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Supporting information for

Specialized pro-resolving mediator Resolvin E1 corrects the altered cystic fibrosis nasal epithelium cilia beating dynamics.

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Supporting text for Material and methods

Primary culture

The human nasal epithelial cells (hNEC) from patients were cultured under ALI as previously described[1]. Briefly, epithelial cells were dissociated from excised polyps and mucosa samples by using 0.01% pronase (Sigma, P5147). Cells were seeded to expand at a density of 2.5 x 10^5 in T25 flasks in complete Pneumacult Expansion medium (StemCell #05008). Epithelial cells were then collected and seeded at a density of 200 000 cells/cm² on permeable filters (Transwell, Corning 3460) bathed in complete Pneumacult Expansion medium, in both apical and basolateral compartments, for 3-4 days. Then apical medium was delicately removed, and basolateral medium was changed for a differentiation air-liquid interface (ALI) medium composed of Pneumacult™-ALI Basal Medium (StemCell #05002). All culture surfaces were previously coated with collagen IV from human placenta (Sigma, C7521) and the media were changed 3-times a week. After a minimum of 4 weeks of ALI culture, a pseudo-stratified epithelium with basal cells, columnar ciliated cells and goblet cells secreting mucus was obtained. When epithelial cells started secreting mucus, apical secretions were washed twice a week before changing the medium by gently adding 200µL of warm medium from the basolateral compartment for one minute and removing it. Primary cell cultures were used for experiment after 4 to 7 weeks of culture.

Thorough mucus washing

In order to study cilia beating over mucus accumulation, the mucus was washed rigorously 24h before the first video acquisitions by following the protocol proposed by Abdullah et al[2]. 200µL of PBS at 37°C were added apically for 10min, and then gently removed. This step was repeated three times, each time by leaving the PBS solution for 1h on the inserts. During each washing step the cultures inserts were put back to incubator.

High speed video microscopy acquisitions

Videos of the beating of cilia at the surface of the nasal epithelia obtained from ALI cultures were acquired using an inverted Axiovert 200M microscope (Carl Zeiss) at magnification 40x (objective NEOFLUAR 40x/0.75) and a high-speed video camera PL-A741 (PixeLink). The microscope was placed on an anti-vibration table. The video camera recorded videos of 400 x 400 pixels, at 158fps, for 10s (1800 frames). One pixel measured 0.334µm[3]. Culture inserts were kept for 5min at room temperature before the acquisitions and then placed on a glass bottom micro-dish (Ibidi 81158). The temperature was recorded in 6 to 8 different locations of the room and the insert placed back to the incubator at 37°C. CF epithelial cultures were treated from the basolateral side with the SPM solution (10nM) or a vehicle control solution in the Pneumacult ALI medium whereas non-CF samples were only treated with the vehicle control solution. For the inhibition of TMEM16A, epithelial cells were exposed to Ani9 (10µM) 30min before SPM's treatment. For every culture insert, videos were taken before SPM's treatment and after 1, 4, 24 and 48 hours.

Calculation of cilia beating parameters

The parameters analysed in this study were the density, the frequency, the synchronisation, and the orientation of cilia beating within video microscopy field recorded from above ALI cultures. The density was evaluated by the percentage of cilia beating over the video. We defined a FIJI macro using the standard deviation projection of the video and considered as movement all pixels above a threshold of 30, for 8-bit encoded images (Supplemental figure 1 A). We used a video analysis algorithm based on multiscale Differential Dynamic Microscopy (multi-DDM) which enables the automatic calculation of the frequency and the degree of spatial and temporal coordination of cilia beating within the overall microscope field[4]. The cilia beat frequency (CBF) was calculated on square boxes of 32 pixels per side (10.7µm) and then averaged over the video (Supplemental figure 1 C). The coordination was described as the λ^2 parameter which corresponds to the surface in μm^2 below which cilia beat in a homogeneous way, and above which the movement starts losing coordination (for more details on the method see[4]. λ^2 was calculated by dividing the video in square boxes ranging from 2.7 µm to 133.6µm, that correspond to 8, 16, 32, 48, 64, 96, 128, 160, 192, 224, 256, 340 and 400 pixels, per side (Supplemental figure 1 D). The heterogeneity of the beating directions (Orientation) over the videos was calculated as the standard deviation of principal beating direction over square boxes of 50 pixels (17 µm) per side (Supplemental figure 1 B). An in-house method was defined. The standard deviation projection of the video revealed the cilia tip trajectories. The image was then divided into boxes of 50 pixels. On every box, FIJI plugin *Directionality* was used to determine the main direction. The plugin used 2D Fourier transform method and 36bins (5 degrees wide steps) to detect the main peak.

Multiparametric analysis using Principal Component Analysis

In order to retain and visualize maximum information from the cilia beating acquisitions, we performed a Principal Component Analysis (PCA). The PCA was calculated over 1659 videos recorded for untreated non-CF samples to be compared with untreated or SPMs treated CF samples. Videos were taken at different time points up to 72h after thorough mucus washing. We analysed five quantitative parameters: the density of movement over the video (Movement), the temperature of acquisition (Temperature), the cilia beating frequency (CBF), the synchronisation of cilia beating (λ^2), the orientation of cilia over the video microscopy field (Orientation). PCA was calculated for the data centred and scaled to unit variance with function *prcomp* of the Stats package in R. Videos with one or more parameters lacking were omitted. We plotted the PC coordinates for untreated samples at different time points to compare the cilia beating phenotypes between CF and non-CF samples. We calculated the ellipses for each condition using multivariate student distribution and 95% confidence interval. The distinction between the localization of the centroid of each group of data was tested using a PERMANOVA test, a non-parametric multivariate test, and the difference in the dispersion of the data with a PERMDISP test.

Secreted mucins visualization

After the video microscopy acquisitions, culture inserts were fixed with Carnoy solution (60% absolute ethanol, 30% chloroform, 10% acetic acid) by removing the culture medium and gently adding 500µL and 1mL of Carnoy in the apical and basolateral compartments respectively. Inserts were kept overnight at 4°C. Epithelia were rinsed 5 times with PBS and permeabilized with 0.4% TritonX-100 for 10min at room temperature (RT). Non-specific sites were saturated for 30min at RT with 3% BSA in PBS blocking solution. Epithelia were incubated for 1h at RT with primary

antibodies, mouse monoclonal anti-MUC5AC (Abcam ab212636) 1:1000, rabbit polyclonal anti-MUC5B (Thermofisher PA5-82342) 1: 500, rat monoclonal anti-α-tubulin (YL1/2) (Thermofisher MA1-80017) 1:500; diluted in 0.1% BSA in PBS. Then, they were incubated for 1h at RT with secondary antibody, Alexa Fluor 488 donkey anti-mouse (A21202), Alexa Fluor 594 goat anti-rabbit (A11012) and Alexa Fluor 647 chicken anti-rat (A21472); diluted 1:200 in 0.1% BSA. Inserts were rinsed 5 times with PBS after each step. Filters were cut from the insert and placed on microscope slides with cells facing the top. About 40µL of prolong diamond antifade medium containing DAPI (Thermofisher 36931) were added on the cells before putting the coverslip. Images of the labelled ALI cultures were acquired using confocal microscopy on an inverted Zeiss Axio Observer 7 with LSM 900 Airyscan 2 with a 20X objective. Z-stacks acquisitions were analysed using FIJI. The mucin layer thickness was measured using the MUC5AC labelling, the red labelling (MUC5B) being more diffuse. Five equally spaced ZX slices and five ZY slices were taken on each slice 5 thickness measures were done. For each acquisition, 50 measures of thickness were taken. Immunofluorescence of secreted mucins was conducted on one F508del homozygous donor and 4 non-CF donors.

Mucin transcripts quantification

Effect of SPMs (10nM) or vehicle treatment for 24 hours on mucin transcripts was evaluated with RT-qPCR on CF and non-CF samples. Mucus on culture inserts was washed thoroughly and treatment was added in the basolateral compartment. After 24h, cells were detached from the insert with trypsin and with the help of a scrapper. After centrifugation and removing the supernatant, the dry pellet was put on ice. The RNeasy Protect Mini Kits (Qiagen 74126) column-based protocol was used to extract RNA. RT was performed on 1µg ARN with reverse transcriptase MMLV (Promega M1705), Oligo(dT)15 (Promega C1101), random primers (Promega C1181) and dNTP (Promega C1181). cDNA at 10ng/µL were used for qPCR. cDNA sequence amplification was achieved using Taqman Master Mix (Thermofisher 4444557). The housekeeping gene was beta-2-microglobulin (Hs00984230_m1). The following primers were used to amplify secreted mucins MUC5B (Hs00861595_m1), MUC5AC (Hs01365616_m1).

ASL layer height analysis

The ASL layer height was measured as previously described[5]. Briefly, 24h before imaging, 8µL of Texas red®- dextran (10,000MW, Invitrogen, Auckland, NZ) at 2mg/mL were added apically at the insert center. Epithelial cells were stained using Calcein-AM (5µM, Invitrogen, Auckland, New Zealand) dissolved in culture medium and introduced to the basolateral compartment. To avoid ASL evaporation, 700µL of Perfluorocarbon-72 (FC-72, 3M, St Paul, USA) immiscible with ASL were added apically. Epithelia were treated in the basolateral compartment with RvE1 10nM for 1 hour. Z-scans of the epithelia were acquired with a Zeiss LSM 510 Meta using a 40X objective. 3 randomly selected fields were chosen. For each image, ASL height was measured on 9 equally spaced points using Zeiss LSM Image Browser. Then, for each culture insert, the ASL height was measured at 27different locations. Acquisitions were performed on primary cultures derived from 3 CF donors (1 F508del homozygous, 1 F508del/394delTT and 1 F508del/R560K).

Reagents

SPMs were purchased from Cayman for LXB_4 (90420) and Vin Resol ID for RvE1 (20010207). They were kept at -80°C to preserve their bioactivity and were used at physiological concentration of 10nM. Ani9 (Sigma, SML1813) was used at 10µM.

Statistical analysis

The number of donors is indicated in Table 1. The difference between the centroids of the PCA "clusters" was tested with a PERMANOVA test based on McArdle & Anderson 2001 principle, by using function adonis2 from the vegan package on R. The distance matrix was calculated with euclidean method. The difference in data dispersion between the two groups was tested with PERMDISP test based on Anderson 2006 work, by using the function betadisper from the vegan package. On bar graphs, data are represented as mean values ± SEM. Statistical significance was tested with an unpaired t-test or an equivalent non-parametric test, Mann-Whitney, using GraphPad InStat software. Statistical significance was defined as $p < 0.05$.

References

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2. Abdullah, L. H., Wolber, C., Kesimer, M., Sheehan, J. K. & Davis, C. W. Studying Mucin Secretion from Human Bronchial Epithelial Cell Primary Cultures. in *Mucins* (eds. McGuckin, M. A. & Thornton, D. J.) vol. 842 259–277 (Humana Press, 2012).
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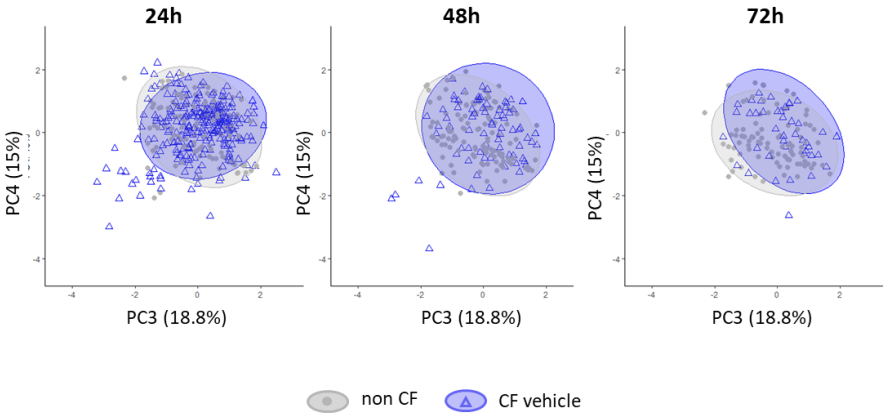
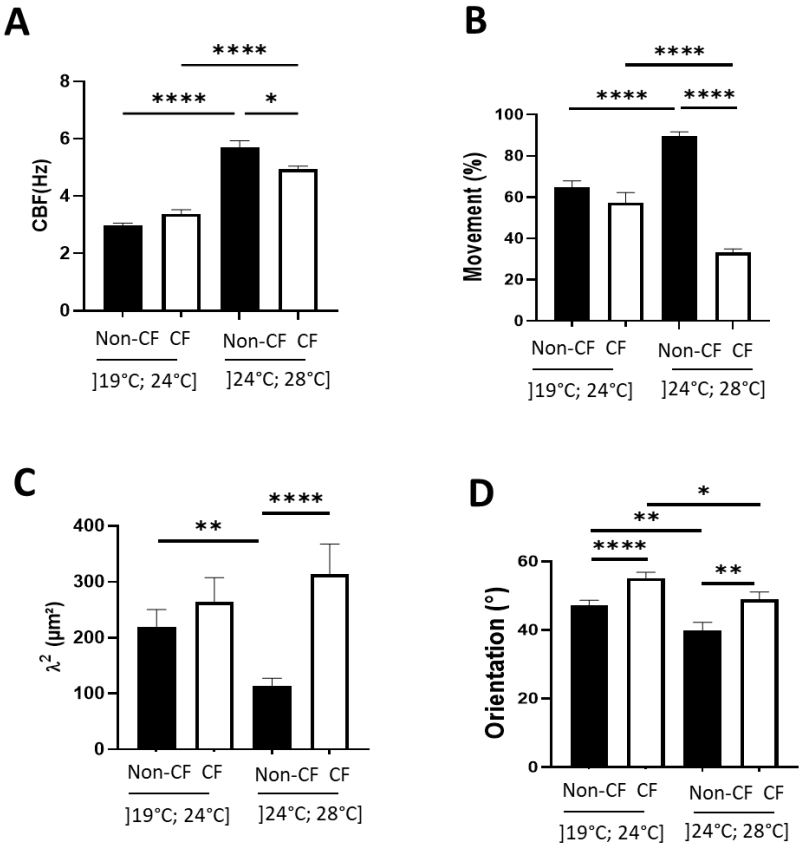
5. Verrière, V. *et al.* Lipoxin A4 Stimulates Calcium-Activated Chloride Currents and Increases Airway Surface Liquid Height in Normal and Cystic Fibrosis Airway Epithelia. *PLoS ONE* **7**, e37746 (2012).

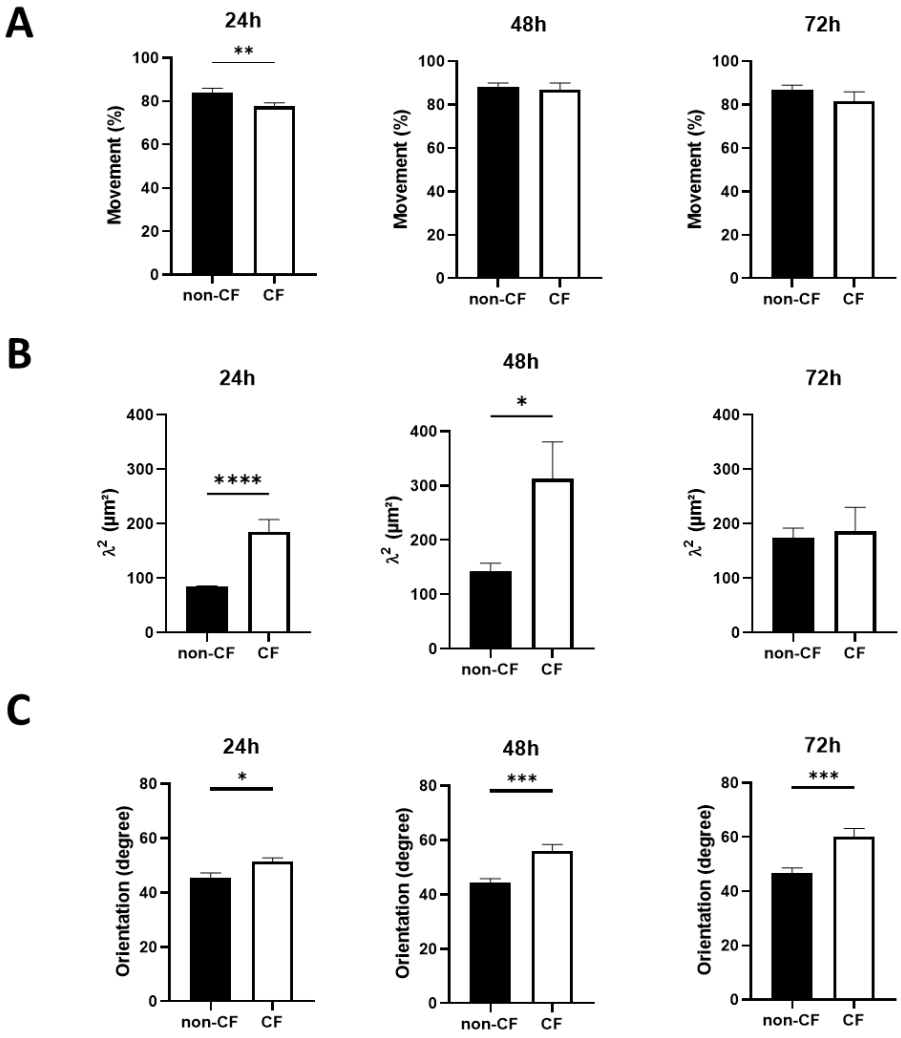
Supplemental figures



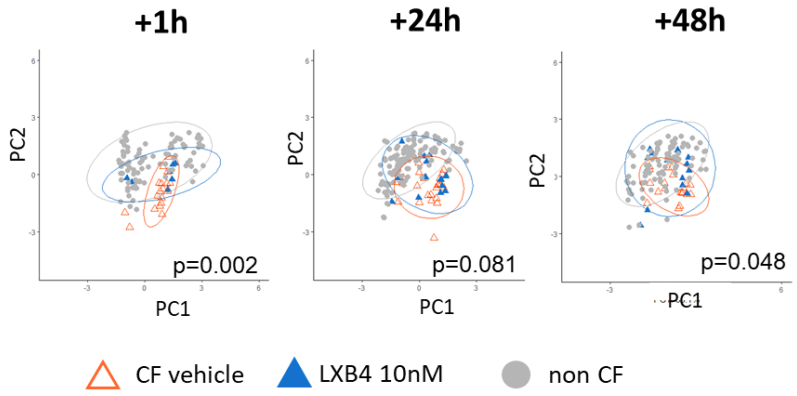
S1: **Beating phenotypes comparison between non-CF and CF samples plotted for PC3 and PC4.** PC1 and PC2 are presented Figure 1. CF and non-CF cultures treated with vehicle control solution, were images 24, 48 and 72h after washing out the mucus. Scatter plot on the first two principal components is displayed. Each point corresponds to one video. Ellipses were drawn using stat_ellipse function from package ggplot2. They are calculated using multivariate Student distribution and 95% confidence interval.



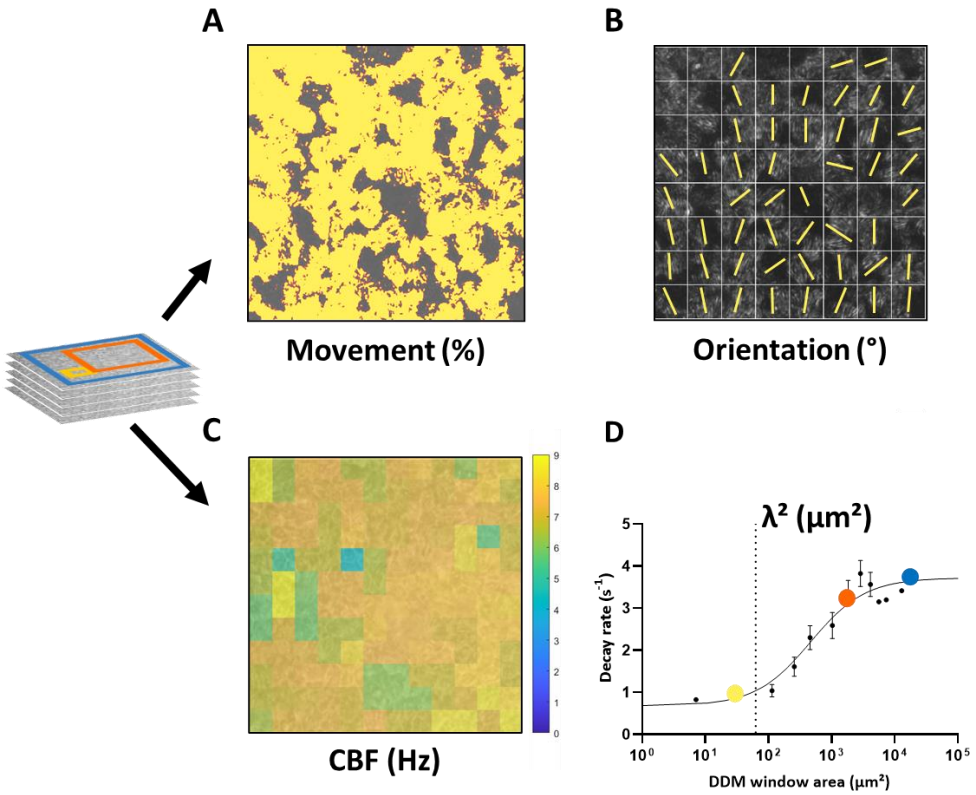
S2 : Cilia beating parameter analysis (24h after washing mucus) for the lower (19°C to 24°C) and the higher (24°C to 28°C) room temperature during acquisition. Mean ± SEM are represented. **A.** Cilia beat frequency (CBF). **B.** Percentage of the video area covered by cilia beating (Movement). **C:** Synchronisation of cilia beating (λ^2). **D.** Measure of the dispersion of cilia beat orientation over the video (Orientation). Statistical analysis by Mann-Whitney tests, * $p<0.05$, ** $p<0.01$,*** $p<0.001$,**** $p<0.0001$.



S3: **Differences between non-CF and CF groups for the parameters contributing to PC2 after restricting the data to videos with more than 50% of movement**. **A.** Percentage of the video area covered by cilia beating (Movement). **B.** Synchronisation of cilia beating (λ^2). **C**. Measure of the dispersion of cilia beat orientation over the video (Orientation). One point per video acquisition and mean ± SEM are represented. Statistical analysis by Mann-Whitney tests, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. The study was conducted on 6 different CF donors and 9 non-CF donors.



S4: Effect of LXB_4 treatment on hNEC CF1 F508del homozygous donor over time. A. Points and ellipses corresponding to hNEC CF1 vehicle and LXB_4 10nM groups are plotted in the PCA space calculated Figure 2. Times correspond to treatment duration. Only PC1 and PC2 are displayed. P values were determined using PERMANOVA test. The study was performed on three independent experiments.



S5: Illustration of the cilia beating parameters. A. Percentage of movement calculated over the standard deviation projection of the video. In yellow the pixels detected as movement. **B.** Cilia beating orientation calculated over the standard deviation projection of the video for boxes of side 50 pixels. The standard deviation of the directions calculated over 1 video is used as the Orientation parameter. **C.** Frequencies calculated on boxes of 32 pixels with the multi-DDM method. The final parameter used for cilia beat frequency is the mean of CBF calculated over the video. D. Illustration of the calculation of the synchronisation parameter, λ^2. Decay rate of the signal calculated with multi-DDM varies according to the window area size. Low decay rate corresponds to zone of synchronized cilia beating. λ^2 marks the transition between correlated and non-correlated zones (for more details see [4].