# EXPRESSION OF THE NF-κB INHIBITOR A20 IS ALTERED IN THE CYSTIC FIBROSIS EPITHELIUM.

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**Online Data Supplement**

## MATERIALS AND METHODS

### *Cell culture and stimulations*

The bronchial epithelial cell lines 16HBE14o- and CFBE41o- (homozygous for the F508del mutation) were obtained from Dr. D. Gruenert (UCSF, CA, USA). Cells were maintained in flasks coated with 1% Purecol (Advanced Biomatrix, CA, USA) in water, and grown in minimal essential medium (MEM) containing glutamine (PAA Laboratories GmbH, Austria) at 37 °C and in 5% $CO_2$ in air. Culture medium was supplemented with 10% heat inactivated fetal bovine serum (FBS), and 100 U/ml penicillin and 100 μg/ml streptomycin and cells were routinely passaged in 10% trypsin (all from PAA). Care was taken to passage cells when 70-80% confluent and all stimulations and experiments were conducted at this density. Cells from passages 6-14 were used for these experiments.

Primary nasal epithelial cells were obtained from CF patients homozygous for F508del and age- and gender-matched control volunteers as previously described [1]. Patient demographics are summarised in Table S1. The study was approved by the Research Ethics Committee of Northern Ireland and all participants provided informed consent (Ethics Number 07/NIR02/23). Nasal epithelial cells were grown in monolayers and maintained in supplemented Airway Epithelial Growth Medium (AEGM, Promocell, USA) until cells were 70-80% confluent. Cells were passaged twice using trypsin and seeded onto 12mm transwell inserts, which were coated with a 1:10 solution of PureCol:sterile $H_2O$, air-dried and exposed to UV light to allow type I collagen fibres to crosslink prior to use. Cells were maintained in Air Liquid Interface (ALI) medium (AEGM with extensive supplements, Promocell, Houston, TX) and fed basolaterally and apically until fully confluent (typically 5 days). The formation of tight junctions was confirmed by measuring transepithelial electrical resistance (TEER) values using an EVOM meter (World Precision Instruments, USA). Once confluent, medium was removed from the apical surface and cultures maintained at ALI for 21 days prior to use in experiments. All cells were treated with *P. aeruginosa*-derived lipopolysaccharide (LPS; Sigma-Aldrich, UK). Primary cells and cell lines

were treated with 50µg/ml LPS for 0 to 24h as indicated in the Figures. A range of LPS concentrations was assessed and 50µg/ml was selected as a concentration which produced a maximal inflammatory response (p65 expression and/or IL-8 release), whilst not inducing a significant change in cellular viability (as determined by MTT and LDH assays; Figure S1).

***Flow cytometry***

Cytoplasmic expression of A20 in 16HBE14o- and CFBE41o- cells was quantified by flow cytometry. Cells ($2.5x10^5$ cells) were detached with enzyme-free cell dissociation buffer, PBS-based (Sigma-Aldrich, UK) and washed twice with PBS. Cells were subsequently fixed with 4 % PFA (Sigma Aldrich, UK), washed with PBS and permeabilized for 15 min in 0.1 % saponin (with 0.5 % BSA) at room temperature. The cells were incubated with a primary antibody against full length A20 (sc-52910, Santa Cruz Biotechnology) for 30 min followed by anti-rabbit FITC conjugate (Sigma-Aldrich, UK) for a further 30 min. All antibodies were diluted in 0.5% BSA containing 0.1 % saponin. Data acquisition and analyses were performed immediately on a flow cytometer (Epics XL, Beckman Coulter, UK). For each sample, cells were gated in the forward angle and side scatter to exclude dead cells or aggregates and a minimum of 10000 events were collected. The threshold of A20 positive cells (expressed as % positive) was set using cells stained with isotype-matched, directly labelled non-specific antibodies in identical concentrations and labelled with the same fluorochrome (BD Pharmingen, UK). Data are expressed as Mean Fluorescence Intensity (MFI) to evaluate the shift in fluorescence intensity of cytoplasmic A20 positive cells compared to isotype controls.

***Quantification of NF-κB expression***

The percentage of epithelial cells positive for the nuclear p50 and p65 subunits of NF-κB was determined using an adapted flow cytometric method [2]. Nuclei were isolated using the CycleTest PLUS DNA Reagent Kit (BD Biosciences, UK) and stained with anti-human NF-κB (p50 or p65)

antibody (Santa Cruz Biological, Germany) or isotype-matched control (Southern Biotech, USA), followed by anti-rabbit FITC conjugate (Sigma-Aldrich, UK) and propidium iodide (PI) [3]. Localisation of nuclei (FS/SC plot) was determined using previously separated epithelial cell nuclei. Acquisition of stained nuclei (min. 5000 events) was carried out on an Epics XL flow cytometer. Single nuclei were gated on the basis of PI staining (FL-3, at 630 nm), after doublet elimination by FL-3 peak vs. integral. Positive p50 or p65 expression (in FL-1 plot) was expressed as % of epithelial cell nuclei. Mean Fluorescence Intensity (MFI) was subsequently calculated to evaluate the shift in fluorescence intensity of nuclear p50/p65 positive cells compared to isotype controls.

***Determination of cytokine concentrations***

The concentration of IL-8 in cell line supernatants and apical and basolateral washings from primary NECs was measured by commercially available ELISA (PeproTech EC Ltd., UK) according to the manufacturer's instructions. Combined IL-8 release from apical and basolateral washings of NECs are presented. To account for interpatient variability in baseline IL-8 release, data for primary NECs is presented as a percentage of the corresponding unstimulated control for each patient.

***Inhibition of p65***

To determine if p65 selectively regulates the production of IL-8 in CFBE41o- cells, cells were pretreated with a commercially available and selective p65 inhibitor (JSH-23, Calbiochem, Merck KGaA, Germany) for 1h prior to challenge with LPS. JSH-23 was resuspended in DMSO to a concentration of 1 mg/ml, further diluted in culture medium and added to cells at a concentration of 30μM. DMSO acted as a vehicle control for the experiments. IL-8 was determined in the supernatants of treated cells by ELISA (PeproTech EC Ltd., UK). The inhibitory action of JSH-23 was found to be specific for p65 as p50 expression was not affected (Figure S2)

***Inhibition of E1 ubiquitin ligases and the 26S Proteasome.***

To inhibit ubiquitin-regulated processes and proteasomal degradation, CFBE41o- cells were pretreated with commercially available inhibitors of E1 ubiquitin activating enzymes (PYR-41, Calbiochem, Merck KGaA, Germany) and the 26S proteasome (MG-132, Calbiochem, Merck KGaA, Germany) for 4h prior to challenge with LPS for a further 24h. Both inhibitors were resuspended in DMSO to a concentration of 1 mg/ml, further diluted in culture medium and added to cells at a concentration of 25μM (PYR-41) and 10μM (MG-132). DMSO acted as a vehicle control for the experiments. A20 mRNA expression after treatment was determined by qPCR as outlined below.

***Quantitative PCR***

Total RNA was extracted from cells using an RNeasy Micro kit (Qiagen, UK), according to the manufacturer's protocol. RNA samples were quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA) and equal amounts of RNA (1 μg for cell lines and 500 ng for primary cells) reverse transcribed into cDNA using a Sensiscript Reverse Transcription Kit (Qiagen, UK). Only RNA with 260/280 and 260/230 ratios between 1.8 and 2.2 were used for these experiments. Primers were designed using gene accession numbers and Primer3 open-source PCR primer design software and obtained from Invitrogen Ltd. (Paisley, UK). Quantitative PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, UK). Multiple house-keeping genes were tested and beta-actin chosen for consistency within cycles and between different samples. An internal calibrator (Jurkat cells) was used to monitor consistency in reaction efficiency between cycles. Primer sequences, gene accession numbers and product sizes are given in Table S2. Relative expression to β-actin was calculated using the ΔΔCt method.

***Immunoprecipitations and Western Blotting***

To extract total protein, ice-cold RIPA buffer (Invitrogen) was added to cells which were then allowed to lyse on ice for approximately 5 mins. Cells were scraped, transferred to a sterile tube and homogenized by repeatedly passing the lysate through a fine gauge needle. Cell lysates were allowed to stand on ice for a further 15 mins, before being centrifuged at 4 ºC for 20 mins. Protein lysates were aspirated and quantified by BCA assay (Thermo Scientific, UK) according to the manufacturer's instructions. For immunoprecipitation (IP) experiments, lysates were precipitated with an antibody against full length A20 (sc-52910, Santa Cruz Biotechnology) using a Direct IP Kit (Thermo Scientific, UK) and following the manufacturer's instructions. Briefly, 5 µg anti-A20 antibody was coupled to the supplied AminoLink Plus Coupling Resin by centrifugation through a Pierce Spin column. Protein lysates (100µg) were subsequently immunoprecipitated to the antibody via a series of centrifugation and wash steps as instructed by the manufacturer. Elution buffer was added to the Pierce Spin column and the immunoprecipitated antigen eluted following a final centrifugation step.A negative control was also included in the experiments and involved the coupling of anti-A20 antibody to the supplied Pierce Control Agarose Resin. The control resin is composed of the same support material as the Amino Link Plus Coupling Resin but is not amine-reactive. As recommended by the manufacturer, an additional control was created for the experiments whereby the supplied Quenching buffer was added in place of anti-A20 antibody.

Lysates were diluted in nuclease free water and Laemmli loading buffer containing 5% β-mercaptoethanol. The samples were boiled, loaded onto 10% Tris-HCl polyacrylamide gels (Thermo Scientific), separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated with primary antibodies against RNF11 (#57180, Abcam, UK), Itch (ab#611198, BD Transduction Laboratories, USA), TAX1BP1 (#ab22049, Abcam, UK) and TRAF6 (#4743, Cell Signalling Technology Inc., USA) overnight at 4$^{o}$C. Horseradish peroxidase-conjugated secondary antibodies (Cell Signalling USA) were applied for 1h at room temperature and the blots visualised on a BioRad Chemi Doc XRS system (BioRad, CA, USA).

***Statistical analysis.***

All data are presented as the means ± SEM. Differences between groups were analysed using the Kruskal-Wallis non-parametric ANOVA with Dunn's post-test and correlations were assessed by Spearman's Rank coefficient. A $p < 0.05$ was considered to be significant. GraphPad Prism was used to plot graphs and to analyse the data.

**Table S1: Patient Demographics**

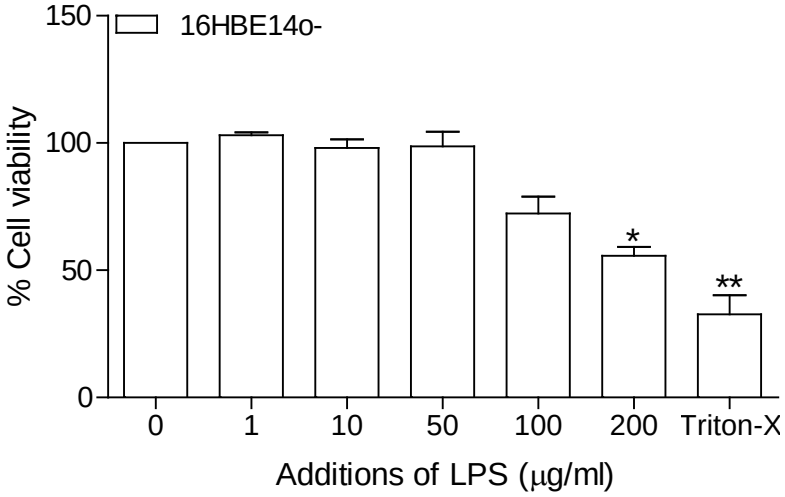
| | Non-CF, healthy individuals | F5808del /F508del CF patients |
|---|---|---|
| % Female | 55 | 60 |
| Median Age | 27 (20, 29.5) | 25 (15, 35) |
| Median BMI | 25.3 (22.4, 26.8) | 22.3 (20.7, 26.3) |
| $FEV_1$ (% predicted) | -- | 50 (36, 62) |
| sputum for +ve *P.aeruginosa* | -- | 100% |
| Smokers | -- | -- |

**Table S2: Primer sequences for qPCR**

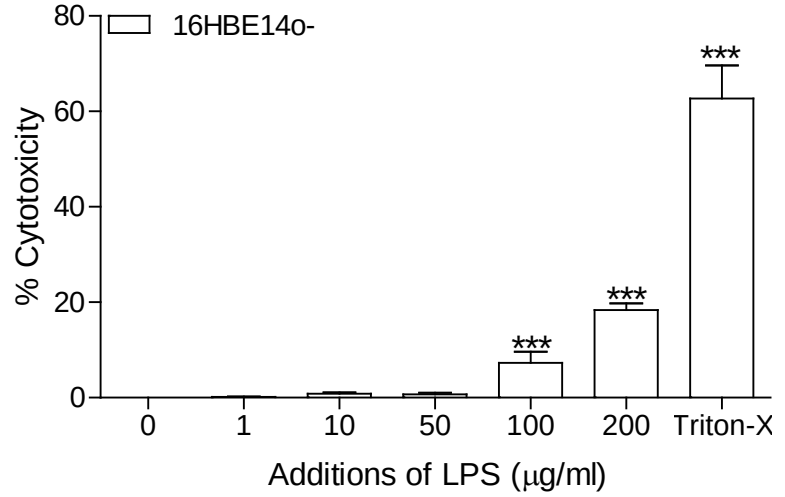
| | Accession Number | Primer Sequence | Product Size |
|---|---|---|---|
| Beta-actin | NM_001101.3 | 5' ctcttccagccttccttcct 3'<br>3' agcactgtgttggcgtacag 5' | 135 |
| A20 (*TNFAIP3*) | NM_006290 | 5' gagagcacaatggctgaaca 3'<br>3' tccagtgtgtatcggtgcat 5' | 155 |
| RNF11 | NM_014372 | 5' tctccctgcttcacgagtct 3'<br>3' gagttgctagccgagtctgg 5' | 144 |
| Itch | NM_031483 | 5' aaggagcaatgcagcagttt 3'<br>3' tctgccattgctgtctgttc 5' | 137 |
| TAX1BP1 | NM_004620 | 5' tttgcccatgtcatctttca 3'<br>3' cacgagcagtactccatcca 5' | 145 |

**Figure S1: Effect of LPS treatment on 16HBE14o- cell viability**

**A.**



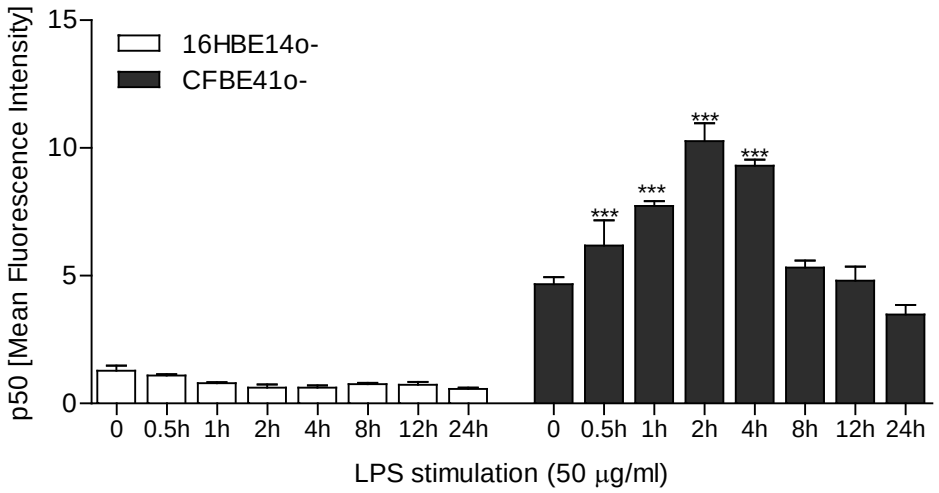

**B.**




To select a suitable concentration of LPS for use in future experiments, we examined the effects of a range of LPS concentrations (1–200μg/ml, 24h stimulations) on cell viability by MTT **(A)** and LDH **(B)** assays. A 10% Triton-X solution acted as a positive control for the experiments. Data are presented as mean ± SEM with n=3 for each assay. *$p$<0.05, *$p$<0.01 and ***$p$<0.001 compared with the corresponding untreated control (0h).

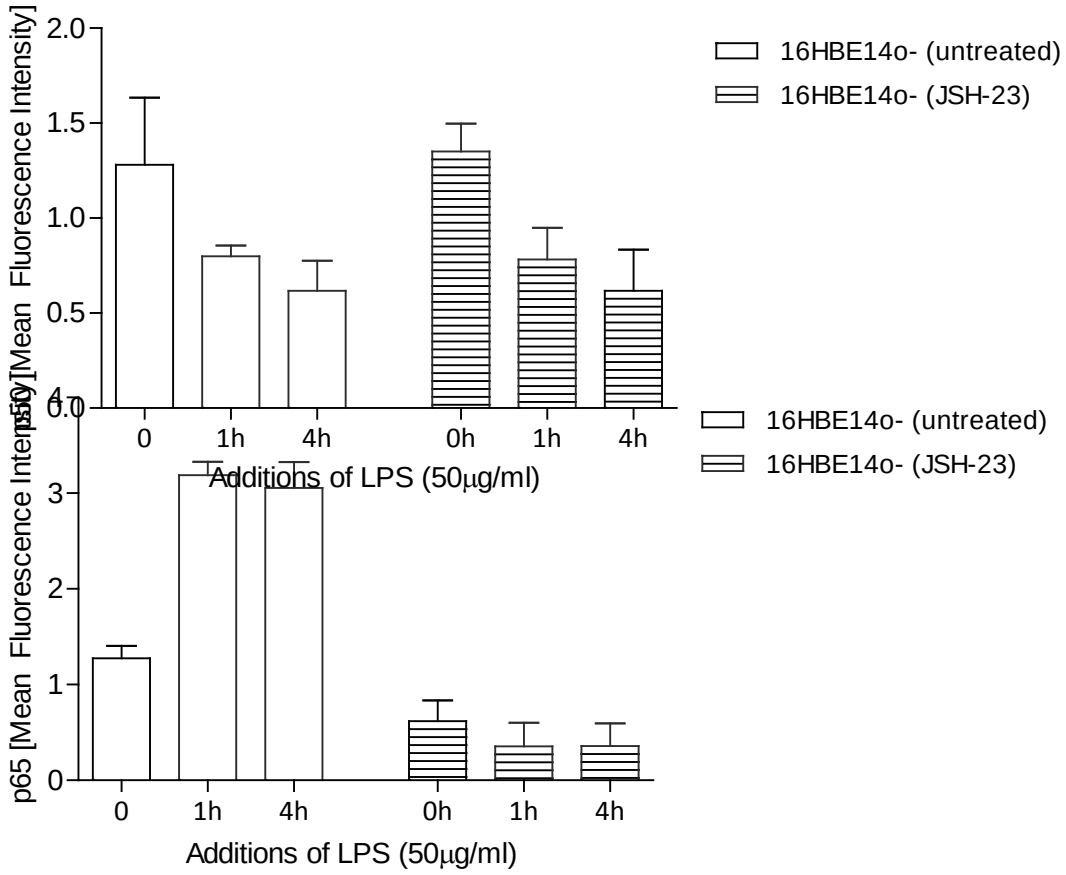
**Figure S2: NF-κB expression and inhibition in airway epithelial cell lines.**

**A.**




**B.**

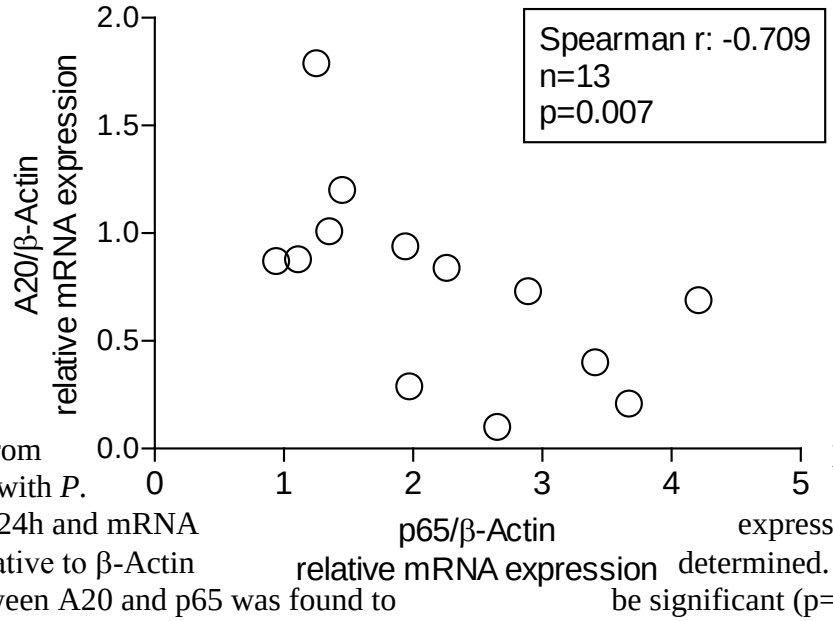
**C.**




16HBE14o- and CFBE41o- cells were exposed to LPS for 0-12h and the percentage of cells positive for the nuclear expression of the p50 **(A)** subunit of NF-κB was determined using an adapted published flow cytometric method with n=5. Data are presented as mean ± SEM with ****p*<0.001 compared with corresponding untreated control (0h) for each cell line. The inhibitory

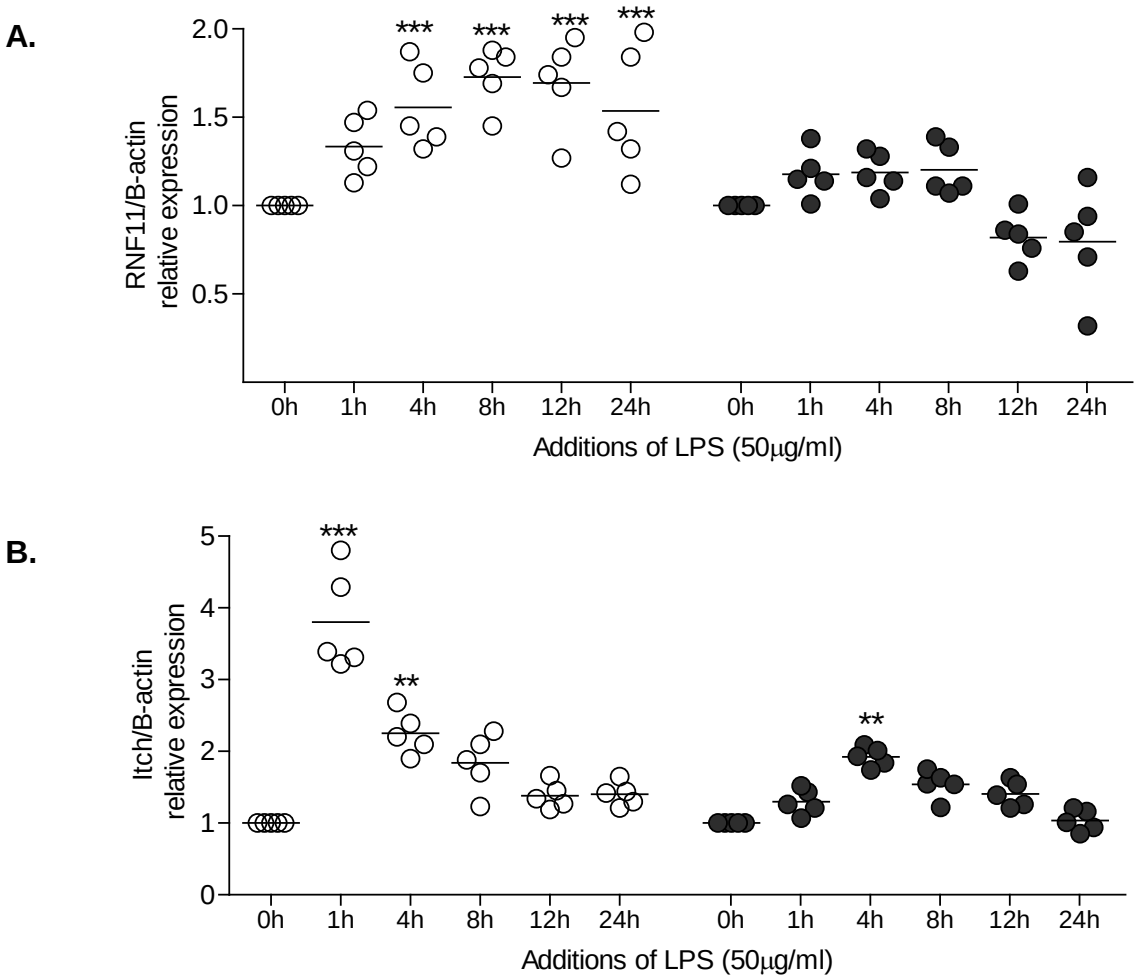
action of JSH-23 was found to be specific for p65, as p50 expression **(B)** was not affected), while p65 expression **(C)** was inhibited in response to LPS**.**

**Figure S3: Correlation of A20 and p65 expression in primary nasal epithelial cells from patients with CF.**

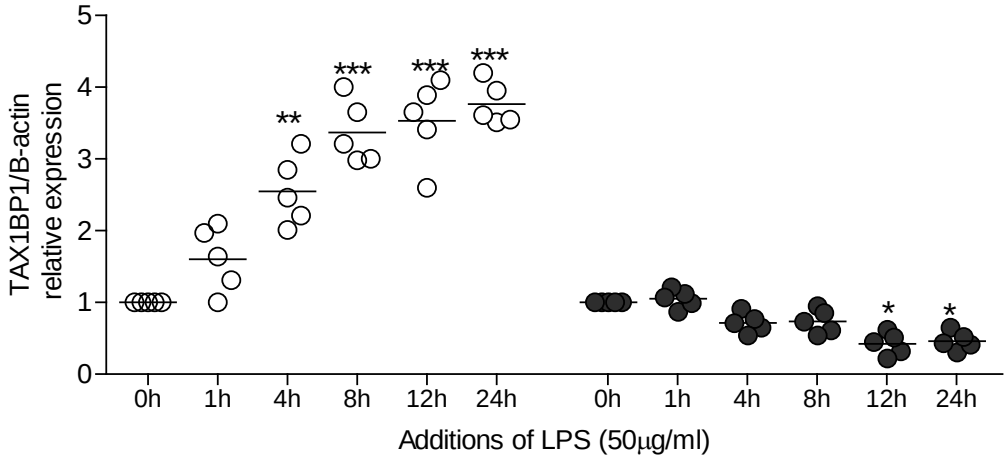



Primary NECs from patients with CF were stimulated with *P. aeruginosa*-derived LPS for 24h and mRNA expression of A20 and NF-κB (p65) relative to β-Actin determined. The inverse relationship between A20 and p65 was found to be significant (p=0.007).

**Figure S4: Full time course of A20 ubiquitin editing complex member expression in cell lines**



**C.**




The mRNA expression of RNF11 **(A),** Itch **(B)** and TAX1BP1 **(C)** was examined in 16HBE14o- (open circles) and CFBE41o- (filled circles) cells by qPCR following stimulation with LPS for 0–24h as indicated. Data are presented as mean ± SEM with n=5. *$p$<0.05, *$p$<0.01 and ***$p$<0.001 compared with the corresponding untreated control (0h) for each individual cell line.

## References

1. O'Brien GJ, Riddell G, Elborn JS, Ennis M, Skibinski G. Staphylococcus aureus enterotoxins induce IL-8 secretion by human nasal epithelial cells. *Respir Res* 2006; 7: 115.

2. Foulds S. Novel flow cytometric method for quantifying nuclear binding of the transcription factor nuclear factor kappa B in unseparated human monocytes and polymorphonuclear cells. *Cytometry* 1997; 29: 182-186.

3. Brown V, Elborn JS, Bradley J, Ennis M. Dysregulated apoptosis and NFkappaB expression in COPD subjects. *Respir Res* 2009; 10: 24.