SUPPLEMENT

To

Phagocytic extracellular traps in children with neutrophilic airway inflammation

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Authors

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SUPPLEMENTARY METHODS

Patient groups

Subjects were recruited from both Monash Children's Hospital (MCH) and Royal Children's Hospital (RCH); both institutions are tertiary referral pediatric centres in Melbourne. All children at both centres were assessed by a respiratory pediatrician prior to the performance of bronchoscopy. Bronchoscopy and BAL at MCH and RCH was performed under general anaesthesia as previously described [1]. A paired blood sample was taken at the time of bronchoscopy.

At MCH bronchoscopies were performed as part of clinical care and part of the BAL sample was taken for research along with a paired blood sample. Generally bronchoscopies were done in the context of exacerbated airways disease, particularly to optimize management in children who had not responded to initial therapy. As bronchoscopy and BAL requires a general anaesthetic this procedure is only performed in our institutions in children who have severe symptoms that have not responded to aggressive medical therapy. Children from RCH were recuited as part of the AREST CF project as previously described [1]. Bronchoscopy was done on annual basis on the child's birthday.

Eleven children with CF were recuited from MCH and 27 from RCH. Their characteristics are listed in Table S1. Twenty three subjects had detectable neutrophil elastase (NE) activity. There were a variety of isolates from the bronchoalveolar lavage (BAL), some of these such as *Haemophilus parainfluenzae* may have relatively low pathogenicity. Upper respiratory tract flora (URTF) was a common finding and was classified as being non-pathogenic. Eleven children with CF at MMC had a bronchoscopy; seven of these were part of an inpatient tune-up with the use of intravenous antibiotics,

three of these were done to optimise therapy after courses of oral antibiotics and one was done as a combined procedure with a change of a percutaneous endoscopic gastrostomy (PEG) tube.

Thirty eight children in the non-CF group had a bronchoscopy performed. Thirty five children were recruited from MCH and three from RCH as a non-CF group. Their characteristics are listed in Table S2. These subjects had a history of chronic cough (> 4 weeks) that was mostly wet (35/38). These subjects generally had prominent neutrophilic airway inflammation as measured by the high neutrophil differential count (which was similar to the CF group). Two subjects had significant underlying neurological disorders (Myasthenia Gravis and Ohdo syndrome). One child in the CF group had confirmed bronchiectasis. However we rarely perform CT scans in young non-CF children due to the risk of radiation and need for sedation/anaesthesia.

Twelve of the 38 CF subjects had abnormalities of their liver function tests; 11 had mild elevation of alanine aminotransferase levels and one had a markedly elevated alkaline phosphatase level.

No child in the non-CF group was on dornase alfa (Pulmozyme) treatment. In the CF group 17 children were on dornase alfa (2.5 gm daily). In all subjects this dornase alfa was given in the evening as a standard regimen. All bronchoscopies were done in the afternoon and no subject received dornase alfa on the day of the bronchoscopy.

We were not able to obtain sufficient BAL samples from children without airway inflammation. Therefore an adult control group that we had previously studied was used (Table S3) [2]. These subjects had a bronchoscopy for the investigation of a cough. They had normal lung function and no definable lung disease. None of these adult subjects had detectable NE activity, NETs or isolated pathogens. These subjects also had BAL IL-6

levels that were 6 fold lower than both the CF and non-CF groups (demonstrating a lower inflammatory state).

General Methods

BAL

BAL samples were obtained as previously described [1]. The right middle lobe (RML) was lavaged with three aliquots of warmed normal saline (1 ml per kilogram of body weight), with one additional aliquot lavaged into the lingua or the most affected lobe identified on X-ray. The first aliquot from each lobe was processed for microbial analysis. The second and third aliquots retrieved from the right middle lobe were pooled and used for analyses of inflammation, as described below.

BAL fluid was processed at either RCH or MCH in the microbiology laboratory for the presence of bacteria, fungi or viruses. BAL and blood samples from the RCH were transported to Monash Health on the day of the bronchoscopy for the analysis of inflammation.

In the adult control group BAL samples had been obtained from the RML as previously described [2]. Three to four 25ml aliquots of warmed saline were lavaged into the RML then aspirated. The BAL was centrifuged to separate cells and supernatant and stored at -80^0C.

Blood samples

Immediately prior to the bronchoscopy, blood was obtained from an arm venepuncture. In six patients it was not possible to obtain blood (3 CF subjects and 3 non-CF subjects).

Sample Processing

BAL samples were spun at 1500rpm for 10 minutes, and supernatant was collected into smaller aliquots and stored at -80C. Cells were washed with phosphate buffered saline (PBS), and resuspended in RF10 media (RPMI (Sigma-Aldrich, USA), supplemented with 10% fetal calf serum and 0.1%L-glutamine (Sigma-Aldrich)). Total cell count and viability as performed manually using a hemocytometer (cells were stained with 0.4% trypan blue). Although total cell numbers varied, cell viability was above 90%. Approximately 5 x 10^4 BAL cells were prepared via cytocentrifugation for later histological analysis and remaining BAL cells were utilised for confocal microscopy.

Serum was obtained from clotted peripheral blood samples by centrifugation and aliquoted and stored at -80^{o}C until use. Neutrophils were isolated from peripheral blood and aliquoted onto cover slips as previously described [2].

NTHi

NTHi was prepared in-house from a previously-collected clinical sample [2-4] and grown on pre-prepared agar plates (Mueller Hinton +5% Horse Blood +20mg/L NAD, Thermo Fisher, USA) kept at 37^{o}C.

Cell histology

Following cytocentrifugation, cells were fixed and stained via a commercial Romanowsky stain (Sigma-Aldrich). Percentages of immune cells were calculated after manually counting at least 500 cells using an Olympus Stereologer on a 40x magnification (with oil) and FIJI image software.

Neutrophil Elastase Assay

An established method was used to determine levels of NE activity in both BAL and blood assay. A specified threshhold that has been shown to be associated with the development of bronchiectasis in children with CF (0.2μg/ml) was used to denote absent or detectable NE activity in the BAL as shown in Figure 1 and Figure S6 [1]. Undiluted, thawed BAL fluid supernatant (or serially diluted human Neutrophil Elastase (Sigma-Aldrich, E7885)) was incubated with an equal volume of substrate N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide (Sigma-Aldrich, M4765) at 37ºC for 60 minutes, and read at 405nm. Substrate was reconstituted in Methyl-2-pyrolidone (Sigma-Aldrich) then diluted in 110mM Tris HCL (pH 8.0) for a final volume of 0.5mM per well.

Confocal microscopy.

Previously described methods were used [2, 5, 6]. A single coverslip (#1.5, 13mm, Thermo Scientific, USA) was placed into each well of a 24 well plate, and incubated in 0.01% of Poly-L-Lysine (Sigma-Aldrich) at 37ºC for 20 minutes. Poly-L-Lysine was removed and coverslips were dried.

Wells were loaded with 3-7 x 10^5 BAL cells suspended in 400μl RF10 media (as above) and incubated at 37ºC for 20 minutes to adhere. Samples were then incubated for 60 minutes: 1) untreated, 2) NTHi (multiplicity of infection (MOI) of 100:1) or 3) NTHi

(MOI 100:1) and 5 international units DNase 1 (Pulmozyme, Genentech, USA). Samples were then fixed with 2% periodate-lysine-paraformaldehyde (PLP) and permeabilized with 0.2% Tween 20. Following PBS wash, samples were blocked with 10% chicken sera in 5% bovine serum albumin (BSA) in OBS for 30 minutes at room temperature. Primary and isotype antibodies (Table E4) were incubated for 16hrs at 4^{o}C in 1% BSA in PBS. Following by PBS wash, samples were incubated in secondary antibody (see below) for one hour at room temperature, then mounted with DAPI prolong gold (Thermo Fisher). Samples were also prepared using only secondary antibody for use as background control.

Images were obtained using Olympus FV1200, excited with 405, 488, 597 and 647 nm lasers. Twenty fields of view per coverslip were obtained for analysis. To obtain representative images a grid pattern was used [5].

Rabbit IgG and Sheep IgG isotype control antibodies were prepared in-house and purified using Protein G columns from sera.

For the first three subjects only METs were measured (1 CF and 2 non-CF) and in the next 43 subjects both METs and NETs were measured. For the samples only a limited number of cells was available. Analyses were prioritised for 1) baseline trap expression, then 2) NTHi-stimulated trap expression, then 3) combined NTHi/DNase 1. Therefore samples analysed for; 1) CF NET expression numbers were 23:22:20, 2) Non-CF NET expression numbers were 20:20:18, 3) CF MET expression numbers were 24:24:22, and 4) Non-CF NET expression numbers were 22:21:17.

Image analysis

Imaris software was used to analyse trap expression uisng previously described methods [2]. NETs were defined by the presence of co-localisation of extracellular chromatin,

H3Cit and NE, whilst METs were defined by co-localisation of extracellular chromatin, H3Cit and MMP9 (and absence of NE staining). Controls for both background and isotype staining were used.

Biomarker Analysis

Biomarker analysis was done both on blood and BAL samples. There were 70 blood samples and 76 BAL samples available for analysis. An initial analysis was done on the first 39 blood specimens for biomarkers with bioplex assay, DNA complexes and CRP. Subsequent analyses were could be done on the 68 available serum samples so there were 68/70 measurements of AAT/CRP/IL-6.

Bioplex assay

Serum samples (diluted 1:4) were examined using Bio-Rad's Bio-Plex Pro human chemokine assays using a MagPix Luminex system, following the manufacturer's instructions. Briefly, standards and samples were incubated overnight with specialised magnetic beads containing antibodies to 40 proteins of interest (ENA-78, CTACK, Eoxtaxin-2, IL-16, SCYB16, SDF-1a+b, 6Ckine, MDC, MIF, MIP-1d, Gro-1a, Gro-1b, IL-8, IP-10, MCP-1, MCP-2, MCP-4, MIG, MIP=3b, Fractalkine, MPIF-1, TARC, TECK, BCA-1, Eotaxin, Eotaxin-3, I-309, I-TAC, MCP-3, GCP-2, GM-CSF, IFN-g, IL-1b, MIP3a, TNF-a, IL-1b, IL-2, IL-6 IL-10, MIP-1a). Following wash (using a handheld magnetic plate washer), standards and samples were incubated for 30 minutes on a shaker with detection antibody. Each detection antibody created an individual fluorescence following incubation with the reporter molecule Streptavidin Phycoerythrin. The MagPix

Luminex system was then used to identify and calculate the quantity of each antibody in the sample, and thus, the concentration its correlating protein of interest.

DNA complexes

DNA complexes were measured using the sandwich ELISA method developed by Kano et al [7]. Wells of a 96-well plate were coated with anti-MPO antibody (Merck Millipore, USA) was diluted to 5ug/ml in M Carbonate-bicarbonate buffer (pH 9.6), incubated overnight at 4C The following morning, wells were washed 3x with PBS, then blocked for 120 minutes with 1% BSA with 0.05% sodium azide in PBS. Following another PBS wash, serum (diluted 1:3 with PBS) and the PBS only control was incubated overnight at 4^{o}C. On the third day, wells were washed with PBS and incubated for 90 minutes at room temperature in peroxidase-conjugated anti-DNA antibody, diluted in the incubation buffer provided (Cell Death Detection Assay, Roche, Switzerland). Wells were washed 3 times with 0.5% Triton X. Photometric colour was provided by ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), prepared according to manufacturer's instructions (Roche, Cell Death Detection Assay), and samples were incubated, with shaking, for 30 minutes for colour development. The optical density (OD) of each well was measured at 405nm, with a reference wavelength of 490nm and calculated as a percentage of the PBS control.

CRP analysis

CRP analysis was performed on serum by the Monash Pathology. Highly sensitive CRP was measured from serum using the routine automated Beckman Coulter AU5812. The quantitative determination of CRP is measured by immuno-turbidimetric method

(Beckman Coulter). CRP reacts specifically with anti-human CRP antibodies coated on latex particles to yield insoluble aggregates and the absorbance of the aforementioned aggregates is directly proportional to the CRP concentration in the sample.

AAT analysis

AAT levels were assessed two methods.

Nephelometry was used to measure AAT levels in the serum by Monash Pathology using the routine Beckman Coulter IMMAGE 800 Protein Chemistry Analyser. The quantitative determination of AAT is measured by rate nephelometry method (Beckman Coulter, USA). The test measures the rate of increase in light scattered from particles suspended in solution as a result of complexes formed during an antigen-antibody reaction. Results were expressed in normal range (or reference interval).

A standardised ELISA (R&D systems, USA) was used to measure AAT levels in the BAL fluid. This method is able to detect the lower levels of AAT present in the BAL fluid (in addition the nephelometry method has only been established for the analysis of blood samples in Monash Pathology). ELISA was performed according to manufacturer's instructions and results were normalised using a positive control.

Functional Assays

BAL macrophage IL-6 production

From BAL fluid 2 to 4 x 10^5 cells were incubated in 24 well plates with 500µl of culture medium and left overnight at 37^{o}C. The next day the media was removed, washed once with PBS and replace media with RPMI (+L-glutamine, no serum). Based on previous

method nearly all the adherant residual cells were then macrophages [2]. Live NTHi was then added to each well at a MOI of 100:1 or as uninfected (control). Samples were collected at 1 and 3 hour timepoints after infection (and matched control samples), spun down (to removed any cells) and were then frozen at -80^{o}C. For final analysis samples were thawed and IL-6 levels measured using ELISA as described above.

Effect of AAT on NE activity

Human serum was obtained and NE activity was measured as described above. Approximately 4ml of serum from a healthy adult donor was mixed with 1ml of CaptureSelect Protein Affinity Resin selective for AAT (Thermo Fisher) and incubated overnight at 4^{o}C on a roller. A column was eqilibrated with PBS, then the resin/serum mixture was added. AAT-free serum was immediately collected, then the column was washed with 10x 10ml of PBS. Concentrated AAT was then collected using approximately 3ml of elution buffer (0.1M glycine, PH3.0). Depleted serum (DS) was then assessed for AAT via the previously described AAT ELISA and found to be undetectable. Likewise, the eluted AAT was assessed to determine the concentration of AAT.

Three preparations of the human serum were used for subsequent experiments 1), whole original serum (WS), 2) eluted AAT and, 3) depleted AAT (DS). Preliminary experiments were done in adults to ascertain doses that significantly reduced/oblitered NE activity that were used in subsequent experiments in children.

A 96 well plate was prepared by adding 50-100µl/well of poly-l-lysine (for neutrophil adherance) and left at 37^{o}C for 20 minutes, then PLL was removed and plates left at 37^{o}C to dry.

Whole blood was lysed with Ammonium Chloride as previously described.[2] Cells were resuspended in culture medium (phenol-free RPMI, Thermo Fisher) and cells counts performed. Neutrophils (5 to 10 x 10^4) were then added to each well (Poly-L-Lysine coated) with NTHi at an MOI of 100:1 to induce NET formation and incubated at 37^{o}C for one hour as previously described (E2,5).

Culture media was removed and fresh culture medium was added for 1) Control (serum-free, phenol-free RPMI), 2) eAAT (approximately 20pg/ml AAT in serum-free, phenol-free RPMI), 3) DNase 1 and 4) DNase 1 and eAAT.

An equal volume of NE substrate was then added to each well. A positive control was also included (0.1mg/ml porcine elastase, as previously described). Plates were read immediately at 405nm and following 1 hour incubation at 37^{o}C. Results were expressed as OD absorbance at the one hour time point.

Further validation experiments were also peformed in which control was compared with 1) whole serum (10% human serum in phenol-free RPMI) and 2) depleted serum (10% AAT-depleted serum in phenol-free RPMI).

Statistics

Data was analysed using paired and unpaired testing with parametric or non parametric methods as appropriate. For contingency analysis, Chi square testing was used. Analysis of three independent groups was performed using the non-parametric Friedman test. Data was analysed using Prism 7 software (Graphpad, USA). After the first 46 samples for NET/MET assessment, an interim analysis was performed; this demonstrated significant differences with large between group changes. Therefore it was decided that further assessment of this parameter was not required and subsequent samples were used to

further define functional assay studies of the extracellular trap pathway (given the small amount of tissue available it was not possible to do both).

SUPPLEMENTARY RESULTS

Staining for NET formation is demonstrated in Figure S1. Panel A shows staining for chromatin (DAPI), B staining for citrullinated histone (H3Cit), C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.

Staining for MET formation is shown in Figure S2. Panel A shows staining for chromatin (DAPI), B staining for H3Cit, C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.

Staining for NET/MET formation is shown using a lower magnification field in Figure S3. Panel A shows staining for chromatin (DAPI), B staining for MMP9, C staining for H3Cit, D staining for NE and E shows the merged image. Insert is isotype control.

A bioplex assay was used to measure 40 potentially relevant biomarkers in serum and assess whether these could be used as a non-invasive way to predict detectable BAL NE activity. Results were compared between subjects who had no measurable NE and those who had detectable NE. Results were analysed from 20 CF subjects and 19 non-CF subjects and both groups were pooled for the analysis. The results showed no significant differencs (data not shown).

DNA complexes were measured in the serum as an indirect measure of circulating extracellular trap activity. This was done in the CF group (20 subjects) and the non-CF group (19 subjects). To increase statistical power the two groups were combined. Results were compared between subjects who had no measurable NE and those who had detectable NE. There were no significant differences (Figure S4).

Serum levels of IL-6 were measured. Most subjects who had measurements performed had detectable IL-6 (82% of CF group and 97% of non-CF group). Levels were similar in both groups (Figure S5).

Serum AAT levels in the CF and non-CF groups were measured using nephelometry with similar results (Figure S6)

Serum CRP and AAT levels (AAT by nephelometry) were measured in subjects with and without detectable neutrophil elastase. CRP levels were higher in subjects who had detectable NE in both CF (Figure S7A) and non-CF groups (Figure S7B). For AAT levels there were no significant differences in the CF group (Figure S7C) or the non-CF group (Figure S7D).

Similar to its effect on NETs, DNase 1 markedly reduced MET expression in the CF group (A) and non-CF group (B). Results are shown in Figure S8.

AAT significantly reduced NE activity in all subjects. It did not obliterate activity in about half of subjects, predominantly in those with higher AAT levels. The dose of AAT used was determined in preliminary experiments in adults. In retrospect a higher dose of AAT would have been required in these children with their active and primed neutrophils to obliterate the NE activity. In addition AAT may be unstable due to a variety of factors and as the assays required a minimum of one hour to do it is possible in some subjects that the function of AAT may have been reduced.

Whole serum (WS) contains multiple anti-proteases of which the most important involved in the inactivation of NE is AAT. To further validate the experiments shown in the main text in Figure 3, the effect of WS on NE was assessed as was the effect of WS that had been depleted of AAT (dAS). The results showed that WS decreased NE activity when compared to control but dAS increased NE when compared to WS. Results assessed

in CF subjects (there were not enough cells to do this in the non-CF subjects). Results are shown in Figure S9.

Of the 23 CF subjects who had NET expression measured by confocal microscopy, 9 subjects were receiving dornase alfa and 14 were not (and this was given the previous day). This dornase alfa group of 9 subjects were older than the other group, 5.1 yrs ± 2.8 versus 3.3 yrs ± 2.3 (p=0.13). The % of neutrophils with NETs was similar in the dornase alfa group 24±16% versus non-Pulmozyme group 21±10% (p=0.53). Dornase alfa is rapidly metabolised in the lung (one study found an 80% reduction in sputum concentration after two hours)[8], and it's half life in serum is reported to be 3-4 hours [9], so it is difficult to draw any conclusions from this data.

We also assessed potentail associations between other NETosis related markers including CFTR status, pathogens and CF medication but were not able to find any definitive associations. This may have been due to the numbers being too small.

Figure S1: NET expression

Staining for NET formation is demonstrated in Figure S1. Panel A shows staining for chromatin (DAPI), B staining for citrullinated histone (H3Cit), C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.

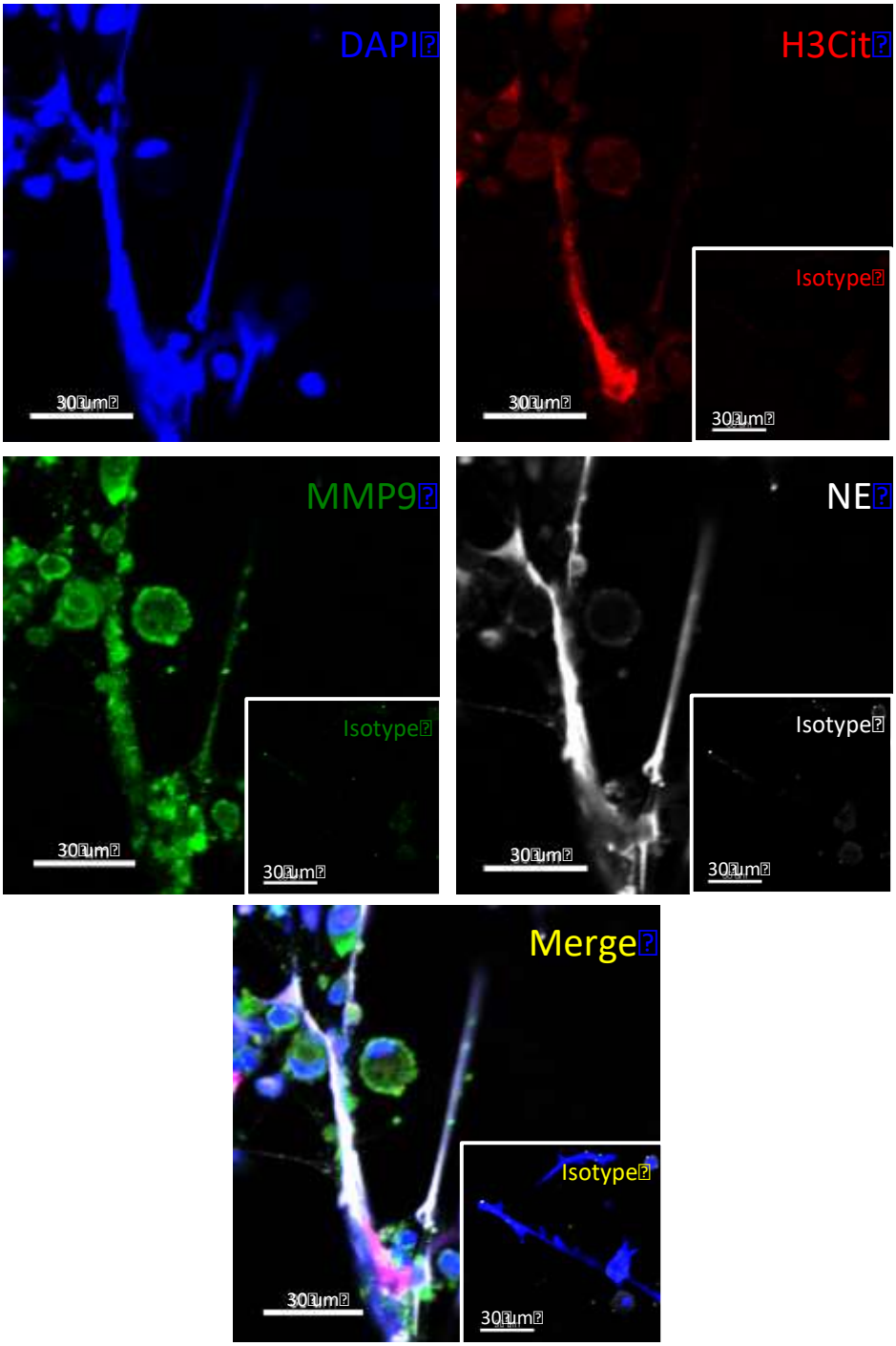

Figure S2: MET expression

Staining for MET formation is shown in Figure S2. Panel A shows staining for chromatin (DAPI), B staining for H3Cit, C staining for MMP9, D staining for NE and E shows the merged image. Insert is isotype control.

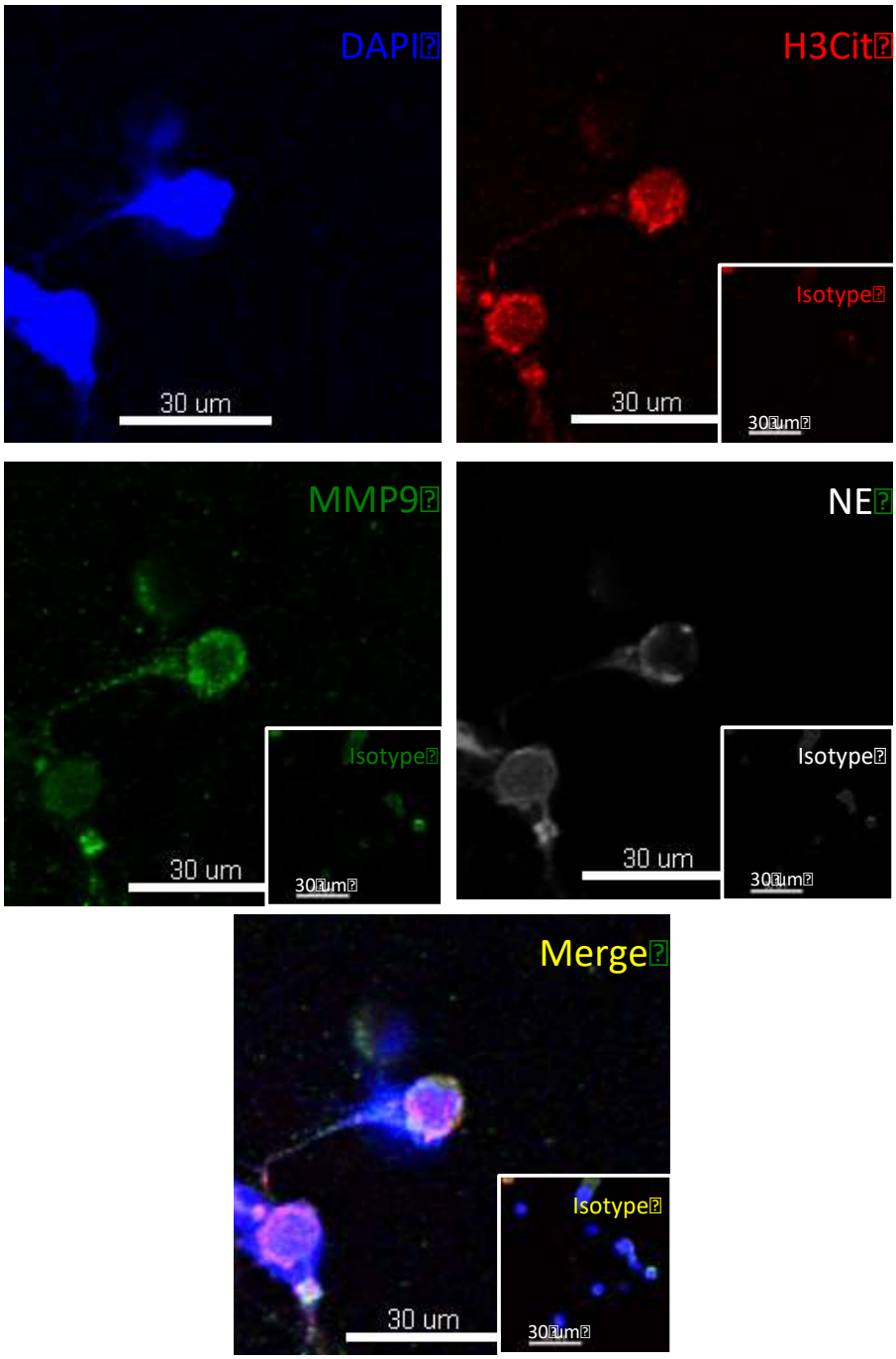

Figure S3: Extracellular trap expression (low power)

Staining for NET/MET formation is shown using a lower magnification field in Figure S3. Panel A shows staining for chromatin (DAPI), B staining for MMP9, C staining for H3Cit, D staining for NE and E shows the merged image. Insert is isotype control.

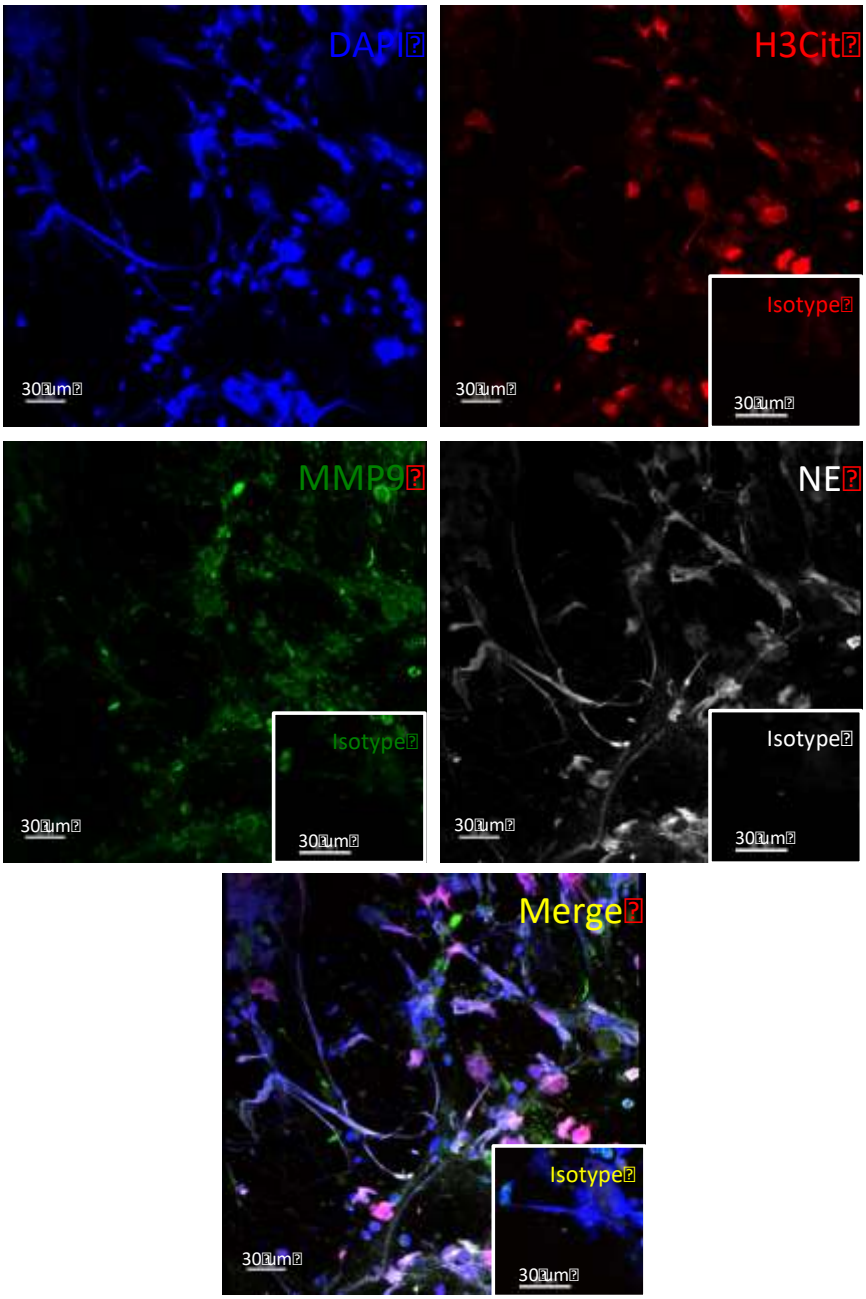

Figure S4: Serum inflammatory mediators and NE levels

A bioplex assay was used to assess if there was a relationship between detectable NE levels. There was no significant association in any of the 40 mediators measured.

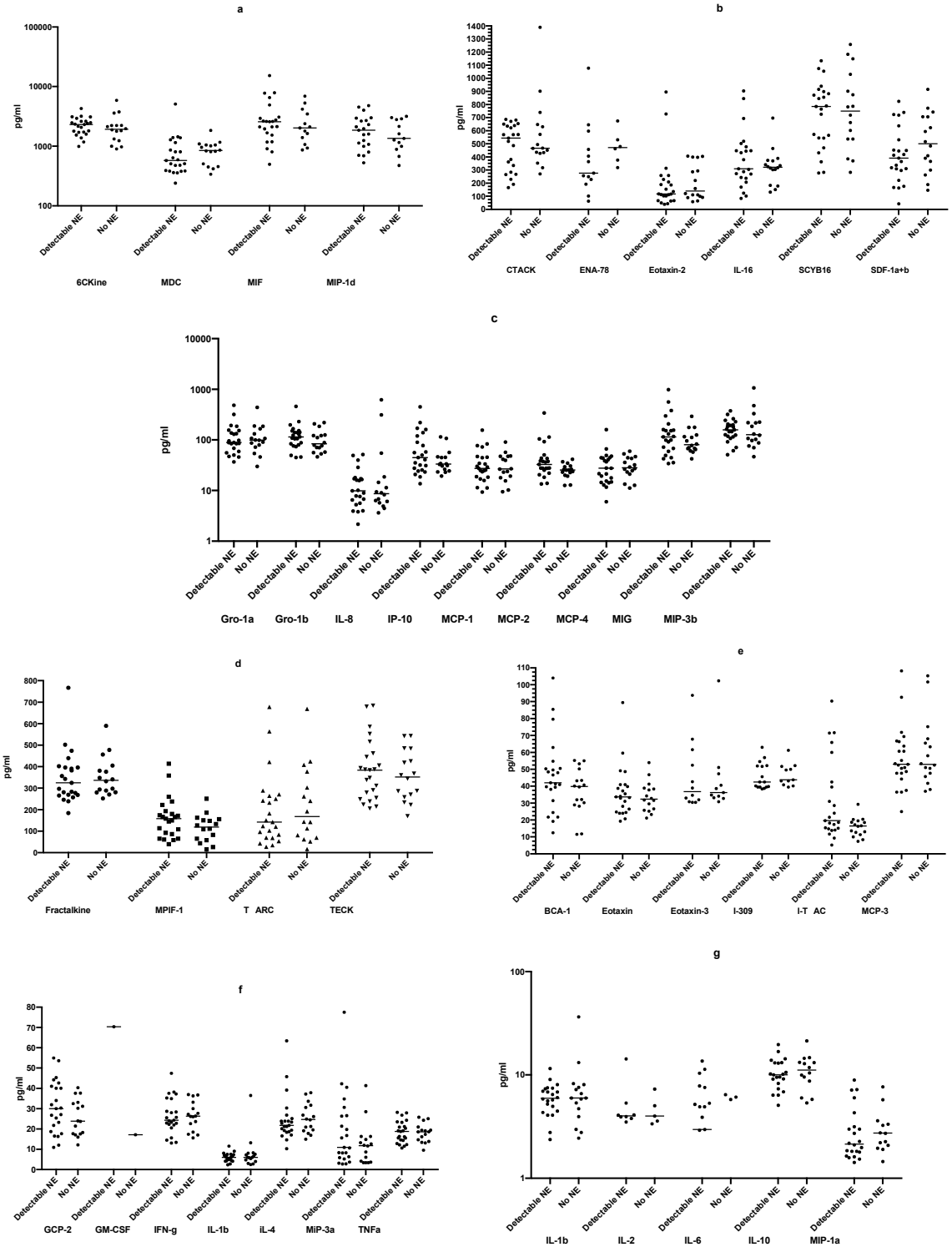

Figure S5: Serum IL-6 Levels

Levels of IL-6 were measured in serum by ELISA in the CF and non-CF groups.

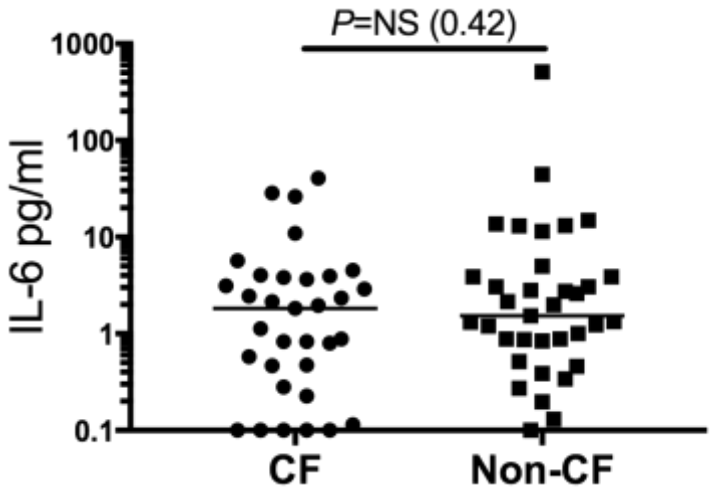

Figure S6: Serum AAT Levels

Serum levels of AAT were measured in the CF and non-CF groups

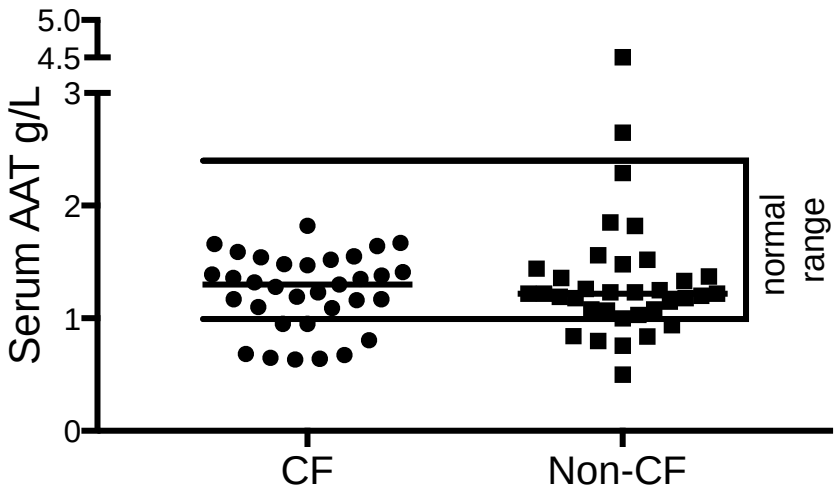

Figure S7: Serum CRP/AAT levels and NE

Levels of CRP and AAT (measured by nephelometry) in the CF and non-CF groups; results were compared between subjects with undetectable and detectable neutrophil elastase (NE). Levels of CRP in CF (A) and non-CF groups (B). Levels of AAT in CF (C) and non-CF groups (D).

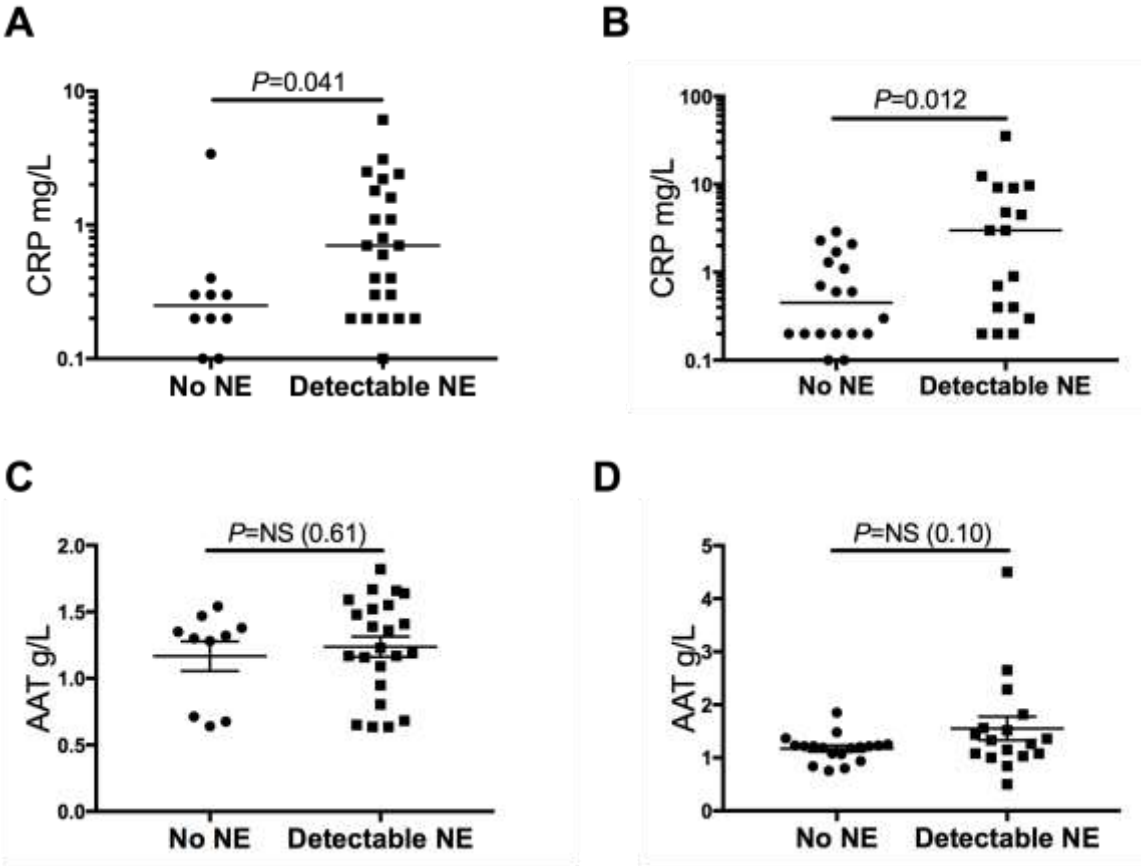

Figure S8: Effect of DNase 1 on MET expression

The effect of DNase 1 on the MET expression of macrophages stimulated with the bacterium NTHi was assessed. Results expressed as % of macrophages expresssing METs. MET expression in CF (A) and non-CF groups (B).

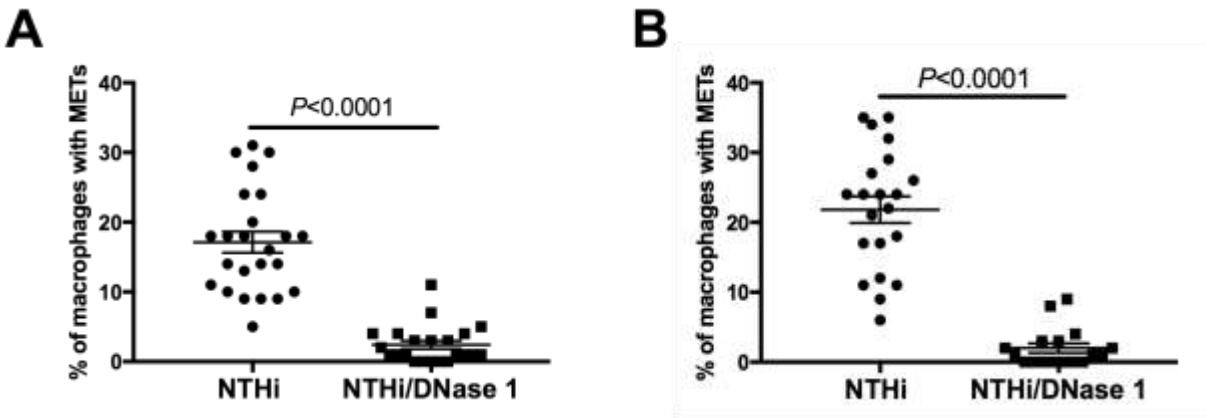

Figure S9: Extra AAT experiments

The effect of whole serum (WS) on NE activity was assessed as was the effect of WS that had been depleted of AAT (dAS). The results showed that WS decreased NE activity when compared to control, but dAS had increased NE activity when compated to WS. Results assessed in CF subjects

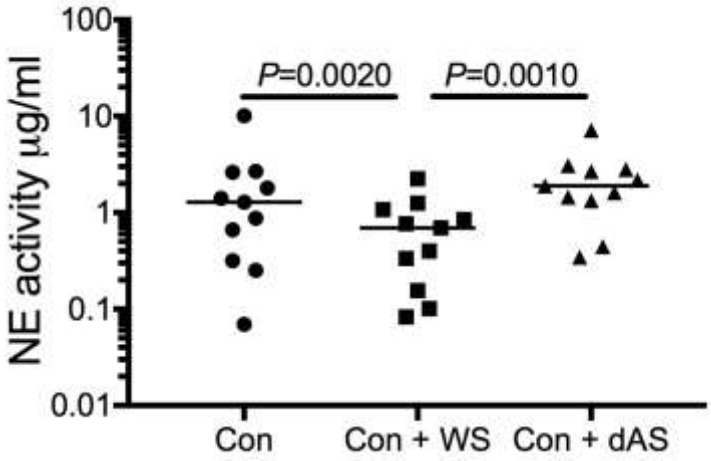

Table S1: CF Group Data

ID	Age	Sex	Dornase alfa	BAL pathogen	CFTR mutation		% neutrophils	NE Activity	MET/NET
					Mutation 1	Mutation 2			
1	8.0	M	Y	*Staphylococcus aureus, Aspergillus fumigatus*	deltaF508	R533X	52	Y	Y*
2	8.6	F	Y	*Streptococcus pneumoniae, S. aureus, Penicillium sp.*	deltaF508	deltaF508	23	Y	Y
3	7.2	M	Y	N	deltaF508	deltaF508	8	N	Y
4	1.0	M	N	*Haemophilus influenzae, S. aureus*	deltaF508	G551D	68	Y	Y
5	4.0	M	N	*Stenotrophomonas maltophilia*	deltaF508	deltaF508	27	N	Y
6	5.4	F	N	*Pseudomonas aureginosa, Aspergillus sp.*	deltaF508	deltaF508	60	Y	Y
7	9.2	M	Y	Rhinovirus (RV)	deltaF508	deltaF508	83	Y	Y
8	3.6	M	Y	*Moraxella catarrhalis*	deltaF508	deltaF508	1	N	Y
9	6.4	M	N	*A. fumigatus*	deltaF508	deltaF508	23	Y	Y
10	1.4	F	Y	*H. parainfluenzae*	c.1521_1523	c.1521_1523 (delCTT)	11	N	Y
11	8.4	M	N	*S. aureus*	deltaF508	deltaF508	26	Y	Y
12	1.7	F	N	*Sten maltophilia*	C.1521_1523	(delCTT)/C.1521_1523 (de	86	Y	Y
13	1.4	M	N	*Haemophilus sp.*	deltaF508	deltaF508	14	N	Y
14	5.2	M	N	*H. parainfluenzae, Escherichia coli, Penicillium sp.*	deltaF508	deltaF508	10	N	Y
15	4.3	F	N	*H. parainfluenzae, H. influenzae*	deltaF508	deltaF508	21	Y	Y
16	4.1	F	Y	*H. parainfluenzae, H. parahaemolyticus*	deltaF508	deltaF508	32	N	Y
17	4.1	F	N	N	p.G551D	p.R117H 5T	5	N	Y
18	6.2	F	Y	*S. aureus*	deltaF508	deltaF508	55	Y	Y
19	2.1	M	N	*Escherichia. coli, S. aureus* , Influenza Virus A (IAV)	deltaF508	deltaF508	17	N	Y
20	6.0	F	Y	*H. parainfluenzae*	deltaF508	deltaF508	16	Y	Y
21	1.0	F	N	N	deltaF508	deltaF508	21	N	Y
22	4.0	F	Y	*S. pneumoniae, M. catarrhalis, H. influenzae*	deltaF508	deltaF508	53	Y	Y
23	1.0	M	N	*Enterobacter cloacae, H. parainfluenzae, Candida sp.*	deltaF508	deltaF508	20	Y	Y
24	1.0	M	Y	N	deltaF508	deltaF508	9	N	Y
25	5.9	M	N	*S. aureus, H. Parainfluenzae, Sten maltophilia*	deltaF508	p.G551D	47	Y	N
26	1.9	M	N	IAV, *Ent. cloacae, H. parainfluenzae, Klebsiella sp.*	deltaF508	deltaF508	18	Y	N
27	3.9	F	Y	*S. aureus* , IAV	p.Arg1158Te	p.Arg1158Ter	56	Y	N
28	1.9	F	Y	*H. parainfluenzae, E. coli*	c.1521_1523	c.1521_1523 (delCTT)	5	N	N
29	3.9	M	N	*H. parainfluenzae, S. aureus*	p.R553X	p.Phe508del	22	Y	N
30	6.0	M	Y	*P. aeruginosa, S. aureus, H. influenzae, Sten. maltophilia*	deltaF508	deltaF508	60	Y	N
31	0.9	M	N	N	deltaF508	c.1521_1523 [delCTT];1646	4	N	N
32	4.0	M	N	*H. influenzae*	2_2657	3insA	41	N	N
33	4.8	F	N	RV	deltaF508	deltaF508	21	Y	N
34	0.9	M	N	N	deltaF508	deltaF508	5	N	N
35	5.0	F	N	*Haemophilus haemolyticus, H. influenza*	deltaF508	deltaF508	11	Y	N
36	4.7	M	Y	N	deltaF508	deltaF508	23	Y	N
37	4.0	M	Y	*S.aurus, Candida sp, Sten.maltophilia*	deltaF508	deltaF508	9	Y	N
38	6.0	M	Y	*H. influenzae* , RV	p.Phe508del	p.Pro99Ala probable path	80	Y	N

Age in years. % neutrophils refers to neutrophil % in the BAL. For MET/NET the * in subject 1 means that only METs were measured. N=no, and Y=yes.

Table S2: Non-CF Group Data

ID	Age	Sex	BAL pathogen	% neutrophils	NET Activity	MET/NET
1	1.7	M	N	5	N	Y*
2	2.7	M	*M. catarrhalis*	20	N	Y*
3	1.5	M	*S. pneumoniae*	82	Y	Y
4	5.2	M	*S. pneumoniae* , RV, RSV	21	N	Y
5	3.1	M	*S. pneumoniae*, *Staph. aureus* , RV,	44	Y	Y
6	1.7	M	N	1	Y	Y
7	4.3	M	*H. influenzae*	13	Y	Y
8	7.3	M	N	27	Y	Y
9	1.5	M	N	36	Y	Y
10	1.3	M	N	20	N	Y
11	2.4	F	RV	2	N	Y
12	3.7	M	*H. influenzae* , Adenovirus	13	N	Y
13	6.5	M	N	12	Y	Y
14	4.0	M	N	48	Y	Y
15	2.2	M	*S. aureus*	25	Y	Y
16	4.3	M	RV	82	N	Y
17	4.8	M	N	72	Y	Y
18	4.6	F	N	43	N	Y
19	1.6	F	*S. pneumoniae*	26	Y	Y
20	3.0	M	Parainfluenza virus	94	Y	Y
21	7.4	F	*H. influenzae*, *S. pneumoniae*	94	Y	Y
22	9.0	M	N	14	N	Y
23	11.1	F	N	8	Y	N
24	14.8	F	*M. catarrhalis*	31	N	N
25	7.3	F	*M. catarrhalis*	12	N	N
26	7.8	M	N	35	N	N
27	1.9	M	*M. catarrhalis* /adenovirus	44	N	N
28	4.6	F	*H. influenzae*	37	Y	N
29	1.3	M	*H. influenzae* , Adenovirus	31	Y	N
30	1.1	m	*M.catarrhalis*, *H. influenzae* , RV	6	N	N
31	1.7	F	RV, Adenovirus	11	N	N
32	3.7	M	N	10	N	N
33	9.6	M	*M. catarrhalis*	17	N	N
34	2.1	f	*H. influenzae*	8	N	N
35	11.7	F	*S. aureus*	35	N	N
36	5.9	F	*S. aureus*, *H. parainfluenzae*	3	N	N
37	3.6	F	*H.influenzae*	2	N	N
38	2.3	M	*H.influenzae*	24	Y	N

Age in years. % neutrophils refers to neutrophil % in the BAL. For MET/NET the * in subjects 1 and 2 means that only METs were measured. N=no, and Y=yes.

Table S3: Adult control group

Characteristic	Study group
	No definable lung disease
Number of subjects	21
Age (mean ± SD)	58 ± 12
Sex	
Male	9
Female	12
Significant smoking history	
Numbers of patients	2
Subjects with pathogens in BAL (%)	0/21 (0)
Subjects with detectable BAL NE (%)	0/21 (0)
Subjects with > !0% neutrophils in BAL	0/21 (0)
Differential cell count (% of cells)	
Macrophages	
Median	91
Interquartile range	79-94
Lymphocytes	
Median	9
Interquartile range	5-16
Neutrophils	
Median	1
Interquartile range	1-5
Eosinophils	
Median	0
Interquartile range	0-1
Spirometry	
FEV_1 (% of predicted value)	
Median	105
Interquartile range	97-105
FVC (% of predicted value)	
Median	107
Interquartile range	98-118
FEV_1/FVC	
Median	80
Interquartile range	75-83

SD = standard deviation, FEV_1 = forced expiratory volume in one second, FVC = forced vital capacity

Table S4: Antibodies used

Primary Antibody	Isotype control	Concentration	Secondary Antibody
Rabbit anti-human H3Cit (Citrulline R26) (Abcam, UK)	Rabbit IgG (in-house)	0.02 mg/ml	Chicken anti-rabbit AF 594 (Life technologies, USA)
Mouse monoclonal anti-MMP9 (abcam, UK)	Mouse IgG2a (BioLegend, USA)	0.02mg/ml	chicken anti-mouse AF 488 (Life technologies, USA)
Sheep anti-human neutrophil elastase (LSBio, UK, LB-B4244)	Sheep IgG (in-house)	0.01mg/ml	Donkey anti-sheep AF 647 (Life Technologies, USA)

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