accessible through the GEO website (GSE234839). Analyses were repeated 3 times with RNA samples derived from cells explanted from different donors.

2.4 | Mice

Hemizygous C57Bl6/N mice overexpressing one transgenic murine βENaC (Scnn1b) allele and wild-type (WT) littermates were bred and genotyped with PCR from tail DNA as previously reported.[11,29] Age- and gender-matched mice were treated for two weeks with RvD1 (100 ng/mouse, I.P., q.a.d.) or ethanol (0.5% vol/vol) as a vehicle control. Mice were monitored daily for clinical signs of disease. Two weeks after the treatment, mice were sacrificed, and BAL samples were collected by injecting/aspirating three aliquots of sterile DPBS i.t. (1 mL each) with a catheter (0.9 × 25 mm) connected to a 1 mL syringe. Total leukocytes in BAL were analyzed with a FACS Canto II flow cytometer and the FACS Diva (BD Bioscience). Samples were stained (30 min, 4°C) with fluorochrome-labeled antibodies (from Sony Biotechnology or Biolegend) against the following antigens: CD45, CD11b, Siglec, F4/80, Ly6G. Concentrations of keratinocyte chemoattractant (KC), tumor necrosis factor (TNF)-α, interleukin (IL)-10, granulocyte-colony stimulating factor (G-CSF), and vascular endothelial growth factor (VEGF) in BAL were measured with a Milliplex multianalyte magnetic bead array. Samples were acquired and analyzed by blind investigators at Transmed Research (https://transmed-research.com). For experiments on bacterial exacerbation, mice were infected with RP73 as previously published by us.[28] Following I.T. inoculum of RP73, mice were monitored daily and treated via gavage with RvD1 or vehicle, using the above doses. Animal protocols were approved by the Ministry of Health (N° 852/2019-PR).

2.5 | Basal indirect calorimetry

Mice were individually placed inside the registration chamber of the calorimetry system (Pan Lab/Harvard Instruments, Spain), with free access to food and water for 48 consecutive hours. After 24 h adaptation, the volume of carbon dioxide production (VCO_2) and the volume of oxygen consumption (VO_2) were registered during the following 24 h using the Oxyletpro calorimeter system (Pan Lab/Harvard Instruments, Spain). VCO_2 production was then used to estimate O_2 consumption and other parameters as previously described.[30]

The analysis of the respiratory quotient (RQ) and the energy expenditure (EE) were performed using the Metabolism software (Pan Lab/Harvard Instruments, Spain) and calculated as follows: $RQ = VCO_2 / VO_2$; $EE = (3.815 + (1.32 \times RQ)) \times VO_2 \times 1.44$.

2.6 | Incremental speed test

The incremental speed test was performed inside a modular system composed by a treadmill apparatus (LE8708TS, OxyletPro system, Pan Lab/Harvard Instruments, Spain), connected with the calorimetry system previously described, allowing the integration of respiratory metabolism (O_2 consumption/CO_2 production).

2.7 | Statistics

Data were reported as median with interquartile range and 25th/75th percentile or as mean and standard error depending on normality (ascertained with Shapiro–Wilk test). Nonparametric Mann–Whitney rank sum test, ANOVA on ranks, or parametric t-test followed by Tukey's

post-hoc test was used depending on normality of data. $p < .05$ were taken as significant.

3 | RESULTS

3.1 | Resolvin D1 reduces airway mucus-driven inflammatory responses of neutrophils and airway epithelial cells

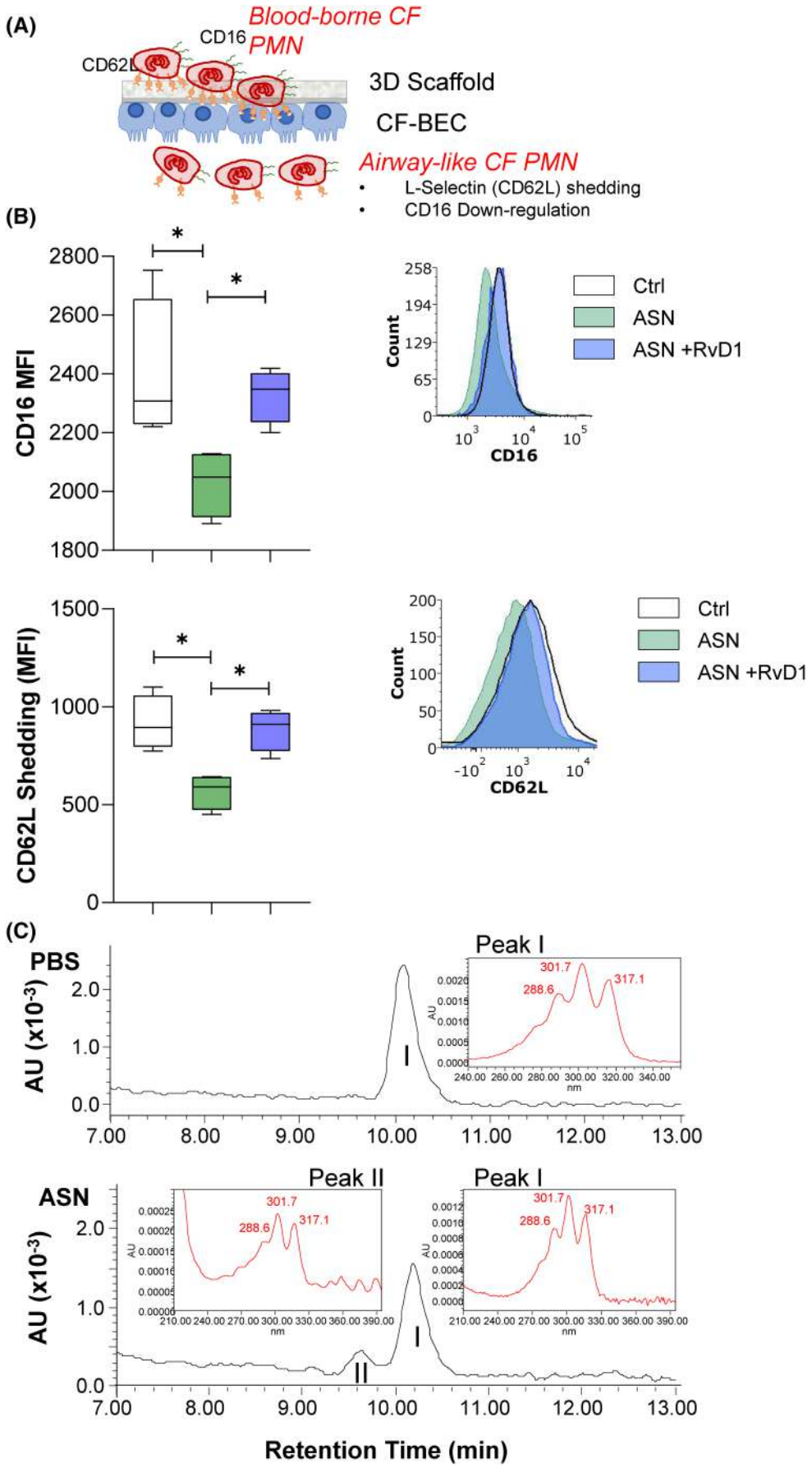
Mucus that accumulates in the airways of people with CF disrupts airway homeostasis and produces pro-inflammatory effects on epithelial cells[31–33] and leukocytes[14,15,34–37] contributing to the development of lung disease.[2] To determine whether RvD1 modified responses of CF leukocytes and CFBEC to mucus, we used organotypic airway chips that recapitulate cell dynamics occurring in lungs of people with CF. CFBE in airway chips were exposed to ASN containing stimulatory factors (e.g., bacterial products and host proteins) present in secretions of patients,[26,38] and CF neutrophils were allowed to migrate in this organotypic model of human airways. We measured loss of L-selectin (CD62L) and of the phagocytic receptor CD16 migrating PMN as a readout of their acquisition of a pathogenic phenotype in CF airways.[14]

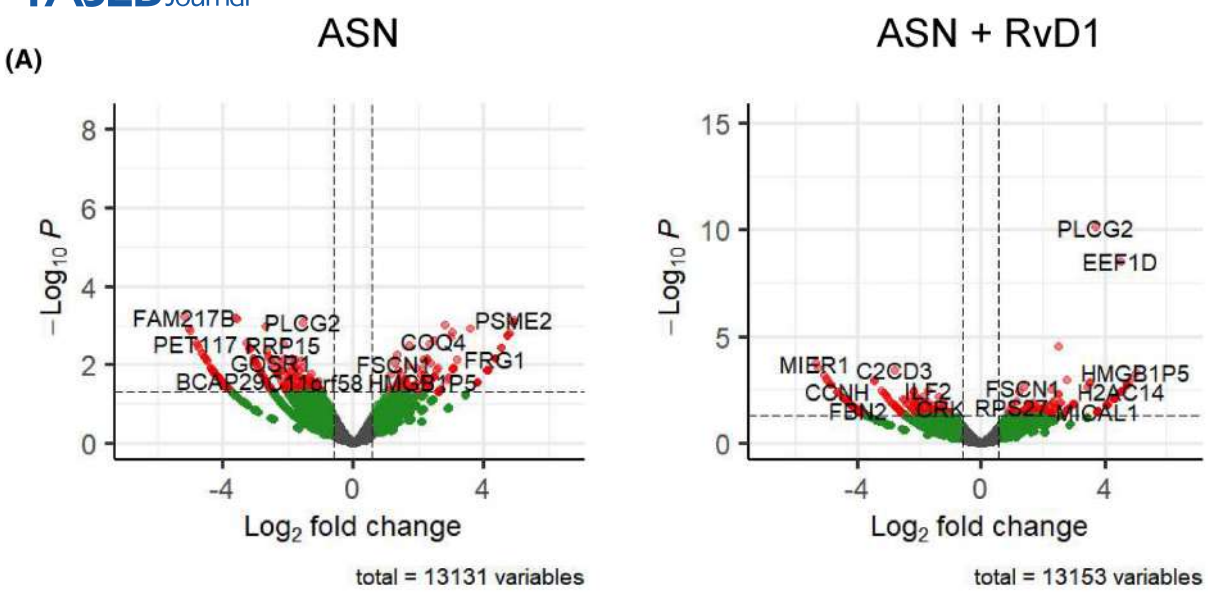
As shown Figure 1, ASN was sufficient to induce a significant CD62L and CD16 membrane loss in transmigrated PMN, confirming its dominant role in priming their pathological condition. On the contrary, treatment with RvD1 (10 nM), significantly diminished L-selectin shedding and CD16 downregulation in CF neutrophils.

Of note, when we assessed chemical integrity of RvD1 in CF ASN (which may contain oxidant species and enzymes from airway cells), we found that ~80% [median = 80.43, 71.82 (25th percentile), 86.46 (75th percentile)] of RvD1 input could be recovered intact after 4 h of incubation as determined with HPLC-UV spectrum analysis and matching of retention time with authentic RvD1 standard (not shown). A major RvD1 further metabolism product with anticipated retention time and conserved UV absorbance was identified following incubation with CF ASN, which is likely to correspond to an "all-trans" RvD1.

Based on these findings, a systematic analysis of transcriptome changes occurring in CFBEC in response to mucus-derived ASN and effects of RvD1 on these changes was of interest. Hence, we carried out RNASeq of transcriptome from CFBEC exposed to ASN alone or upon treatment with RvD1. Among the 13131transcripts that were mapped and showed variances in ASN-treated CFBEC vs control (CFBEC exposed to buffer devoid of ASN), we identified 142 and 245 genes significantly (adjusted $p < .05$) up- or downregulated ($\log_2$ fold changes > |0.58|) in response to ASN(Figure 2A and Table S1). RvD1 treatment resulted in a significant upregulation of 122 genes and downregulation of 245 transcripts (out of 13 152 mapped variables) compared to ASN (Figure 2A and Table S2). IPA-based bioinformatics analysis revealed that biochemical pathways and functions associated with AMPK signaling, synthesis and uptake of carbohydrates, insulin signaling, and RNA transcription were significantly inhibited by ASN (IPA z-score < −1.5). In contrast, cell proliferation, sirtuin signaling, and formation of actin stress fibers were among the pathways increased (z-score > 1.5) by ASN (Figure 2B). For instance, ASN significantly altered expression of glucose-6-phosphatase C3 (G6PC3), glucose-6-phosphate dehydrogenase (G6PD), and several phosphatidylinositol 3-kinases (PIK3) leading to the inactivation of sugar uptake and biosynthesis and sirtuin, while it activated stress fiber formation by modifying (among other genes) the kinases BRAF and phosphatidylinositol 4-kinase (PI4K2A) and the phosphates signal regulatory protein alpha (SIRPA) and inositol polyphosphate-5-phosphatase (INPP5K) (Figure 2C). In contrast, IPA analysis demonstrated that RvD1 determined global shifts of pathways activated or inhibited in CFBEC exposed to ASN. For example, RvD1 blunted formation of stress fibers, cell proliferation, and sirtuin singaling pathways (Figure 2B) and restored the AMPK by regulating several kinases, including PIK3CB,

FIGURE 1 RvD1 regulates CF PMN pathological conditioning. (A) In vitro model mimicking PMN pathological changes (CD62L shedding and CD16 downregulation) in CF airways based of transepithelial transmigration (see Ref.[14]). Primary CFBEC grown (2–4 weeks) as in Ref.[26] at air-liquid interface on porous 3D scaffolds (Alvetex) were placed with the apical side exposed to medium (M) alone or mixed with airway supernatants (ASN) from CF volunteers (1:30) and/or RvD1 (10 nM). Blood PMN (1×10^6/well) from volunteers with CF were loaded on the upper compartment in medium or RvD1 (10 nM). (B) Surface expression of CD62L and CD16 determined with FACS analysis of CF PMN transmigrated (3 h) across CFBEC exposed to ASN. Results are median and interquartile range ($n = 4$ experiments with cells from different volunteers). *$p < .05$ (Kruskal-Wallis test). Representative flow cytometry histograms are in right inserts. (C) HPLC analysis of RvD1 after incubation in ASN obtained from CF donors. Spontaneous sputum (~5 mL) collected from volunteers with stable CF (FEV1 > 70%) was gently dissociated after addition of 10 mL of PBS and centrifuged (300 *g*, 7 min; 3000 *g*, 10 min) to generate a cell/bacteria free airway supernatant. RvD1 (50 ng) was incubated in 0.5 mL ASN at 37°C for 4 h, after which 1 mL of MeOH was added and samples were taken to dryness prior injection into a C18-HPLC system for determining RvD1 isomerization as we described previously.[39] RvD1 Representative HPLC chromatograms are shown in the inserts displaying UV-spectra of the corresponding peaks.





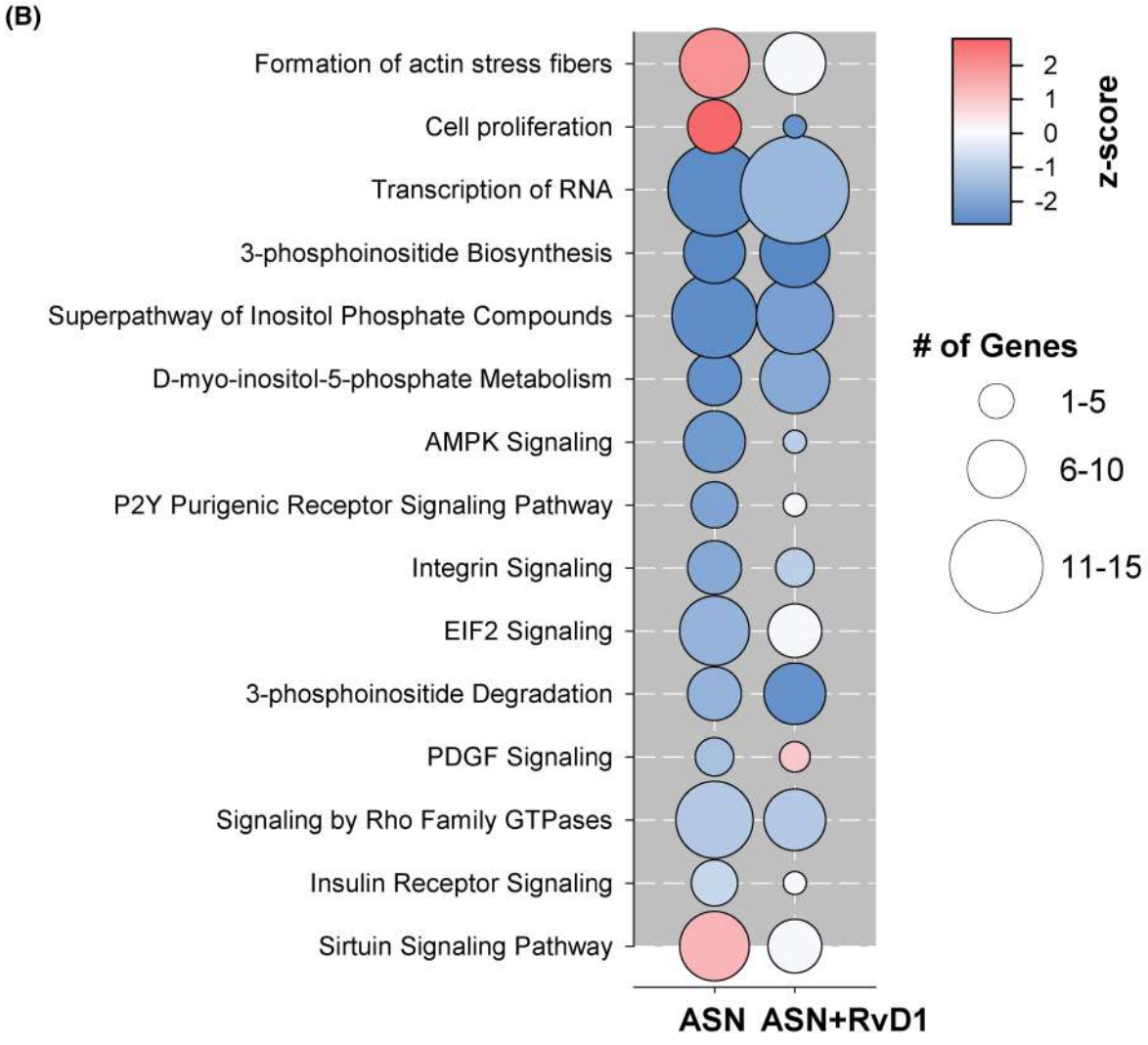


FIGURE 2 RvD1 restores CF bronchial epithelial cell homeostasis disrupted by ASN. (A) Volcano plots of differentially expressed genes identified with transcriptomic analysis alone or with RvD1 compared to CFBEC exposed to cell culture medium devoid of ASN. Genes below the $\log_2$ fold change > |0.58| and $p < .05$ (with false discovery rate <0.1) cut-off are shown in red. (B) Pathway analysis of differentially expressed genes derived from the IPA bioinformatic analysis tools. Pathways are color-coded and sized based on the IPA z-score and number of beneath genes. (C–E) Functional roles and interactions of differentially expressed genes in CFBEC exposed to ASN (C) or ASN + RvD1 (D and E) identified using the IPA analysis tools. Symbols are colored based on differential expression (red = upregulated genes; green = downregulated genes; blue lines and symbols = blocked pathways and functions; yellow-orange lines and symbols = activated pathways and functions). Results are mean from analyses carried out 3 times with samples derived from cells different donors treated in separate experiments.

PIK3C3, and protein kinase AMP-activated (PRKAG1) (Figure 2D). Moreover, RvD1 treatment resulted in the inactivation of pathways involved in inflammation, including NF-κB, interferons, engulfment of leukocytes, and immune responses (Figure 2E).

Overall, these results indicate that RvD1 abates inflammatory responses induced by CF mucus-derived factors on neutrophils and epithelial cells.

3.2 | Resolvin D1 reduces airway inflammation due to mucus obstruction in vivo

Given these findings, we sought to investigate RvD1 actions on lung inflammation driven by mucus accumulation in vivo. To this end, we treated with RvD1 specific pathogen-free βENaC-Tg mice, which have dehydrated airway surface liquid, impaired mucus transport, and spontaneous mucus obstruction with lung disease that shared key features with people suffering from CF including chronic neutrophilic airway inflammation.[11] As expected from previous studies,[11] adult (10-week-old) βENaC-Tg mice had significantly higher numbers of leukocytes in BAL compared to age- and sex-matched WT littermates, with increased airway neutrophilia and MΦ (Figure S1).

To determine if RvD1 could stop the development of CF-like lung disease triggered by mucus obstruction, βENaC were given RvD1 or vehicle from 6 to 8 weeks of age. Treatments of βENaC mice with RvD1 resulted in a significantly lower number of infiltrated total leukocytes and PMN at the end of the two weeks of administration (Figure 3). Since Siglec$^-$ MΦ are known as a subset of airway leukocyte subsets highly involved in the development of chronic respiratory diseases and persistence of inflammation,[40] we analyzed if RvD1 modified this MΦ subtype in βENaC-Tg mice. As shown, RvD1 selectively decreased the number of Siglec$^-$ MΦ, whereas total BAL MΦ were not significantly different when comparing RvD1-treated vs veh-treated mice (Figure S2).

Subsequently, based on these indications of homeostatic roles of RvD1 in early phases of lung disease in βENaC mice, we sought to determine if RvD1 could revert this lung dysfunction in older mice. To this end, we treated βENaC-Tg mice at 10–12 weeks of age with RvD1. In these mice, we still observed reduction in total leukocytes, PMN, and Siglec$^-$MΦ compared to vehicle (Figure 4A). Moreover, RvD1 significantly decreased BAL concentrations of KC (the main PMN attractant chemokine), TNF-α, G-CSF (main MΦ-derived pro-inflammatory cytokines), and VEGF (key pro-angiogenic factor), which were higher in βENaC-Tg mice treated with vehicle compared to WT, while it increased the anti-inflammatory protein IL-10 (Figure 4B).

Hence, these results indicate that RvD1 reduces cellular and soluble components of mucus-induced airway inflammation in βENaC-Tg mice at different ages.

3.3 | Resolvin D1 reduces *P. aeruginosa* infection in βENaC tg mice

Given the essential role of *P. aeruginosa* infections in pulmonary exacerbations and the progression of CF lung disease,[41] we tested βENaC mice with a chronic infection with the RP73, a clinical strain isolated from a patient with CF at a late stage of infection.[28] As shown in Figure 5, following inoculum with RP73, βENaC mice had a significantly higher loss of body weight, bacterial load, and inflammatory cells in BAL compared to WT, confirming that in βENaC mice, like in people with CF, inflammation that predates infection is uncapable to clear pathogens.

Administration of RvD1 significantly improved weight recovery following infection and diminished bacterial load, total leukocyte, PMN number in BAL. Moreover, both total and interstitial MΦ were significantly decreased in βENaC mice treated with RvD1. Thus, RvD1 reduces mucus-driven lung inflammation exacerbated by *P. aeruginosa* superinfection in βENaC mice.

3.4 | Resolvin D1 improves physical activity of βENaC mice

Since people with CF have a marked intolerance to physical exercise related to both pulmonary function and infection,[42] we determined energy expenditure (EE) and physical activity of βENaC compared to mice that

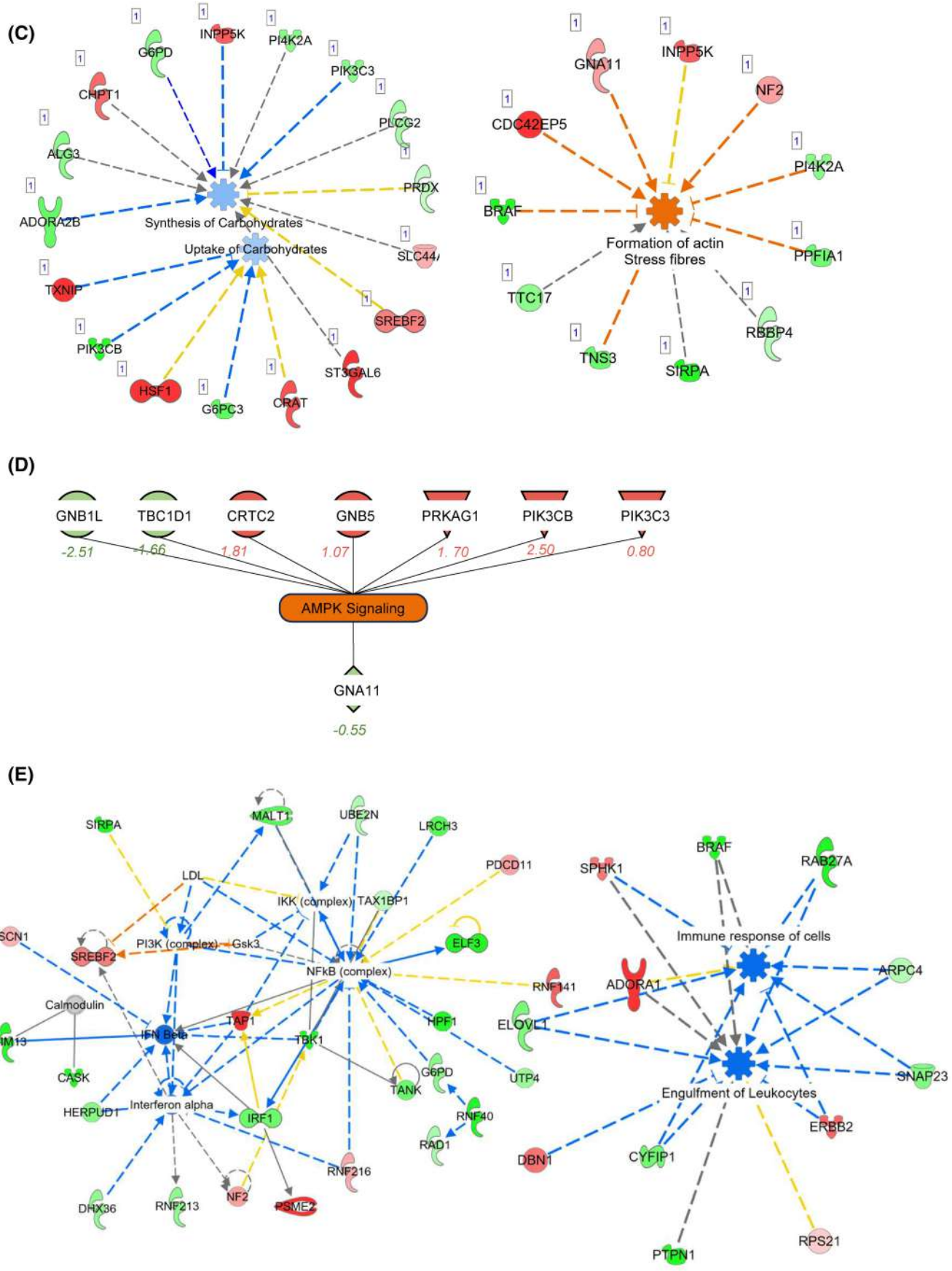


FIGURE 2 (Continued)

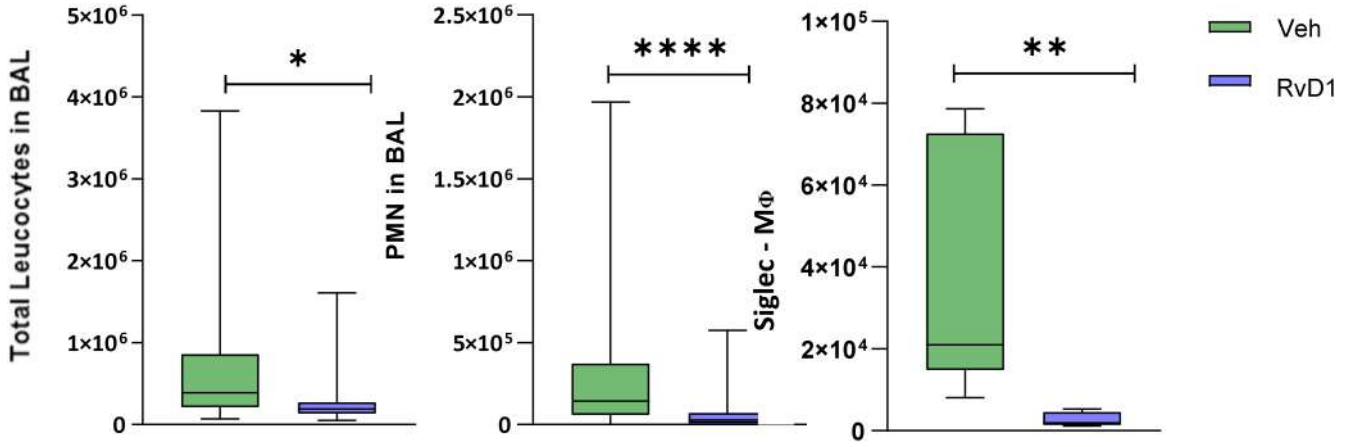


FIGURE 3 Effects of RvD1 treatment on lung disease development in βENaC mice. Total leukocyte count, PMN number, and MΦ in BAL from 6-week-old βENaC mice treated with vehicle (0.5% EtOH) or RvD1 (100 ng). Mice received treatments q.a.d for 2 weeks via I.P. Box–whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from $n = 15$ mice/group. *$p < .05$; **$p < .01$; ****$p < .0001$ (Mann–Whitney test).

do not have lung disease. Results from indirect calorimetry indicated that βENaC mice have increased VO_2 consumption (Figure 6A,D) compared to WT [area under the curve (AUC) VO_2 603.70 ± 28.88 mL/min/kg$^{0.75}$ vs. 508.80 ± 13.18 mL/min/kg$^{0.75}$]. This increase was maintained during all day (dark and light cycle), suggesting a higher O_2 demand of βENaC to produce energy even in resting conditions. VCO_2 was significantly higher in βENaC mice than WT (Figure 6B,E) (AUC CO_2 444.10 ± 20.42 mL/min/kg$^{0.75}$ vs. 383.80 ± 13.72 mL/min/kg$^{0.75}$). Respiratory quotient (RQ), which is the ratio between VCO_2 produced and VO_2 consumed, is indicative of the principal energetic substrate utilized by mitochondria to produce energy.[43] RQ values shift depending to the energetic substrate metabolized, because the VCO_2/VO_2 ratio is higher in glucose than in lipids: a ratio between 0.7 and 0.8 indicates the prevalent use of lipids and proteins as substrates, whereas a ratio above 0.8 indicates increasing use of carbohydrates as main source of energy. Here, no differences were found in RQ between βENaC-Tg and WT mice (Figure 6C,F), 0.73 ± 0.01 vs. 0.75 ± 0.02. These results were reinforced by EE data (Figure 6G), showing an average increase of 40% in βENaC mice during all circadian cycle when compared to WT (178.40 ± 7.29 Kcal/day/kg$^{0.75}$ vs. 152.40 ± 4.31 Kcal/day/kg$^{0.75}$). The data collected indicate that mitochondria from βENaC mice require more O_2 and effort to provide to the basal energetic demands of the body. However, the absence of RQ value shifts towards a more lipid-oriented metabolism to supply the increased energetic demand, suggesting that a balance in the energetic substrate use is still maintained.

Next, we assessed tolerance to aerobic exercise of βENaC mice, subjecting them to an incremental speed test during which O_2 consumption and CO_2 production were finely registered to assess respiratory metabolism during physical activity. As shown in Figure 7A, βENaC-Tg mice displayed lower tolerance to exercise than WT. Indeed, the distance run by βENaC mice was lower (379.20 ± 15.23 m vs. 507.8 ± 33.55 m) than WT. Moreover, the maximal speed reached during the test by βENaC was inferior to that reached by WT mice (Figure 7B) (25.00 ± 2.17 m/min vs. 29.4 ± 3.41 m/min). On the contrary, βENaC-Tg mice displayed enhanced EE per meters run than WT (Figure 7C) (7.22 ± 0.24 (Kcal/day/kg$^{0.75}$)/m vs. 6.27 ± 0.41 (Kcal/day/kg$^{0.75}$)/m). Therefore, results from incremental speed tests confirmed and reinforced those obtained in standard housing conditions, demonstrating that βENaC mice clearly show an excessive O_2 demand and EE during the running, enhancing their intolerance to exercise in comparison to WT mice, mirroring the clinical hallmarks of people with CF.

The same incremental speed test performed in βENaC-Tg mice (10-week-old) after a 2-week treatment with RvD1 (100 ng q.a.d, I.P.) demonstrated that RvD1 ameliorates exercise tolerance of βENaC mice. In details, RvD1 treated mice covered a distance 17% higher than non-treated mice (Figure 7D) (446.10 ± 22.55 m vs. 379.20 ± 15.23 m). Moreover, the maximal speed was increased by about 10% (Figure 7E) (27.43 ± 0.78 m/min vs. 25.00 ± 0.56 m/min) by RvD1, which also rectified the augmented EE in βENaC-Tg mice (5.42 ± 0.28 Kcal/day/kg$^{0.75}$/m vs. 7.22 ± 0.24 Kcal/day/kg$^{0.75}$/m) (Figure 7F). Therefore, RvD1 positively modifies EE reducing the energy demand required to sustain the running task, improves physical exercise, and lowers airway inflammation in βENaC-Tg mice.

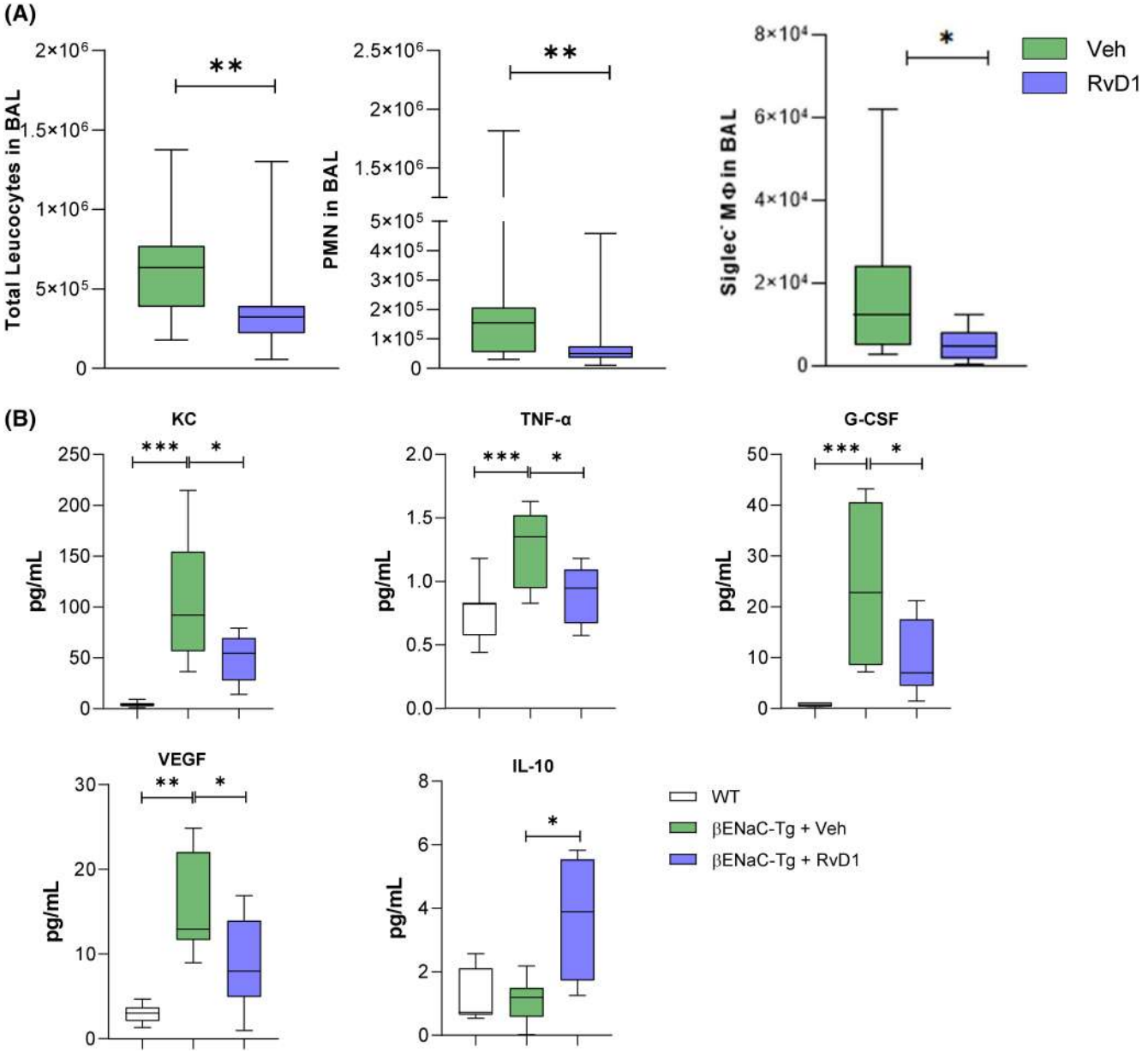


FIGURE 4 RvD1 corrects lung disease in old βENaC mice. (A) Total leukocyte count, PMN number, MΦ in BAL from 10-week-old in WT, or βENaC mice with vehicle (0.5% EtOH) or RvD1 (100 ng). (B) soluble mediators from 10-week-old in WT, or βENaC mice with vehicle (0.5% EtOH) or RvD1 (100 ng). Mice were treated q.a.d. for 2 weeks via I.P. Box–whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from $n = 15$ mice7group. *$p < .05$; **$p < .01$, $p < .001$ (Mann–Whitney test or ANOVA on Ranks).

4 | DISCUSSION

In the present study, we report that RvD1 dampens mucus-driven inflammatory responses in human cells from people with CF and in βENaC mice with CF-like muco-obstructive lung disease. RvD1 also improves exacerbation of lung inflammation during *P. aeruginosa* infection and restores physical activity of βENaC mice.

The classical model of CF pathophysiology points towards defects in CFTR activity as the underlying cause of altered Cl^- and water homeostasis in the airway. This is believed to lead to the thickening and stasis of the mucus that entraps bacteria and impairs mucociliary clearance contributing to a perpetual self-feeding cycle of infections, secondary inflammation, and collateral tissue damage. However, a series of observations have demonstrated that inflammation and mucus plugging are already evident prior to detectable bacterial colonization in young children with CF,[4] in tracheal grafts from CF fetuses,[6] and in mice with CF-like airway surface dehydration,[11] indicating that sterile CF-mucus per se constitutes a sufficient environmental inciting

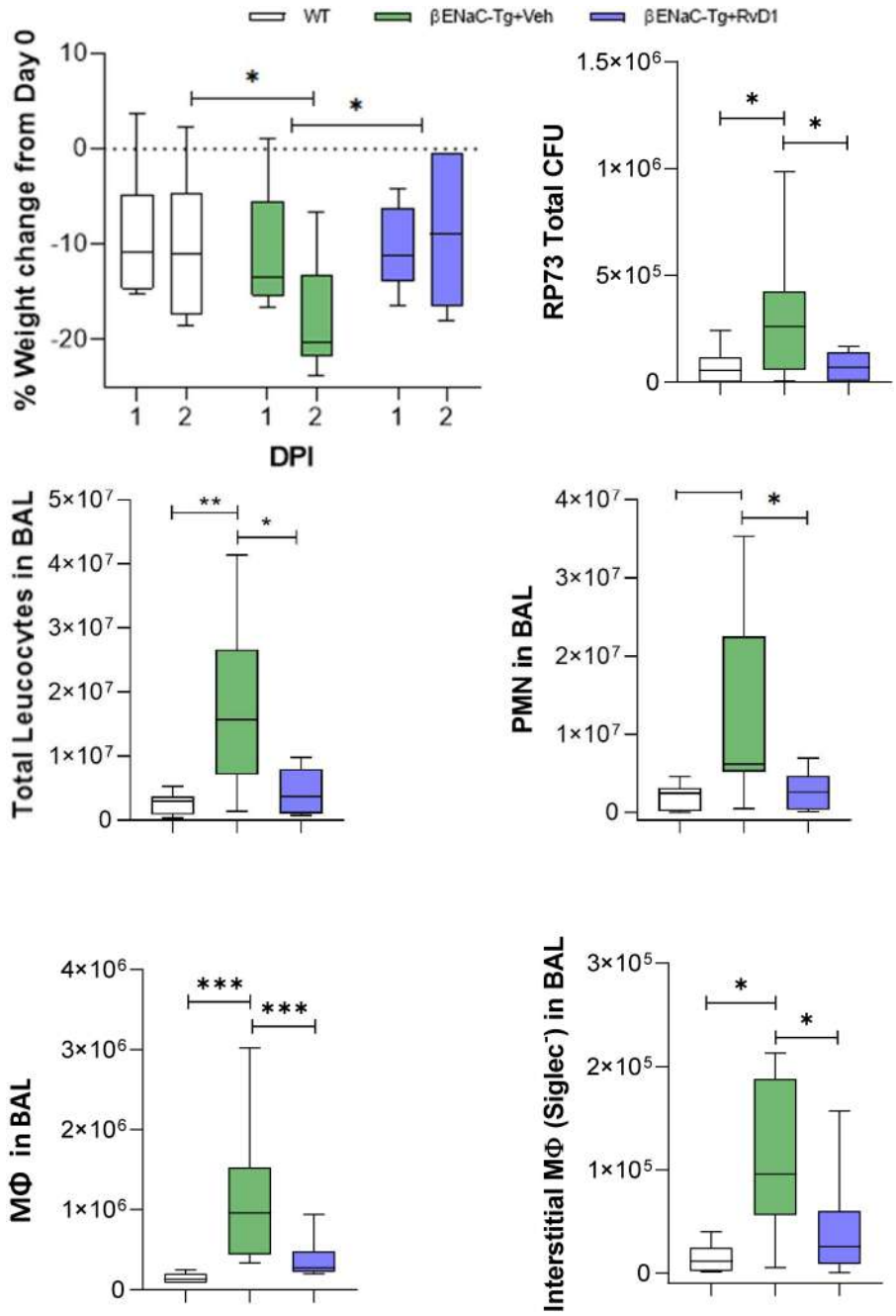


FIGURE 5 RvD1 resolves exacerbation of lung inflammation in βENaC mice following *P. aeruginosa* infection. Body weight, bacterial counts, BAL total and differential cell numbers from WT or βENaC mice infected with RP73. Mice were treated with RvD1 (100 ng) or vehicle (0.5% EtOH) I.P. once a day starting 3 h post infection. Bacteria and leukocytes were quantified 2 days post infection (DPI). Box–whisker plots show 25th and 75th percentile range (box) with 95% confidence interval (whiskers) and median values (transverse lines in box) from $n = 7$ mice/group. *$p < .05$; **$p < .01$; ***$p < .001$ (Mann–Whitney test).

of factor airway inflammation that precedes pathogen colonization. Although PMN are terminally differentiated leukocytes, several studies[14,15,44] indicate that CF mucus modify phenotypes of neutrophils recruited into the airways via miRNA and protein synthesis, including increase of anabolic pathway and downregulation of antimicrobial genes. Using CFBEC exposed to CF ASN, we found that the molecular components of mucus alter epithelial cellular and biochemical pathways that impact their structure (e.g., induction of stress fibers), proliferation, and metabolism (e.g., reduction in carbohydrate synthesis and uptake) converging towards inflammatory mechanisms.

Hence, these results contribute towards a better understanding of basic pathophysiological mechanisms of CF associated with mucus accumulation.

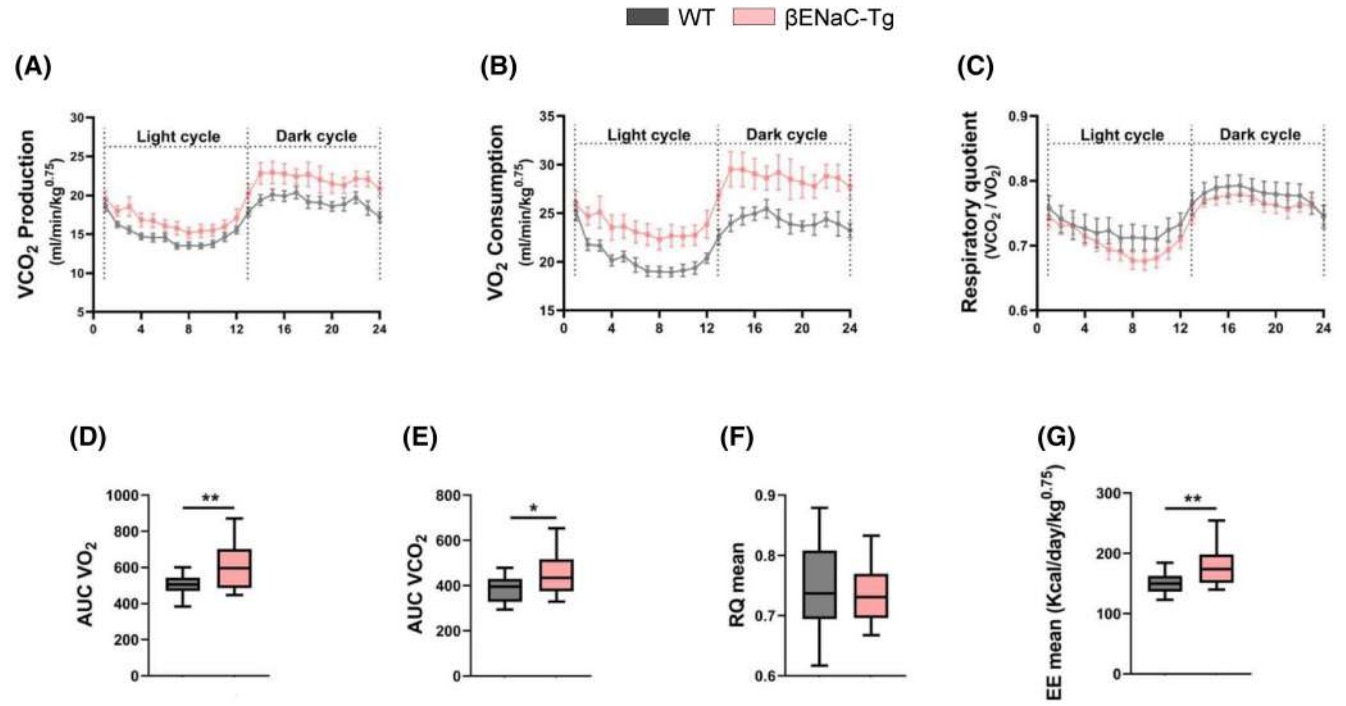


FIGURE 6 Altered VO_2 consumption, CO_2 production, respiratory quotient, and energy expenditure in βENaC mice. (A and B) Oxygen consumption and CO_2 production expressed as mL/min/kg$^{0.75}$. (C) Respiratory quotient (RQ) expressed as VCO_2/VO_2 during 24 h. (D and E) Area under the curve (AUC) of O_2 consumption (panel D) and CO_2 production (panel E). (F and G) Mean values of Respiratory Quotient (RQ) and Energy expenditure (EE). Legend: Light cycle (8 AM to 7:59 PM); dark cycle (8 PM to 7:59 AM). Data are shown as mean ± SEM (WT $n = 16$; βENaC $n = 19$) of data recorded for 24 h. *$p < .05$ as evaluated by Student's t test followed by Tukey's post-hoc test. n = number of mice.

Here, we present that RvD1 is chemically stable in CF ASN and rescues loss of L-selectin and CD16 by CF neutrophils while dampening their degranulation and transmigration across CF epithelia (Figure 1), which are key determinants of PMN-mediated, relentless, and tissue damaging lung inflammation in people with CF. RvD1 originally identified in vivo in resolving murine exudates, is present in human plasma and airway exudates from patients with CF as demonstrated by two independent studies.[25,26] In their work, Eickmeier and colleagues hypothesized that sputum RvD1, rather than a bystander, could identify patients with CF having a better control of neutrophil accumulation in their lungs and respiratory function. RvD1 reduces leukocyte infiltration in the airways in COPD,[19] stops PMN transepithelial migration[23] and dampens upregulation of CD11b (integrin αM) in human neutrophils as a mechanism regulating their recruitment in inflammatory loci.[27]

We also show here that RvD1 blunts inflammatory signaling responses of CFBEC given by ASN that include alteration of cell growth, cellular stress, and NF-κB activation (Figure 2). Several studies have unveiled that inactivation of NF-κB is an integral component of RvD1 signaling pathway and mode of action in different disease settings. For instance, RvD1 targets NF-κB in human MΦ and in vivo during peritonitis and pneumonia through microRNAs (e.g., miR-146b, miR-21, miR-155) and the histone-modifier protein CARM1.[28,45,46] Moreover, RvD1 counter-regulates NF-κB and downstream genes (e.g., cytokines and chemokines) in vivo and in human MΦ inflamed with bacteria or cigarette smoke.[19,47] Similarly, RvD1 down-regulates NF-kB in human peripheral blood monocyte-derived CF MΦ and in fully differentiated CFBEC subjected to *P. aeruginosa* infection.[26] Importantly, we also found a similar modulation of the NF-kB signaling pathway in human PMN co-cultured with tumor cell lines,[48] further underlying a common mode of action of RvD1 in distinct inflammatory conditions. RvD1 also restores the AMPK pathway, which is inhibited by ASN, in CFBEC. Previous studies have demonstrated that RvD1 activates AMPK in MΦ, leading to enhancement of necroptotic cell clearance[49] and in adipocytes, where it mediates anti-diabetic functions.[50] AMPK is a kinase originally described as a sensor of cell energy demand activated during physical exercise and more recently recognized as engaged in maintenance of epithelial tight junctions. Alterations of AMPK are associated with chronic intestinal inflammatory diseases and post-pneumonia lung injury.[51] In addition, In RvD1 countered the ASN effects on other metabolic

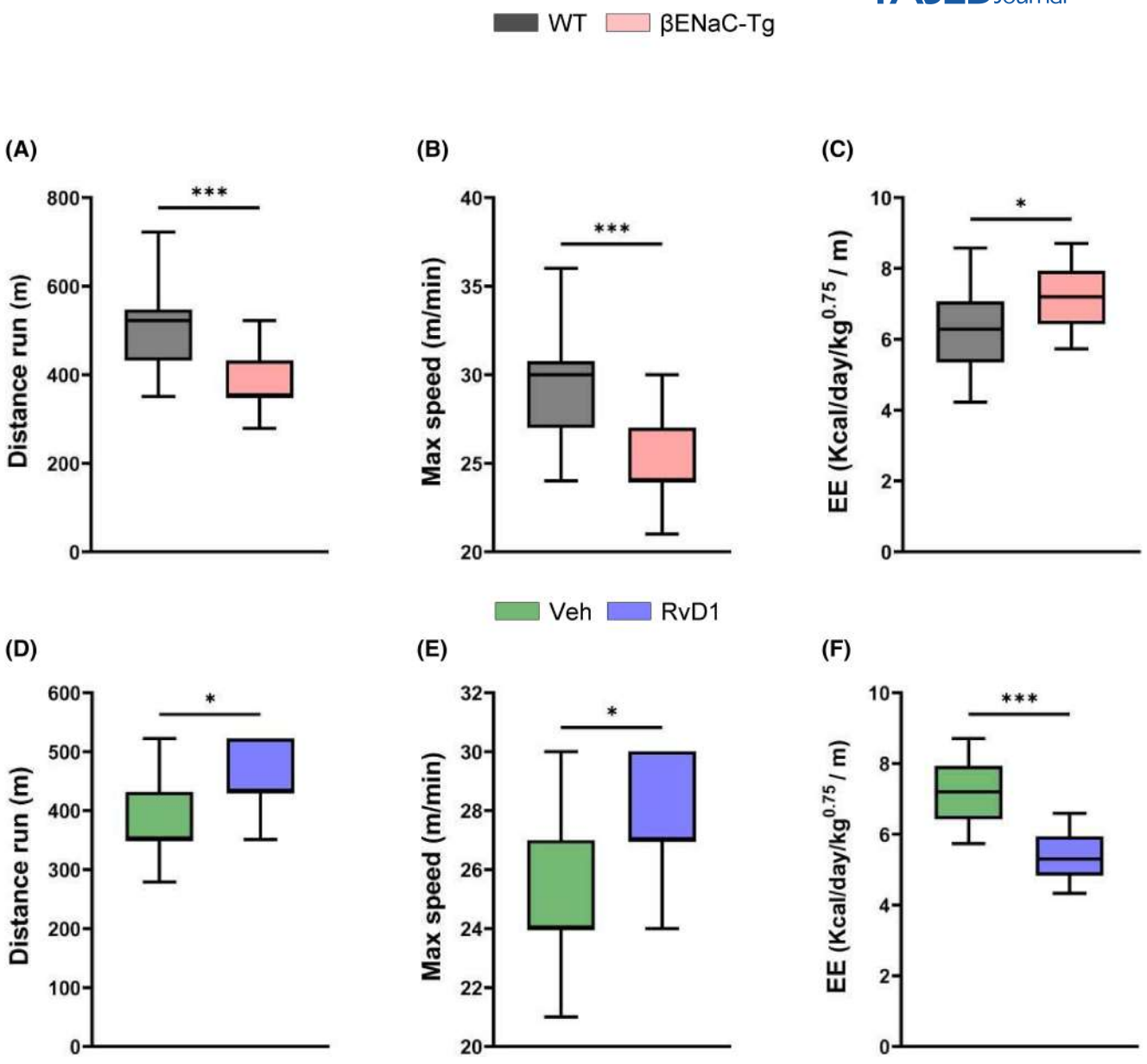


FIGURE 7 Impaired physical activity of βENaC mice during incremental speed test is improved by RvD1. (A) Distance run by WT or βENaC-Tg mice during the test. (B) Maximal speed reached by each mouse during the test. (C) Energy expenditure (EE) per meters run expressed as (Kcal/day/kg$^{0.75}$)/m. (D) Distance run by βENaC mice treated with vehicle (0.5% EtOH) or RvD1 (100 ng q.a.d. for 2 weeks via I.P). during the test. (E) Maximal speed reached by each mouse during the test. F) Energy expenditure (EE) per meters run expressed as (Kcal/day/kg$^{0.75}$)/m. Data are shown as mean ± SEM (WT $n = 16$; βENaC $n = 19$). *$p < .05$ as evaluated by Student's t test followed by Tukey's post-hoc test.

pathways in CFBEC, including carbohydrate uptake and synthesis, and sirtuin signaling by acting on PIK3 and glucose-metabolizing enzymes (Figure 2). Overall, these results unveil genes and pathways regulated by RvD1 in CFBEC exposed to ASN that are overlapping (e.g., NF-κB, AMPK) or unique (e.g., sugar metabolism and sirtuin) compared with the regulation profiles of RvD1 in different experimental diseases.

RvD1 carries many biological actions on epithelial tissues. In CF airway epithelial cells, RvD1 lowers cytokine secretion and inflammatory genes downstream *P. aeruginosa* infection[26,52] and reduces bronchial hyperplasia in vivo in mice bearing chronic bacterial infection.[28] Of interest, Ringholz and coworkers demonstrated that RvD1 enhances Cl^- secretion in CFBEC while diminishing ENaC-mediated Na^+ absorption, thus determining a marked improvement in airway surface liquid height, a key mechanism of airway mucus clearance.[52] Thus, results shown here provide a functional role and action for RvD1, which is

produced[25,26] and remains active in airway secretions, in regulating airway inflammation in CF.

Here, we also demonstrate beneficial effects of RvD1 on mucus-derived lung inflammation in vivo with βENaC -Tg mice (Figures 3 and 4). The mouse colony utilized in this study is suitable for preclinical studies, as it permits for in vivo studies with aseptic lung infection and inflammation exhibiting cellular, biochemical, and histological characteristics (including mucus plugging, heightened inflammatory reactions, and defective bacterial clearance) similar to that seen in patients with CF.[11] Previous studies with these mice showed that therapies with hypertonic (3 or 6%) saline (which is thought to enhance mucus hydration and kinetics) reduce airway mucus accumulation but have limited effects on inflammation,[53] a finding reproduced in children with CF,[54] suggesting that improvement in mucus clearance provides limited improvements to people with CF.

In contrast, targeting the βENaC channel with amiloride proved to dampen airway inflammation as well as mucus obstruction if administered to 5-day-old Tg mice but not at a later age (i.e., 4 weeks),[55] which supports the notion that βENaC is not the only contributor to mucus dehydration and stagnation in CF.

More recently, Addante and coworkers showed the synthetic thiol-saccharide MUC-031, which acts by disrupting disulfide crosslinks between mucins, decreases total airway leukocytes and MΦ in adult βENaC-Tg mice following chronic (twice-a-day, 14 days) local treatment,[56] supporting the hypothesis that mucus-driven chronic inflammation can be targeted pharmacologically.

Herein, we demonstrate that the administration of nanogram doses of RvD1 to βENaC-Tg mice alleviates pulmonary symptoms in vivo, markedly reducing total leukocytes, PMN and MΦ. These results are consistent with known bioactions of RvD1, widely demonstrated also in vivo on smoke-induced emphysema and chronic bronchitis[19] and lung transplantation.[57] RvD1 actions are dependent upon engagement of ALX/FPR2 and DRV1/GPR32 on leukocytes and tissue-resident cells.[27] Receptor-dependent actions of RvD1 and other endogenous ligands, which include active counter regulation of leukocyte migration, chemokine/cytokine secretion, and NF-κB activation, have been confirmed in vivo in peritonitis,[58] arthritis,[59] pneumonia,[18] and atherosclerosis.[60]

Thus, the present results are in agreement with the concept of "resolution agonists" (i.e., actively stopping excessive leukocyte and PMN influx in inflamed tissue and curbing MΦ activation) and provide evidence for RvD1 as a pro-resolution therapeutic strategy to reduce the burden of inflammation derived from mucus obstruction.

It is known that airway clearance following infections is impaired in CF.[1] Current strategies generally rely on mucociliary clearance and aggressive treatment of infections to improve life expectancy for people with CF. Hence, we exacerbated the pulmonary status of βENaC mice by infection with the clinical strain RP73 *P. aeruginosa* and found that the pulmonary exacerbation was reduced after RvD1 treatment (Figure 5) with an improved weight recovery and reduced *P. aeruginosa* titer and BAL leukocyte numbers. Previous works from this laboratory were the first to show the pro-resolving effects of RvD1 treatment in chronic lung inflammation associated with persistent infection in COPD and CF.[26,28] A comprehensive analysis of bioactive lipid mediators from critically ill patients with COVID-19 vs patients who had resolved identified that plasma concentrations of select SPM were upregulated in patients with mild COVID-19 and are downregulated in those with severe disease.[21] Hence, along with RvD1, other SPM proved multipronged, anti-inflammatory, organ-protective, and antimicrobial actions in the lungs[18,20,61] that are important to preserve this organ essential for life.

An important fining here is that RvD1 had distinct effects on lung MΦ of βENaC on sterile conditions versus under infection. Indeed, in specific pathogen-free mice, RvD1 selectively reduced Siglec$^-$ MΦ, which are primarily responsible for driving continuous PMN infiltration and lung remodeling in chronic respiratory disease like CF.[40] On the contrary, in *P. aeruginosa*-infected mice RvD1 reduced both Siglec$^-$ and total MΦ, which are effector of innate immunity during infections. Hence, RvD1 modifies MΦ-dependent lung immune responses in a stimulus specific manner: reducing tissue-damaging lung leukocytes while improving antimicrobial responses of the airways as demonstrated by the reduction in *P. aeruginosa* colonies in βENaC mice.

CF is a multisystem disease and patients develop a progressive exercise intolerance that has been identified as an independent predictor of mortality.[62] Chronic exposure to pro-inflammatory mediators accumulating locally in inflammatory loci or circulating in blood, such as TNF-α and other chemokines, long-term infections, and reduced FEV1%, has long been known as a determinant of reduced exercise capacity in people, mainly in children, with CF.[9] In this study, we provide the first evidence that βENaC-Tg mice bearing a chronic obstructive lung pathology have the same impaired physical tolerance seen in people with CF, corroborating the pathophysiological link between reduced lung function and chronic inflammation and exercise intolerance. Moreover, we demonstrate the beneficial effects of a short treatment with RvD1 in reverting this pathological reduction exercise capacity associated with lung

obstruction (Figures 6 and 7). A recent study conducted by Markworth et al. reports that RvD1 increases skeletal muscle maximal force and regeneration following injury.[63] Along with them, our present findings suggest that RvD1 could be beneficial for supporting physical resistance in people suffering from debilitating diseases like CF. They also prompt further investigations to ascertain direct functions and actions on skeletal muscles.

In summary, here we demonstrate regulatory effects of RvD1 on mucus-induced inflammation on human CFBEC and neutrophils. We also prove therapeutic effects of RvD1 in resolving chronic airway inflammation and physical impairment associated with mucus obstruction, which are highly clinically relevant in CF and other muco-obstructive lung diseases.

AUTHOR CONTRIBUTIONS

Antonio Recchiuti conceived and coordinated the overall study and wrote the final version of the manuscript; Giulia Ferri, Matteo Serano, Elisa Isopi, Matteo Mucci, Domenico Mattoscio, and Romina Pecce carried out experiments, collected and analyzed results; Giulia Ferri wrote the first draft of the manuscript; Domenico Mattoscio, Matteo Serano, Marcus A Mall, Feliciano Protasi, and Mario Romano contributed to the writing of the manuscript.

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DISCLOSURES

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon reasonable request from the corresponding author. Further materials and methods can be found as online supplements.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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