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Cystic fibrosis neutrophils have normal intrinsic reactive oxygen species generation

Damian J McKeon[1,2,#], Karen A Cadwallader[1], Saad Idris[1], Andrew S Cowburn[1], Mark C Pasteur[2], Helen Barker[2], Charles S Haworth[2], Diana Bilton[2,§], Edwin R Chilvers[1*] and Alison M Condliffe[1*]

[1]Respiratory Medicine Division, Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, UK. [2]Adult Cystic Fibrosis Centre, Papworth Hospital, Cambridge, UK.

[#]Current address: Faculty of Medical Sciences, Bangor University, Gwynedd, Wales.

[§] Current address: Department of Respiratory Medicine, Royal Brompton Hospital, London.

*contributed equally to the manuscipt

Short title: Neutrophil NADPH oxidase in CF

Address for correspondence:

Dr A M Condliffe, Respiratory Medicine Division, Department of Medicine, Box 157, Cambridge University Hospitals NHS Foundation Trust, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK

Telephone/Fax: (44) 1223 762007, email: amc46@cam.ac.uk

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Abstract

Previous studies have identified abnormalities in the oxidative responses of the neutrophil in cystic fibrosis (CF), but it is unclear whether such changes relate to loss of membrane cystic fibrosis transmembrane conductance regulator (CFTR) or to the inflammatory environment present in this disease.

Objective: To determine whether neutrophils from CF patients demonstrate an intrinsic abnormality of the respiratory burst.

Methods: The respiratory burst activity of neutrophils isolated from stable ΔF508 homozygote CF patients and matched healthy controls was quantified by both chemiluminscence and cytochrome C reduction. Expression of NADPH oxidase components and CFTR was determined by Western blotting and reverse transcriptase polymerase chain reaction.

Results: The oxidative output from neutrophils from CF in response to receptor-linked and particulate stimuli did not differ from that of controls. Expression of NADPH oxidase components and was identical in CF and non-CF neutrophils. While low levels of CFTR mRNA could be identified in the normal human neutrophil, we were unable to detect CFTR protein in human neutrophil lysates or immunoprecipitates.

Conclusions: CFTR has no role in controlling neutrophil oxidative activity; previously reported differences in neutrophil function between CF and non-CF subjects most likely relate to the inflammatory milieu from which the cells were isolated.

Abstract Word count: 200

Key Words: cystic fibrosis transmembrane conductance regulator; inflammation; NADPH oxidase; neutrophil.

Introduction

Cystic fibrosis (CF) is the commonest genetic disease affecting Caucasians and confers a reduced (but improving) life expectancy, estimated at 50 years for the 2000 birth cohort [1]. Mortality is predominantly due to pulmonary disease, where unrelenting cycles of infection and inflammation destroy the airways and lung parenchyma, thereby leading to respiratory failure. Neutrophils are recruited to the lung by pro-inflammatory mediators such as leukotriene B_4, interleukin-8 (IL-8) and granulocyte-macrophage colony stimulating factor (GM-CSF) [2] and release proteases and reactive oxygen species (ROS); this response fails to clear the infecting bacteria [3] but instead contributes to pulmonary damage and further cycles of inflammation [4].

The phenotype of CF relates primarily to the absence of CFTR from epithelia1 cells, resulting in ductal disease. There is also evidence that the immune response in CF is abnormal and that inflammation may predate infection. Neutrophils are the principal effectors of the innate immune response [5] and underlie the over-exuberant inflammatory response in CF, but it is unclear whether this is due to a primary abnormality of neutrophil function or whether CF neutrophils are responding appropriately to elevated pro-inflammatory stimuli. Reported abnormalities of neutrophil function in CF encompass chemotaxis [6, 7], adhesion [8], degranulation [9] and respiratory burst activity [10, 11]. However, in many of these studies the patient groups were heterogeneous in terms of age, genetic defect, infection status, colonising organism or medication, all variables which may modulate neutrophil responsiveness. Whilst mRNA encoding CFTR has been reported to be present at low

copy number from preparations of human neutrophils [12], it is unclear whether this message originates from the neutrophils themselves or from other cells such as monocytes or lymphocytes present in small but significant numbers in conventional neutrophil isolates. Likewise, there are conflicting reports as to whether CFTR is expressed by neutrophils at the protein level [13, 14].

Animal models and *in vivo* human studies have also demonstrated an important role for the neutrophil in mediating lung damage in CF, but have not established whether this is a primary or secondary phenomenon. In an elegant series of experiments, Tirouvanziam *et al* [15] showed that sterile human foetal CF small airway grafts implanted into severe combined immunodeficiency mice developed neutrophil-mediated inflammatory changes, leading to progressive lung tissue destruction: matched non-CF grafts did not suffer this fate. Khan *et al* [16] demonstrated neutrophilic inflammation in bronchoalveolar lavage from CF infants (mean age 6 months) even in the absence of infection, together with elevated levels of IL-8, suggesting that the excessive neutrophil recruitment could be secondary to cytokine generation.

We have addressed whether there is any intrinsic abnormality in the expression, organisation or function of the neutrophil NADPH oxidase in a stable (assessed clinically and by inflammatory markers) homogeneous (homozygous ΔF508, colonised with Pseudomonas aeruginosa) adult CF population. The macrolide azithromycin has clinical efficacy in this situation [17-20] but since it accumulates dramatically in phagocytic cells [21] and has been reported to affect the respiratory burst *in vitro* and *in vivo* [22, 23], we excluded patients taking this antibiotic from our

study. We have found no significant differences in receptor or particle-induced oxidative burst activity (assessed by cytochrome C reduction, and both luminol- and lucigenin-dependent chemiluminescence) between CF and control neutrophils, and no detectable expression of CFTR protein in the normal (non-CF) human neutrophil.

Methods

Patients

CF patients were all clinically stable ΔF508 homozygotes colonised with *Pseudomonas aeruginosa* recruited from the Adult CF Centre, Papworth Hospital, Cambridge, UK. Those taking oral steroids or macrolide antibiotics (including azithromycin) were excluded. Patients were age and sex matched with healthy volunteers. The study was approved by the Local Research Ethics Committee.

Neutrophil isolation and measurement of ROS generation

Neutrophils (10^7/ml in PBS) isolated from venous blood using plasma/Percoll gradients [24] were primed (or not) with GM-CSF (100 ng/ml, 37ºC, 30 min) and stimulated with phosphate buffered saline (PBS), n-formyl-methionyl-leucyl-phenylalanine (fMLP, 100 nM), phorbol myristate acetate (PMA, 10 ng/ml), opsonised (heat-inactivated pooled human serum, Sigma, 30 min 37ºC) zymosan (5-20 particles/neutrophils, 1 h 37ºC) or heat-killed *Staphylococcus aureus* (12.5-50 particles/neutrophil, 1 h 37ºC) in the presence of cytochrome C (1.2 mg/ml), lucigenin (0.25 mM) or luminol (1 mM). The superoxide dismutase-inhibitable reduction of cytochrome C was quantified [25]; lucigenin dependent (Luc-DCL) and luminol (Lum-DCL) dependent chemiluminescence were recorded from triplicate wells using

a Berthold CentroPhago luminometer. All stimuli produced a maximal or near-maximal response. Flow cytometric-based analysis of whole blood neutrophil shape-change was performed exactly as described [26]. For assessment of phagocytosis, following the completion of the oxidase assay (1 h incubation with the phagocytic prey at 37ºC), neutrophils were aspirated, cytospun and stained (Quick-Diff, Dade-Behr); the percentage of cells ingesting particles and the phagocytic index were quantified (light microscopy under oil immersion) by an observer who was blinded to the experimental conditions, counting at least 100 cells/condition (all conditions assayed in triplicate).

For the preparation of highly purified neutrophils, cells isolated over plasma/Percoll gradients as above were subjected to negative selection by incubation with Human Granulocyte Enrichment Cocktail (StemCell Technologies, Vancouver, Canada) and dextran-coated magnetic colloid, followed by magnetic separation over a StemSep 0.3μm negative selection column prior to elution and washing as described above**.**

Western Blot Analysis of NADPH Oxidase Components

Control and CF neutrophils were treated with 7 μM di-isopropylfluorophosphate (DIFP, Sigma, UK) and samples (5 x 10^6 cells) were pelleted and lysed (0.1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 3 mM β-glycerophosphate, 30 mM NaF, 0.2% cholate and leupeptin, aprotitin and antipain all at 2.5 μg/ml. Samples (50 μg protein, BCA protein assay, Pierce) were separated on 10% SDS-polyacrylamide gels, transferred to PVDF and the membranes were subjected to Western blotting using the following primary antibodies: mouse monoclonal anti-$p47^{phox}$ and mouse monoclonal anti-$p67^{phox}$(BD Transduction Labs, 1 in 2000), mouse monoclonal anti-$p40^{phox}$ (Upstate, New York, 1 in 1000), rabbit polyclonal

anti-gp91^{phox} (Upstate, New York, 1 in 250), rabbit polyclonal anti-p22^{phox} (Santa Cruz, New York, 1 in 750) and anti-Rac-2 rabbit antiserum (Upstate, New York. 1 in 7500). Equal protein loading was confirmed using mouse monoclonal antibodies to β-COP and GAPDH (gifted by Dr Nick Kstakis, Babraham Institute). HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Biorad) were diluted 1 in 3000, and proteins detected using ECL (Amersham Pharmacia).

RT-PCR of CFTR mRNA

Neutrophils were isolated as over plasma percoll gradients as described above. To ensure that any detected products were not due to the small number of contaminating mononuclear cells in these preparations, we also employed an additional monocyte depletion step to obtain neutrophils at >99.9% purity , since even low levels of mononuclear cell contamination has been shown to be physiologically relevant[27]. RNA isolated using RNeasy spin columns (Qiagen, Sussex, UK) was transcribed using oligo(dT) primers) and reverse transcriptase (Stratagene, La Jolla, USA). PCR amplification (50 cycles) was performed using primers specific for CFTR (sense: CAA GGA GGA ACG CTC TAT CCG; antisense: GCC TTC CGA GTC AGT TTC AG; 558 bp product) and ampliTaq DNA polymerase (Bioline, London, UK).

Determination of CFTR protein expression

Neutrophils ($6x10^7$) were lysed (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% CHAPS, plus protease inhibitors [Complete tablets, 1 per 50 ml, Roche, UK plus di-isopropylfluorophosphate 7 μM, Sigma, Poole, UK]), incubated on ice (30 min), sonicated and spun (5 min, 15,000*g*). Supernatants were immunoprecipitated with protein A-sepharose and anti-human CFTR C-terminus

monoclonal antibody (R&D Systems MAB25031, 1:250) for 14 h at 4°C. Samples were analysed by SDS/PAGE. For fractionation experiments, cells were lysed (10 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride plus protease inhibitors), incubated on ice (30 min), sonicated and spun (5 min, 15,000*g*). Supernatants were ultracentrifuged (100,000*g*, 30min), and pellets re-suspended in membrane lysis buffer (50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.5% CHAPS, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride plus protease inhibitors). Membrane fractions were immunoprecipitated and Western blotted as above. Additionally, protein was prepared from freshly isolated neutrophils ($3x10^7$) by trichloro-acetic acid (TCA) precipitation as described [14]. The specificity of the CFTR antibody was confirmed using lysates, immmunoprecipitates or TCA precipitates prepared in parallel from T84 colonic cancer epithelial cells (ATCC, Middlesex, UK).

Statistical analysis

Values are mean ± sem from (*n*) independent experiments. Where biological parameters did not follow a Gaussian distribution paired results were analysed with non-parametric (Mann-Whitney) calculations of significance (StatView 4.5, Abacus Concepts Inc). Differences were considered significant when $p<0.05$.

Results

Patients

CF patients were all clinically stable ΔF508 homozygotes colonised with *Pseudomonas aeruginosa* (mean FEV_1 2.76 l – 67.7%predicted, mean BMI 21.8); those taking oral steroids or macrolides were excluded. 4 of the 23 patients studied

also grew *Staphylococcus aureus* on at least one occasion in the year of participation. Patients were age and sex matched with healthy volunteers (Table 1). All subjects had a normal full blood count, serum electrolytes and renal function; the mean serum alkaline phosphatase was slightly elevated at 154 U/l in the CF subjects and the mean (range) CRP values were 2.2 (2-3) mg/l in controls, and 6.0 (2-21) mg/l in CF patients (see Table 2).

Expression and localisation of oxidase components

Patients with CF and chronic granulomatous disease (CGD) are both susceptible to infection with *Staphylococcus aureus* and *Burkholderia cepacia* [28]. This, plus the known sensitivity of this organism to oxidative killing, suggests that abnormalities of the NADPH oxidase may also exist in CF. However, Western blotting confirmed equal expression of cytosolic ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, rac2) and membrane ($p22^{phox}$, $gp91^{phox}$) components of the oxidase in CF and non-CF neutrophils (Fig1 A-I). Furthermore, immunofluorescence demonstrated identical recruitment of $p47^{phox}$ to the phagosomal membrane in CF and non-CF cells (data not shown).

ROS generation

Measurement of ROS generation by cytochrome C reduction (extracellular superoxide anion generation, Fig. 2A), lumi-DCL (intra- and extracellular ROS, Fig. 2B&C) and luci-DCL (extracellular ROS, not shown) demonstrated identical fMLP-stimulated oxidant function in neutrophils from CF individuals compared to healthy controls. Lack of baseline neutrophil priming was confirmed by the minimal ROS response to fMLP alone and lack of basal shape change in unstimulated neutrophils (Fig. 2E); this may explain why we did not recapitulate the exaggerated fMLP-stimulated lumi-DCL

respiratory burst response reported by Witko-Sarsat and co-workers [11]. Priming with GM-CSF enhanced the responsiveness of neutrophils to subsequent stimulation with fMLP as expected, and again there was no difference in the magnitude of this response between control cells and those derived from CF patients. Extracellular ROS production elicited by PMA did not differ between CF and control cells (Fig. 2A), and likewise there was no statistical difference in the PMA generated lumi-DCL response (area under the curve, Mann-Whitney test, Fig. 2D). Likewise, although there was a trend towards an increased responsiveness of CF neutrophils to particulate stimuli (particularly at higher particle:neutrophil ratios) this did not reach statistical significance (Mann-Whitney test, Figure 3A and 3B). The phagocytic capacity of CF neutrophils for zymosan particles and *Staphylococcus aureus* was equivalent to that of cells derived from healthy volunteers (Fig 3C and 3D, and data not shown).

CFTR mRNA but not CFTR protein is detectable in human neutrophils

Reverse transcriptase polymerase chain reaction (rtPCR) demonstrated abundant CFTR mRNA in T84 colonic epithelial cells; CFTR mRNA (confirmed by sequence analysis) could be amplified from normal human neutrophils and monocyte-deplete highly purified neutrophils [27], but only after 50 PCR cycles, suggesting very low level expression of CFTR mRNA (Fig. 4A-B). Using qPCR we could again identify CFTR mRNA in normal and monocyte-depleted human neutrophil preparations from healthy volunteers only at very high (>45) cycle numbers; message in T84 cells was 50,0000-100,000 fold more abundant than in neutrophils (data not shown). CFTR protein has been found in cells other than epithelia [12] including lymphocytes [29] and erythrocytes [30], but the expression of functional CFTR protein in the neutrophil is uncertain [13,14]. Despite identifying low level CFTR mRNA we were unable to

detect CFTR protein either in neutrophil lysates or by immunoprecipitation of whole cells or membrane-enriched fractions (all samples derived from healthy non-CF individuals, Fig. 4C-E). Likewise we could not identify CFTR in samples prepared by TCA precipitation from up to $5x10^7$ normal human neutrophils (data not shown). CFTR was readily detectable in all of the above fractions prepared from T84 cells. Of note, CFTR protein from T84 cells spiked into neutrophil lysates was not broken down under the experimental conditions employed (Fig. 4E), suggesting that proteolysis does not explain the failure to identify CFTR protein under these conditions. Thus CFTR protein is either not expressed in normal human neutrophils or is present at levels below the threshold of detection with the methods employed.

Discussion

In patients with CF, chronic infection with bacterial and non-bacterial pathogens combined with profound airway inflammation results in progressive suppurative lung disease, respiratory compromise and premature death. A characteristic 'march' of pathogens is seen, with initial isolates of *Haemophilus influenzae* and *Staphylococcus aureus*, progressing to *Pseudomonas aeruginosa* , and in some cases *Burkholderia cepacia*, atypical mycobacteria and aspergillus. . Paradoxically, these pathogens survive and multiply within the CF lung despite the presence of an abundant neutrophilic infiltrate, indicating that there is a significant impairment of the normal bactericidal mechanisms operating within this environment. Whether failure to kill pathogens results from a primary abnormality of CF neutrophil function, a secondary neutrophil defect related to the CF pulmonary microenvironment, a neutrophil-independent factor, or from a combinatorial effect, is at present unclear.

The neutrophil respiratory burst, which results in the generation of potentially injurious oxygen radicals, has been reported to be elevated in cells isolated from CF patients [10]; in other studies the response has been shown to vary according to the infecting pathogen [31] or to the method employed to detect respiratory burst activity [11], and secretory products of *Pseudomonas aeruginosa* have been reported to suppress neutrophil respiratory burst activity [32]. In this study, we have employed three methods (cytochrome C reduction, luminol-dependent chemiluminescence and lucigenin-dependent chemiluminsecence) to quantify the respiratory burst activity of neutrophils isolated (by a method demonstrated to cause minimal disturbance of neutrophil function, [24]) from a macrolide-naïve homogeneous patient group (ΔF508 homozygotes colonised with *Pseudomonas aeruginosa*) and have demonstrated unequivocally that in response to soluble ligand stimulation, oxidant output is identical to that from neutrophils from healthy volunteers. Since the CF patients were clinically stable and had normal or near-normal inflammatory indices it is likely that this reflects a lack of circulating pro-inflammatory mediators, entirely consistent with the complete lack of basal shape change seen in the CF neutrophils. Furthermore, the expression of both membrane and cytosolic components of the NADPH oxidase, and the recruitment of cytosolic components to the phagosomal membrane was entirely equivalent in normal and CF neutrophils, again arguing against a primary abnormality of neutrophil function in CF. Whilst there was a trend for increase intracellular oxidant generation in response to particulate stimuli, this did not reach statistical significance and was less apparent at lower (more clinically relevant) particle:neutrophil ratios. In keeping with data reported by Morris *et al* [13], we

found no difference in the phagocytic capacity of neutrophils isolated from the peripheral blood of normal and CF subjects.

Also in keeping with previous data in the literature [12], we were able to demonstrate mRNA for CFTR at low copy number, even in highly purified (monocyte depleted) neutrophil preparations. Despite this finding, a diligent search failed to reveal the presence of CFTR protein in samples derived from normal human neutrophils. CFTR could not be detected on direct Western immunoblotting or by immunoprecipitation of either whole cell lysates, or enriched membrane or cytosolic fractions; CFTR could be detected readily under identical conditions from T84 colonic carcinoma cells. The antibody used in this study was used in the studies referenced above, was able to identify bands consistent with newly-synthesized, non-glycosylated (~130 kD), ER-glycosylated (~150 kD) and mature (fully glycosylated ~180 kD) CFTR in T84 lysate (not shown), and in addition we were unable to detect CFTR in human neutrophil lysates using a range of other commercially available CFTR antibodies (not shown). Neutrophils possess a wide array of powerful proteolytic enzymes; to exclude proteolysis as the cause of our failure to detect CFTR from neutrophil lysates, we used a potent anti-protease cocktail, varied the immunoprecipition conditions (1-14 hours at 4ºC, and compared a variety of lysis buffers, data not shown), and demonstrated the neutrophil lysates under these conditions did not break down CFTR protein 'spiked' into the sample. We were also unable to demonstrate CFTR protein in neutrophil TCA precipitates as described by Painter et al. [14]. Our results agree with those of Morris *et al*. [13] and Di *et al*. [33], but not with those of Painter *et al.* [14]; the latter group reported detection of CFTR protein in TCA precipitates from HL60 cells and human neutrophils, and at present we are unable to explain this

discrepancy, although differences in neutrophil maturity or preparative techniques might be relevant.

If there is no primary defect in the NADPH oxidase in CF neutrophils, what other factors might explain the failure of neutrophils within the CF lung to kill pathogenic organisms? Bacterial killing is a complex process, and both systemic inflammation and the CF pulmonary microenvironment may affect bacterial growth and modulate innate immunity. Systemic inflammation may modify the function of circulating neutrophils in CF, both by priming the oxidative burst as described above, and by up- or down-regulating cell surface receptor expression. Reduced expression of the pattern-recognition receptor TLR2 has been reported on peripheral blood neutrophils from CF patients and this was correlated with increased systemic TNFα, but more dramatic effects on TLR expression are seen on transmigrated CF neutrophils [reviewed in 36], suggesting an important acquired effect secondary to factors within the lung microenvironment. Different groups have reported either normal [34] or somewhat reduced [35] ability of isolated peripheral blood CF neutrophils to kill *Pseudomonas aeruginosa in vitro*; importantly Hartl *et al*. [34] also found that CF neutrophils obtained from sputum or BAL had a dramatically attenuated bactericidal capacity with respect to comparable cells from healthy subjects or to the peripheral blood CF cells, strongly implicating an acquired rather than an intrinsic defect.

The composition of the airway surface liquid (ASL) is profoundly altered in CF, with altered ion fluxes and fluid transport contributing to dehydration of airway mucus and impaired mucociliary transport. The activity of endogenous antimicrobial factors in ASL is inhibited by high ionic strength [37], and the antibacterial activity of the CF ASL is also impaired independently from the effects of ionic strength [38]. Abnormal ASL/mucus composition may also impair neutrophils function; reduced phagocytic

capacity has been reported [13], perhaps related to the physical constraints imposed by concentrated mucus [39]. *Pseudomonas aeruginosa* (and perhaps other pathogens) exist as biofilms in the CF airway [40], and organisms within biofilms are resistant to killing by both antibiotics and neutrophils [41]. Quorum sensing-controlled release of rhamnolipid has been reported to induce neutrophils necrosis [42], and factors released from necrotic neutrophils may promote biofilm formation [43, 44] setting up a vicious cycle favouring bacterial persistence. Other factors released by degranulating or necrotic neutrophils may also negatively regulate their function; neutrophil-derived proteases in CF sputum have been shown to cleave neutrophil CXCR1, disabling TLR5-mediated effector pathways and impairing the killing of P. aeruginosa, [34]; more recently Human Neutrophil Peptides present in high concentrations in CF sputum have been shown to reduce neutrophil phagocytic capacity, actin remodelling and degranulation [45]. Importantly, airway-specific overexpression of the sodium channel ENaC in mice resulted in CF-like lung disease (including mucus plugging, neutrophilic inflammation and susceptibility to bacterial infection), demonstrating that such changes may be produced by accelerated Na^+ transport in the airways alone [46].

In summary we have demonstrated that circulating neutrophils isolated from clinically stable CF patients do not differ from cells from matched healthy controls with regard to their capacity to mount an oxidative burst to soluble or particulate stimuli. Systemic inflammation and factors within the pulmonary microenvironment may modulate both neutrophil function and other aspects of innate immunity to impair host defence. Therapeutic strategies which target the pulmonary microenvironment such as antiproteases [47]ref) or agents which modulate quorum sensing [48,49] or biofilm formation [50] may augment treatments which promote innate immune function [51].

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Figure legends

Figure 1

Expression of phox proteins

A-I. Normal or CF neutrophils (10^6/lane) were sonicated in Laemmli sample buffer and separated by SDS-PAGE prior to Western blotting with antibody to A $p47^{phox}$ (Upstate), B rac2 (Upstate), C $gp91^{phox}$ (Upstate), D $p22^{phox}$ (Santa Cruz), E $p40^{phox}$ (Upstate), F $p67^{phox}$ (Upstate), G GAPDH (gifted by Dr N Ktistakis) and H βCOP (gifeted by Dr N Ktistakis). A representative blot of n=3 performed in duplicate is shown. Densitometry (Image J) data (mean ±standard deviation) for n=3 experiments performed in duplicate normalised to control values shown in I.

Figure 1

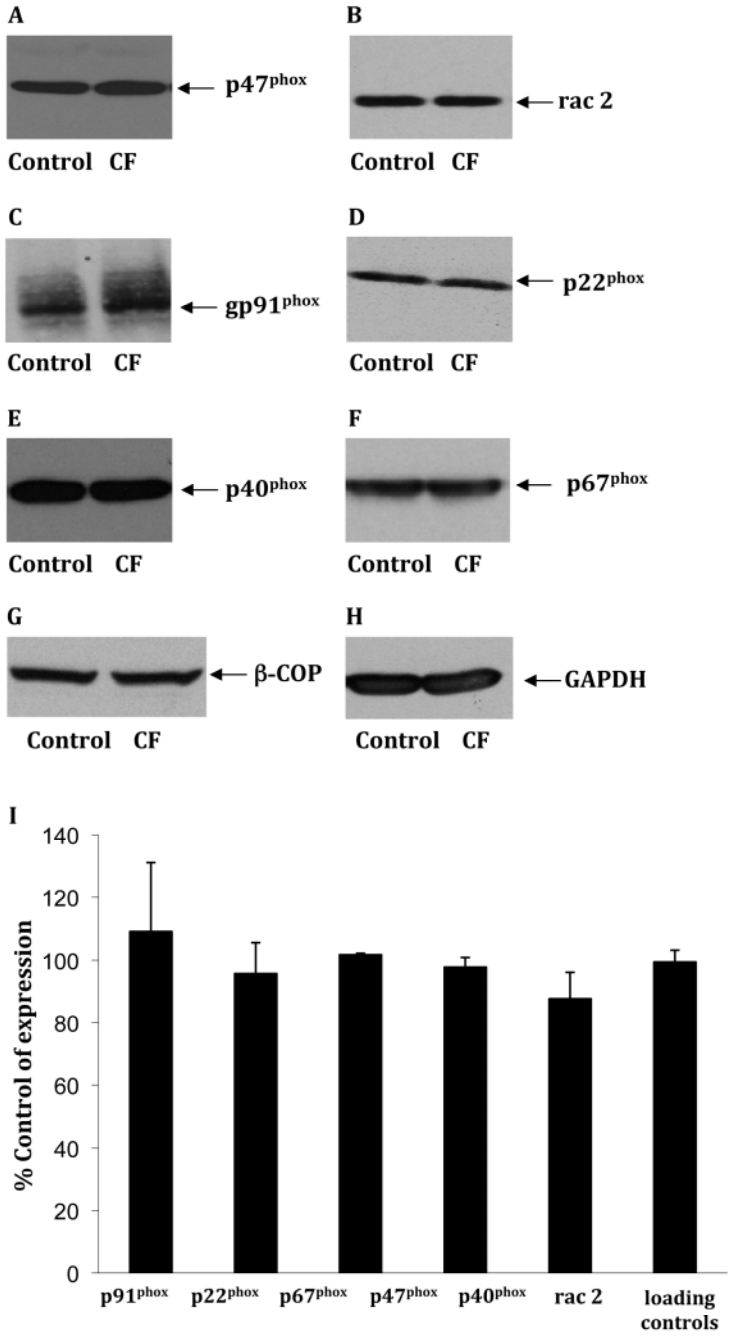


Figure 2

Receptor-mediated ROS generation and shape change in CF and normal neutrophils

2A. Neutrophils from healthy volunteers (open bars) or CF patients (closed bars) were treated with GM-CSF 100ng/ml or PBS (30 min, 37°C) followed by PBS, fMLP 100nM or PMA 1ng/ml for 10 min in the presence of cytochrome C. Superoxide generation was quantified by the superoxide dismutase-inhibitable reduction of cytochrome C. Data represent mean ± sem of 4 independent paired experiments each performed in triplicate.

2B-D. Neutrophils from healthy volunteers (open symbols) or CF patients (closed symbols) were incubated with PBS (B, D) or GM-CSF 100ng/ml (C) 30 min, 37°C and stimulated with fMLP 100nM (B, C) or PMA 10 ng/ml (D) in the presence of 1mM luminol. Lumi-DCL was recorded every 25 s for 1775 s. Data represent mean ± sem of 9 independent and paired experiments performed in duplicate. Values obtained in unstimulated cells are omitted for clarity but amounted to <10% of the fMLP values and did not differ between CF and control subjects.

2E. Venous blood (5 ml) from paired donors was incubated with PBS or 100 nM fMLP (37°C, 30 min); red cells were lysed (ammonium chloride) and the cells fixed (Cell Fix 1:40, BD Biosciences, New Jersey, USA). Forward scatter (FSC) was quantified by flow cytometry (Becton Dickinson, New Jersey, USA). Data are from 5 independent paired experiments each performed in triplicate.

Figure 2

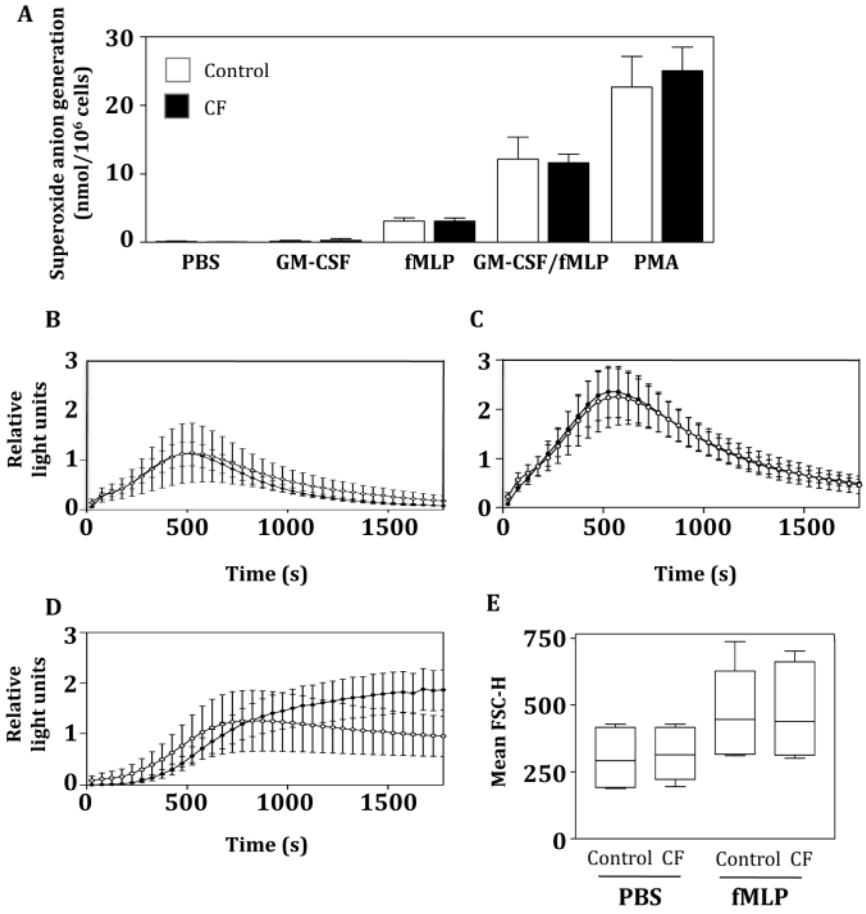


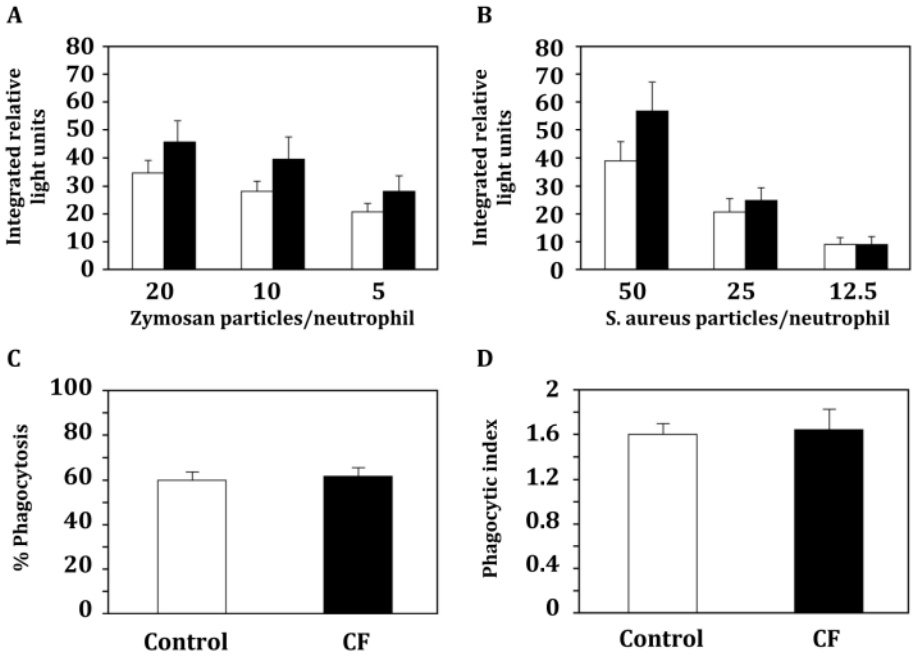
Figure 3

Particle-induced ROS generation and phagocytosis in CF and normal neutrophils

3A. Neutrophils from control subjects (open bars) or CF patients (filled bars) at $5x10^6$/ml were incubated with serum-opsonised zymosan (5-20 particles/ml) in the presence of 1 mM luminol for 60 min. Lum-DCL was recorded over 1 hour. Data are total oxidative output (area under curve) for n=6 separate experiments each performed in triplicate.

3B. Neutrophils from control subjects (open bars) or CF patients (filled bars) at $5x10^6$/ml were incubated with heat-killed, serum-opsonised S. aureus (12.5-50 particles/ml) in the presence of 1 mM luminol for 60 min. Lum-DCL was recorded over 1 hour. Data are total oxidative output (area under curve) for n=6 separate experiments each performed in triplicate.

Figure 3



3C-3D. Following the experiments described in 3A, neutrophils were aspirated, cytospun and stained with Quick-Diff prior to assessment by light microscopy. Data

are expressed as percentage of cells with ingested particles (C) or mean number of particles/cell (D) from n=4 experiments each performed in triplicate.

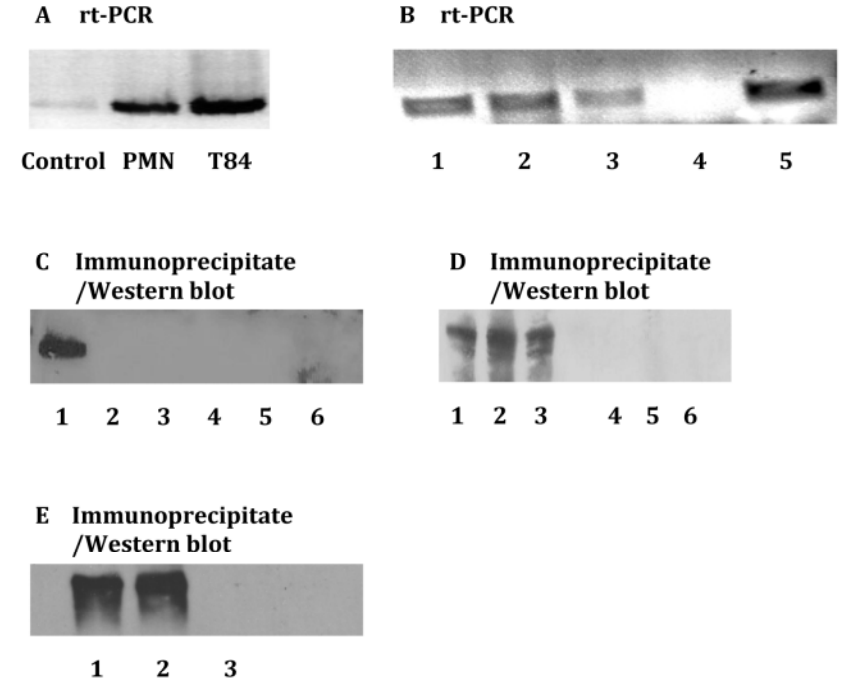
Figure 4

Expression of CFTR

4A-B. rt-PCR for CFTR was performed using RNA extracted from normal human neutrophils and T84 cells. Sequence analysis confirmed the 558 bp product as CFTR mRNA. (A) Lane 1 - control, Lane 2 - neutrophils, Lane 3 - T84 cells. (B) Lane 1 - neutrophils, Lanes 2 + 3 - monocyte depleted neutrophils, Lane 4 - control, Lane 5 - monocytes. Data are representative of 3 independent experiments.

4C-E. Lysates and immunoprecipitates from whole cells and membrane fractions of both normal human neutrophils and T84 cells were prepared and analysed for CFTR by Western blotting as described. (C) Lane 1 - T84 whole cell lysate (10^6 cells), Lane 2 - neutrophil whole cell lysate ($2x10^7$ cells), Lane 3 - neutrophil whole cell lysate ($4x10^7$ cells), Lanes 4-6, immunoprecipitates from neutrophil whole cell lysates, $2x10^7$, $4x10^7$ and $6x10^7$ cells respectively. (D) Lanes 1-3 - T84 cells (10^6 cells): whole cell lysates, immunoprecipitates from whole cell lysates and immunoprecipitates from membrane fractions respectively. Lanes 4-6 - neutrophils ($6x10^7$ cells): whole cell lysates, immunoprecipitates from whole cell lysates and immunoprecipitates from membrane fractions respectively. (E) Lane 1 - T84 whole cell (10^6) immunoprecipitate. Lane 2 - T84 cell (10^6) plus neutrophil ($6x10^7$) immunoprecipitates. Lane 3 - neutrophil ($6x10^7$) immunoprecipitate.

Figure 4


A rt-PCR
Control PMN T84
B rt-PCR
1 2 3 4 5
C Immunoprecipitate /Western blot
1 2 3 4 5 6
D Immunoprecipitate /Western blot
1 2 3 4 5 6
E Immunoprecipitate /Western blot
1 2 3

Variable	**Cystic Fibrosis**	**Control**
Sex	Male (13) Female (7)	Male (13) Female (7)
Age (years)	Mean 26.4 Range 18-36	Mean 30.3 Range 23-34
BMI	Mean 21.8 Range 19-23.3	Mean 27 Range 23-34
Genotype	ΔF508/ΔF508 (23)	Not genotyped
Sputum	*Pseudomonas aeruginosa* (23) *Staphylococus aureus* (4)	N/A
CF-related diabetes	2	N/A
CF-related liver disease	2	N/A
FEV1	Mean 2.76 l (67.7%predicted) Range 1.6-3.6 l (45.3-103.6% predicted)	Not available
VC	Mean 4.89 (88.8% predicted) Range 3.0-5.75 (63.6-112.7% predicted)	Not available

Table 1. Demographics of CF patients versus control subjects

Parameter	**Cystic Fibrosis**	**Control**	Normal Range
Haemoglobin (g/dl)	Mean 14.8 Range 14.3-15.6	Mean 14.8 Range 13.5-16.2	13-18
Total white cell count (10^9/l)	Mean 8.3 Range 5.4-13.3	Mean 5.3 Range 3.7-6.6	4-11
Neutrophil count (10^9/l)	Mean 5.1 Range 2.6-7.3	Mean 2.5 Range 2.2-2.8	2-7.5
Serum alkaline phosphate (U/l)	Mean 154 Range 53-260	Mean 79 Range 53-96	30-135
C-reactive protein (mg/l)	Mean 6.0 Range 2-21	Mean 2.2 Range 2-3	0-6

Table 2 Biochemical and haematological parameters in CF patients and normal controls