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# Cystic fibrosis neutrophils have normal intrinsic reactive oxygen species generation

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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. The aim of the present study was to determine whether neutrophils from CF patients demonstrate an intrinsic abnormality of the respiratory burst.**

**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 RT-PCR.**

**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 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.**

**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.**

**KEYWORDS: Cystic fibrosis transmembrane conductance regulator, inflammation, NADPH oxidase, neutrophil**

Cystic fibrosis (CF) is the most common genetic disease affecting Caucasians and confers a reduced (but improving) life expectancy, estimated at 50 yrs 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 (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] and instead contributes to pulmonary damage and further cycles of inflammation [4].

The phenotype of CF relates primarily to the absence of CF transmembrane conductance regulator (CFTR) from epithelial 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 that may modulate neutrophil responsiveness. While 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

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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 fetal 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 (lum-DCL and luc-DCL)) 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 ($10^7$ $mL^{-1}$ in PBS) isolated from venous blood using plasma/Percoll gradients [24] were primed (or not) with GM-CSF (100 $ng \cdot mL^{-1}$, 37°C, 30 min) and stimulated with PBS, n-formyl-methionyl-leucyl-phenylalanine (fMLP; 100 nM), phorbol myristate acetate (PMA; 10 $ng \cdot mL^{-1}$), opsonised (heat-inactivated pooled human serum; Sigma, Poole, UK; 30 min, 37°C), zymosan (5–20 particles per neutrophil; 1 h, 37°C) or heat-killed *Staphylococcus aureus* (12.5–50 particles per neutrophil; 1 h, 37°C) in the presence of cytochrome C (1.2 $mg \cdot mL^{-1}$), lucigenin (0.25 mM) or luminol (1 mM). The superoxide dismutase-inhibitable reduction of cytochrome C was quantified [25]; luc-DCL and lum-DCL were recorded from triplicate wells using a Berthold CentroPhago luminometer (Berthold Technologies Ltd, Harpenden, UK). All stimuli produced a maximal or near-maximal response. Flow cytometry-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 37°C), neutrophils were aspirated, cytospun and stained (Quick-Diff; Dade-Behring, Newark, NJ, USA); 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 per 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, BC, Canada) and dextran-coated magnetic colloid, followed by magnetic separation over a StemSep 0.3 μm 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 diisopropylfluorophosphate (DIFP; Sigma) and samples ($5 \times 10^6$ 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 $\mu g \cdot mL^{-1}$. Samples (50 μg protein, BCA protein assay; Pierce, Fisher Scientific, Loughborough, UK) were separated on 10% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride and the membranes were subjected to Western blotting using the following primary antibodies: mouse monoclonal anti-$p47^{phox}$ and mouse monoclonal anti-$p67^{phox}$ (1 in 2,000; BD Transduction Labs, Oxford, UK), mouse monoclonal anti-$p40^{phox}$ (1 in 1,000; Upstate, New York, NY, USA), rabbit polyclonal anti-$gp91^{phox}$ (1 in 250; Upstate), rabbit polyclonal anti-$p22^{phox}$ (1 in 750; Santa Cruz, New York, NY, USA) and anti-rac2 rabbit antiserum (1 in 7,500; Upstate). Equal protein loading was confirmed using mouse monoclonal antibodies to β-coatomer protein and glyceraldehyde 3-phosphate dehydrogenase (gifted by N. Ktistakis, Babraham Institute, Babraham, UK). Horse radish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Biorad Laboratories, Hemel Hempstead, UK) were diluted 1 in 3,000, and proteins detected using enhanced chemiluminescence (Amersham Pharmacia, Little Chalfont, UK).

### RT-PCR of CFTR mRNA

Neutrophils were isolated 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, CA, 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 ($6 \times 10^7$) were lysed (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% CHAPS, plus

**TABLE 1** Demographics of cystic fibrosis (CF) patients *versus* control subjects

| Variable | CF | Control |
|---|---|---|
| **Sex** | | |
| Males | 13 | 13 |
| Females | 7 | 7 |
| **Age yrs** | 26.4 (18–36) | 30.3 (23–34) |
| **BMI kg·m$^{-2}$** | 21.8 (19–23.3) | 27 (23–34) |
| **Genotype** | | |
| ΔF508/ΔF508 | 23 | Not genotyped |
| **Sputum** | | |
| *Pseudomonas aeruginosa* | 23 | Not available |
| *Staphylococus aureus* | 4 | Not available |
| **CF-related diabetes** | 2 | Not available |
| **CF-related liver disease** | 2 | Not available |
| **FEV$_1$ L** | 2.76 (1.6–3.6) | Not available |
| **FEV$_1$ % pred** | 67.7 (45.3–103.6) | Not available |
| **VC L** | 4.89 (3.0–5.75) | Not available |
| **VC % pred** | 88.8 (63.6–112.7) | Not available |

Data are presented as n or mean (range). BMI: body mass index; FEV$_1$: forced expiratory volume in 1 s; % pred: % predicted; VC: vital capacity.

protease inhibitors (Complete tablets, 1 per 50 mL; Roche, Welwyn Garden City, UK; plus DIFP 7 μM; Sigma)), 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 (MAB25031, 1:250; R&D Systems) 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*, 30 min), 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 ($3 \times 10^7$) by trichloroacetic 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 presented as 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., Berkley, CA, USA). Differences were considered significant when $p<0.05$.

## RESULTS

### *Patients*

CF patients were all clinically stable ΔF508 homozygotes colonised with *Pseudomonas aeruginosa* (mean forced expiratory volume in 1 s 2.76 L, 67.7% predicted; mean body mass index 21.8 kg·m$^{-2}$); those taking oral steroids or macrolides were excluded. Four 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$^{-1}$ in the CF subjects and the mean (range) C-reactive protein (CRP) values were 2.2 (2–3) mg·L$^{-1}$ in controls, and 6.0 (2–21) mg·L$^{-1}$ in CF patients (table 2).

### *Expression and localisation of oxidase components*

Patients with CF and chronic granulomatous disease 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 ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and rac2) and membrane ($p22^{phox}$ and $gp91^{phox}$) components of the oxidase in CF and non-CF neutrophils (fig. 1). 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), lum-DCL (intra- and extracellular ROS; fig. 2b and c) and luc-DCL (extracellular ROS; data not shown) demonstrated identical fMLP-stimulated oxidant function in neutrophils from CF individuals compared to healthy controls. Lack of baseline

**TABLE 2** Biochemical and haematological parameters in cystic fibrosis (CF) patients and normal controls

| Parameter | CF | Control | Normal range |
|---|---|---|---|
| **Haemoglobin g·dL$^{-1}$** | 14.8 (14.3–15.6) | 14.8 (13.5–16.2) | 13–18 |
| **Total white cell count ×10$^9$ L$^{-1}$** | 8.3 (5.4–13.3) | 5.3 (3.7–6.6) | 4–11 |
| **Neutrophil count ×10$^9$ L$^{-1}$** | 5.1 (2.6–7.3) | 2.5 (2.2–2.8) | 2–7.5 |
| **Serum alkaline phosphate U·L$^{-1}$** | 154 (53–260) | 79 (53–96) | 30–135 |
| **C-reactive protein mg·L$^{-1}$** | 6.0 (2–21) | 2.2 (2–3) | 0–6 |

Data are presented as mean (range), unless otherwise stated.

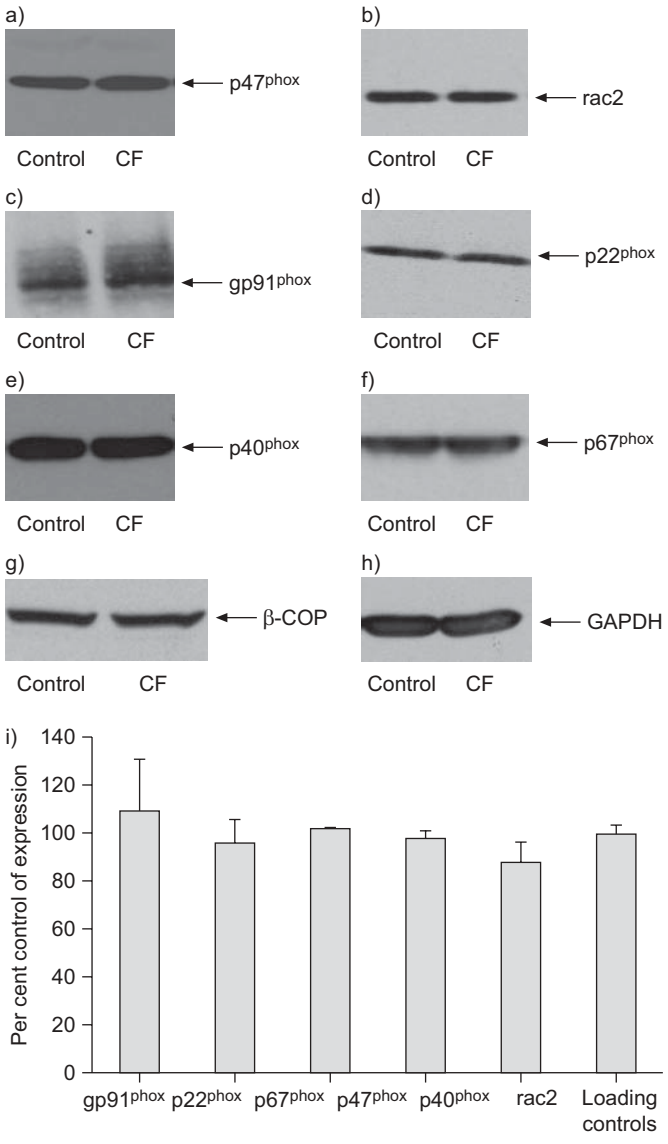


**FIGURE 1.** Expression of phox proteins. Normal or cystic fibrosis (CF) neutrophils were lysed, sonicated in Laemmli sample buffer and separated by SDS-PAGE prior to Western blotting with antibody to a) $p47^{phox}$ (Upstate, New York, NY, USA), b) rac2 (Upstate), c) $gp91^{phox}$ (Upstate), d) $p22^{phox}$ (Santa Cruz, New York, NY, USA), e) $p40^{phox}$ (Upstate), f) $p67^{phox}$ (Upstate), g) β-coatomer protein (β-COP) (gifted by N. Ktistakis, Babraham Institute, Babraham, UK) and h) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (gifted by N. Ktistakis). A representative blot of three performed in duplicate is shown. i) Densitometry (Image J) data (mean±SD) for three experiments performed in duplicate normalised to control values.

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 lum-DCL respiratory burst response reported by WITKO-SARSAT *et al.* [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 lum-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; fig. 3a and b). 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 d).

### *CFTR mRNA but not CFTR protein is detectable in human neutrophils*

RT-PCR demonstrated abundant CFTR mRNA in T84 colonic epithelial cells; CFTR mRNA (confirmed by sequence analysis) could be amplified from normal human neutrophils and monocyte-depleted highly purified neutrophils [27], but only after 50 PCR cycles, suggesting very low level expression of CFTR mRNA (fig. 4a and b). Using real-time PCR 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 50,000–100,000 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 $5 \times 10^7$ 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 nonbacterial 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.

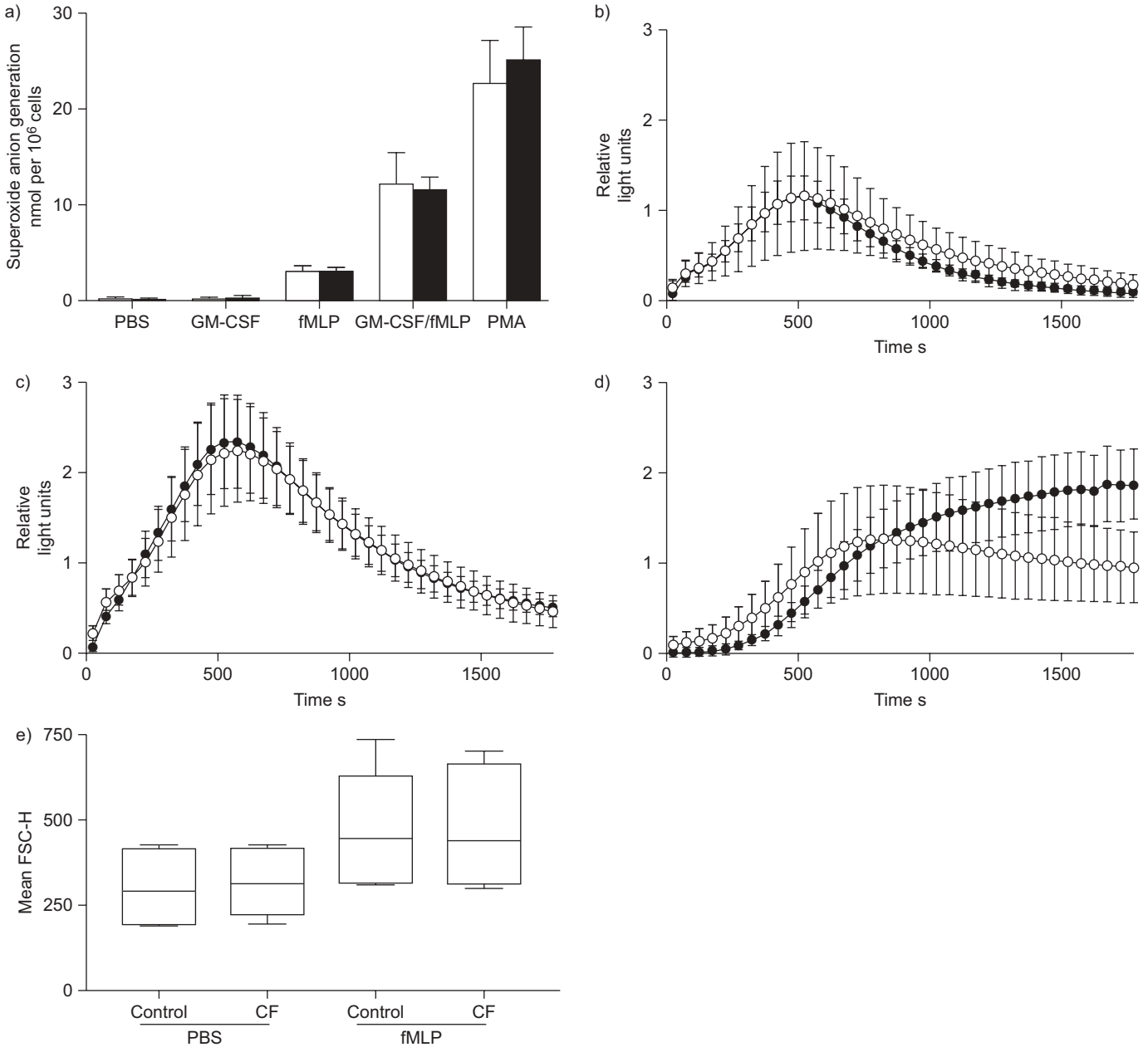


**FIGURE 2.** Receptor-mediated reactive oxygen species generation and shape change in cystic fibrosis (CF) and normal neutrophils. a) Neutrophils from healthy volunteers (□) or CF patients (■) were treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) 100 ng·mL$^{-1}$ or PBS (30 min, 37°C) followed by PBS, n-formyl-methionyl-leucyl-phenylalanine (fMLP) 100 nM or phorbol myristate acetate (PMA) 1 ng·mL$^{-1}$ 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 four independent paired experiments each performed in triplicate. b–d) Neutrophils from healthy volunteers (○) or CF patients (●) were incubated with PBS (b and d) or GM-CSF 100 ng·mL$^{-1}$ (c) for 30 min at 37°C and stimulated with fMLP 100 nM (b and c) or PMA 10 ng·mL$^{-1}$ (d) in the presence of 1 mM luminol. Luminol-dependent chemiluminescence was recorded every 25 s for 1,775 s. Data represent mean±SEM of nine 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. e) 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, Franklin Lakes, NJ, USA). Forward scatter (FSC) was quantified by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). Data are from five independent paired experiments each performed in triplicate. Boxes represent medians and interquartile ranges, and whiskers indicate ranges.

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

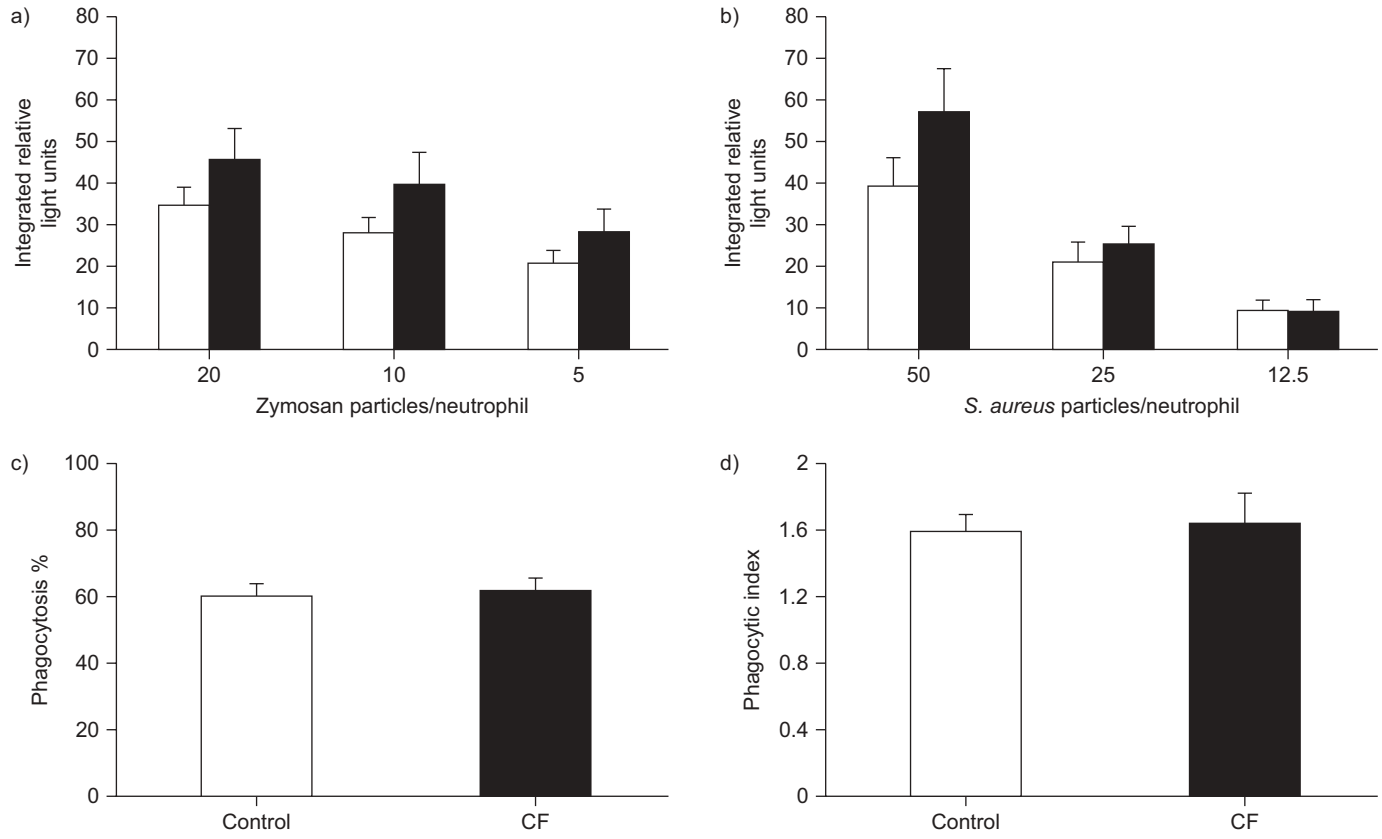


**FIGURE 3.** Particle-induced reactive oxygen species generation and phagocytosis in cystic fibrosis (CF) and normal neutrophils. a) Neutrophils from control subjects (□) or CF patients (■) at $5 \times 10^6$ per mL were incubated with serum-opsonised zymosan (5–20 particles per mL) in the presence of 1 mM luminol for 60 min. Luminol-dependent chemiluminescence (lum-DCL) was recorded over 1 h. Data are total oxidative output (area under curve) for six separate experiments each performed in triplicate. b) Neutrophils from control subjects (□) or CF patients (■) at $5 \times 10^6$ per mL were incubated with heat-killed, serum-opsonised *Staphylococcus aureus* (12.5–50 particles per mL) in the presence of 1 mM luminol for 60 min. Lum-DCL was recorded over 1 h. Data are total oxidative output (area under curve) for six separate experiments each performed in triplicate. c and d) Following the experiments described in part a, neutrophils were aspirated, cytospun and stained with Quick-Diff (Dade-Behring, Newark, NJ, USA) 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 four experiments each performed in triplicate.

have employed three methods (cytochrome C reduction, lum-DCL and luc-DCL) 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. While 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 synthesised, non-glycosylated (~130 kD), endoplasmic reticulum-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

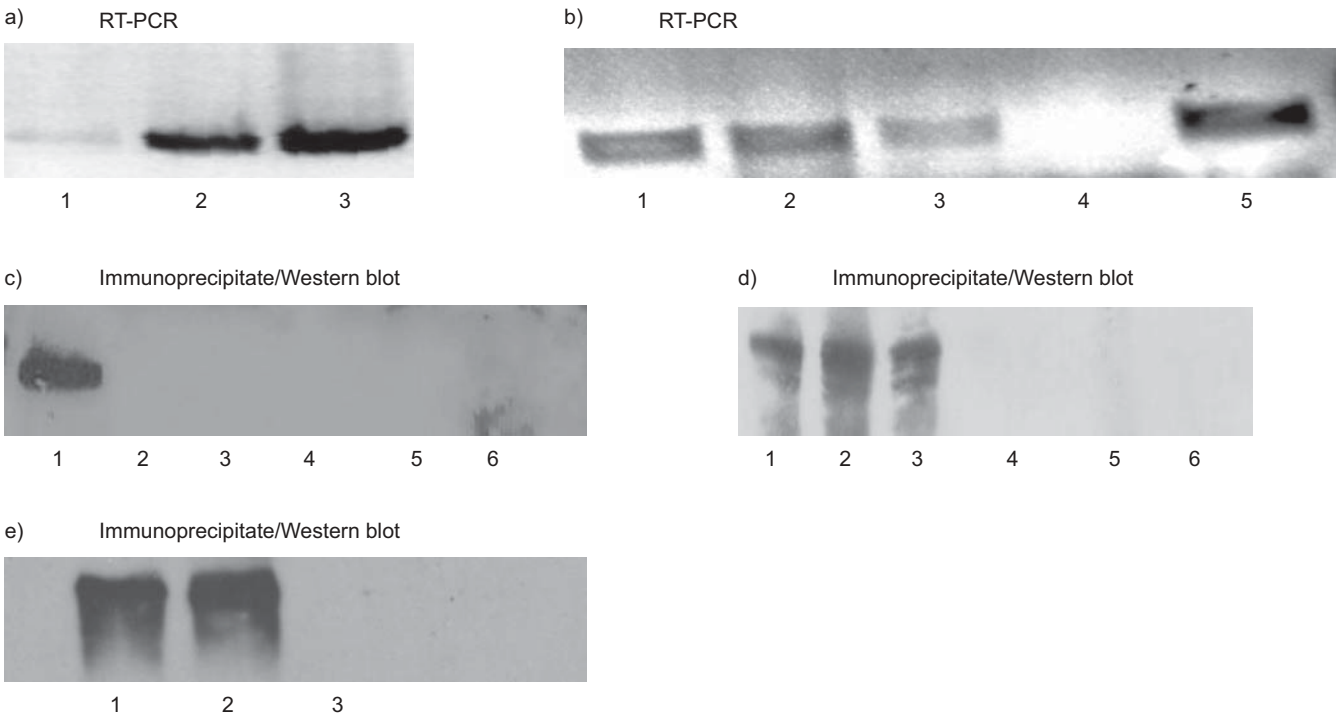


**FIGURE 4.** Expression of cystic fibrosis transmembrane conductance regulator (CFTR). a and b) RT-PCR for CFTR was performed using RNA extracted from normal human neutrophils and T84 cells. Sequence analysis confirmed the 558 bp product as CFTR mRNA. a) Lane 1: control; lane 2; neutrophils; lane 3: T84 cells. b) Lane 1: neutrophils; lanes 2 and 3: monocyte-depleted neutrophils; lane 4: control; lane 5: monocytes. Data are representative of three independent experiments. c–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 ($2\times10^7$ cells); lane 3: neutrophil whole cell lysate ($4\times10^7$ cells); lanes 4–6: immunoprecipitates from neutrophil whole cell lysates, $2\times10^7$, $4\times10^7$ and $6\times10^7$ 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 ($6\times10^7$ 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 ($6\times10^7$) immunoprecipitates; lane 3: neutrophil ($6\times10^7$) immunoprecipitate.

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 h at 4°C) and compared a variety of lysis buffers (data not shown), and demonstrated that 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 Toll-like receptor (TLR)2 has been reported on peripheral blood neutrophils from CF patients, and this was correlated with increased systemic tumour necrosis factor-α, but more dramatic effects on TLR expression are seen on transmigrated CF neutrophils [34], suggesting an important acquired effect secondary to factors within the lung microenvironment. Different groups have reported either normal [35] or somewhat reduced [36] ability of isolated peripheral blood CF neutrophils to kill *Pseudomonas aeruginosa in vitro*; importantly, HARTL *et al.* [35] also found that CF neutrophils obtained from sputum or bronchoalveolar lavage 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 neutrophil 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 neutrophil 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 CXC chemokine receptor 1, disabling TLR5-mediated effector pathways and impairing the killing of *Pseudomonas aeruginosa* [35]; 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 that target the pulmonary microenvironment, such as anti-proteases [47], or agents that modulate quorum sensing [48, 49] or biofilm formation [50], may augment treatments that promote innate immune function [51].

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## STATEMENT OF INTEREST

None declared.

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