



# *Aspergillus*-induced superoxide production by cystic fibrosis phagocytes is associated with disease severity

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**ABSTRACT** *Aspergillus fumigatus* infects up to 50% of cystic fibrosis (CF) patients and may play a role in progressive lung disease. As cystic fibrosis transmembrane conductance regulator is expressed in cells of the innate immune system, we hypothesised that impaired antifungal immune responses play a role in CF-related *Aspergillus* lung disease.

Peripheral blood mononuclear cells, polymorphonuclear cells (PMN) and monocytes were isolated from blood samples taken from CF patients and healthy volunteers. Live-cell imaging and colorimetric assays were used to assess antifungal activity *in vitro*. Production of reactive oxygen species (ROS) was measured using luminol-induced chemiluminescence and was related to clinical metrics as collected by case report forms.

CF phagocytes are as effective as those from healthy controls with regards to phagocytosis, killing and restricting germination of *A. fumigatus* conidia. ROS production by CF phagocytes was up to four-fold greater than healthy controls ( $p < 0.05$ ). This effect could not be replicated in healthy phagocytes by priming with lipopolysaccharide or serum from CF donors. Increased production of ROS against *A. fumigatus* by CF PMN was associated with an increased number of clinical exacerbations in the previous year ( $p = 0.007$ ) and reduced lung function (by forced expiratory volume in 1 s) ( $p = 0.014$ ).

CF phagocytes mount an intrinsic exaggerated release of ROS upon *A. fumigatus* stimulation which is associated with clinical disease severity.



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**Excessive superoxide production by CF phagocytes against *A. fumigatus* is associated with clinical disease severity** <http://ow.ly/Elwy30i8mLe>

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## Introduction

Cystic fibrosis (CF) is the most common life shortening inherited disease among Caucasians with an estimated prevalence of 0.74 per 10000 in Europe [1]. Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) in epithelial cells affect mucus fluid dynamics and immune function, which promotes pathogen survival and lung inflammation [2]. CFTR expression has also been demonstrated in cells of the innate and adaptive immune system [3, 4] and is critical for normal function [5]. *In vitro* studies have demonstrated that CF monocytes and neutrophils display reduced killing activity against *Pseudomonas aeruginosa* [6]. The underlying molecular mechanisms appear to be phagocyte specific and include impaired chloride transport, abnormal complement activation and defective autophagy [6–8]. Reduced killing of *Burkholderia cepacia* by CF macrophages has also been reported and linked to defective autophagy [9].

To date, exploration of the innate immune response of CF phagocytes has focussed on typical CF bacterial pathogens, such as *P. aeruginosa* and *B. cepacia* [6–9]. More recently, however, it has been suggested that the ubiquitous environmental fungus *Aspergillus fumigatus* may play a critical role in CF lung disease. *A. fumigatus* can cause a wide range of disease as a function of the host response. Pulmonary aspergillosis ranges from angio-invasive disease with a high fungal burden in patients with a profoundly weakened immune system, to allergic disease with exaggerated inflammatory responses damaging the host tissue as seen in allergic bronchopulmonary aspergillosis (ABPA) [10]. It has been reported that up to 58% of CF patients are colonised with *A. fumigatus* [11] and that an estimated 47.7% of CF adults are affected by either ABPA or *Aspergillus* infection [12]. In addition, persistent *A. fumigatus* infection in CF is adversely correlated with lung function and hospitalisation [13, 14]. The aim of the current study was to explore the antifungal defence mechanisms of CF phagocytes, to compare them with those of healthy controls and to relate them to clinical metrics of CF disease severity.

## Material and methods

A single centre cross-sectional study was conducted. Additional details on the methods are provided in the supplementary material.

### Participants

Patients attending the adult CF clinic at Aberdeen Royal Infirmary (Aberdeen, UK) were invited to participate. The inclusion criteria were: CF-related lung disease, aged  $\geq 18$  years, stable clinical condition for  $>4$  weeks. Lung-transplant recipients were excluded as well as those on steroids or other anti-inflammatory therapy. Healthy control participants were drawn from the staff working in the Institute of Medical Sciences (University of Aberdeen). The study was approved by the North of Scotland Research Ethics Service (14/NS/1072) and all participants provided written informed consent. Participants were asked to provide a venous blood sample (minimum 10 mL and maximum 40 mL) using standard procedures. Clinical data from each participating CF patient was collected and included: CF genotype, forced expiratory volume in 1 s (FEV<sub>1</sub>), body mass index (BMI), age, CF-related diabetes, *Aspergillus* IgG and IgE, days in hospital, pulmonary exacerbations, Ivacaftor treatment, sputum culture results and antimicrobials prescribed over the previous year.

### Human peripheral blood phagocytes

Peripheral blood mononuclear cell (PBMC) and polymorphonuclear cell (PMN) fractions were isolated from EDTA blood samples by density gradient centrifugation. Monocytes (MNC) were purified from the PBMC fraction using CD14+ microbeads (Miltenyi Biotec, UK) according to the manufacturer's protocol.

### Microorganisms

Conidia of the V45-07 (clinical isolate) and dsRed A293-*A. fumigatus* strains of *A. fumigatus* were used. The lab reference strain of *P. aeruginosa* was PA01 (see supplementary material).

### Live-cell video microscopy

The dsRed A293-*A. fumigatus* was stained with AF633 (Fluorescent *Aspergillus* Reporter (FLARE) conidia) as previously described [15]. To an eight well glass-bottomed microslide was added PMN or MNC ( $1.2 \times 10^5$ ), which was then stimulated with FLARE conidia ( $3.6 \times 10^5$ ). Imaging was performed with a spinning disk confocal microscope.

### Fungicidal activity

XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay was used to assess fungal viability [18]. A 96-well filter plate was seeded with PBMC ( $5 \times 10^5$ ), MNC ( $5 \times 10^5$ )

or PMN ( $1 \times 10^5$ ). Subsequently, *A. fumigatus* conidia ( $1 \times 10^5$ ) was added and the whole incubated for 18 h. Cells were lysed, incubated for 2 h with XTT–Menadione solution and absorbance was measured.

#### **Oxidative burst**

Oxygen radical production was evaluated by luminol-based chemiluminescence as previously published [16]. PMN, PBMC or MNC ( $5 \times 10^5$ ) were incubated with *A. fumigatus* conidia ( $5 \times 10^7$  per mL), latex beads ( $5 \times 10^7$  per mL), depleted zymosan ( $200 \mu\text{g}\cdot\text{mL}^{-1}$ ), lipopolysaccharide (LPS;  $50 \text{ ng}\cdot\text{mL}^{-1}$ ) or *P. aeruginosa* ( $5 \times 10^7$  per mL).

#### **Statistical considerations**

Nonparametric tests (Mann–Whitney, Spearman's rank correlation coefficient (Spearman's rho), Jonckheere–Tepetra) were used for all analyses using IBM SPSS Statistics v24.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism v5.04 (GraphPad Software, La Jolla, CA, USA). Reactive oxygen species (ROS) production over time from CF and control cells was analysed using repeated measures ANOVA with Greenhouse–Geisser correction.

## **Results**

### **Clinical characteristics of participants**

Of the 60 eligible patients attending the clinic, 30 CF patients participated in the study (median age 32 years (range 18–67 years); 69% male). All of the participants with CF were either homozygous or heterozygous for the Phe508del mutation. Median BMI was 22.3 (range 18.3–29) and median FEV<sub>1</sub> was 50% predicted (range 26–92% predicted). Three patients received Ivacaftor, 17 received anti-*Pseudomonas* inhalation antibiotics, and one received antifungal therapy. Sputum grew *A. fumigatus* and *P. aeruginosa* in 36% and 72% of the patients, respectively. Healthy participants were between 18 and 60 years of age.

### **Phagocytic and antifungal activity of cystic fibrosis phagocytes**

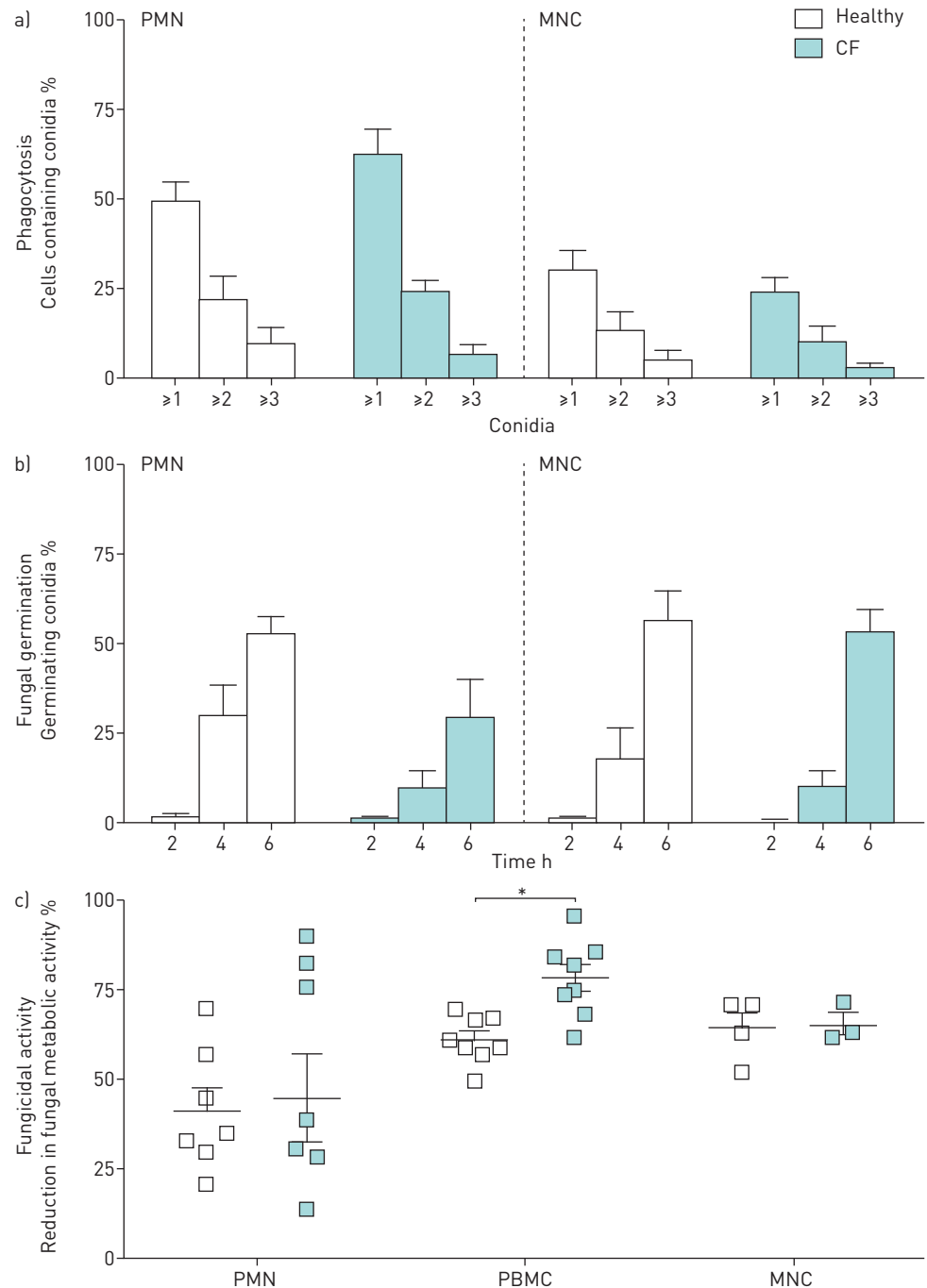
PMN and MNC from CF and control subjects were co-incubated with *A. fumigatus* conidia and uptake of conidia was imaged with live-cell video microscopy. No differences were observed between CF and healthy phagocytes in either the proportion of cells containing conidia (figure 1a) or in the number of conidia contained within each cell. To determine the ability of phagocytes to kill *A. fumigatus*, live-cell imaging using FLARE conidia was performed. FLARE conidia were not killed within a 6-h incubation period with either neutrophils or monocytes for both healthy and CF donors (data not shown). No differences were observed in the proportion of germinating conidia when co-incubated with either PMN or MNC at any of the measured time points (figure 1b). XTT colorimetric assay demonstrated that CF PBMC had a significantly higher mean fungicidal activity ( $78.6\% \pm 3.8\%$ ) compared to healthy PBMC ( $61.5\% \pm 2.3\%$ ) ( $p=0.002$ ). No significant differences were observed in the fungicidal activity of PMN ( $45\% \pm 12\%$  versus  $41.5\% \pm 6.4\%$ ) or MNC ( $65.7\% \pm 2.9\%$  versus  $64.4\% \pm 4.6\%$ ) from CF and healthy controls, respectively (figure 1c).

### **Reactive oxygen species production by cystic fibrosis phagocytes**

ROS production after incubation with *A. fumigatus* conidia was significantly increased by CF PMN, PBMC and MNC when compared to healthy controls (figures 2a–d). CF PMN, PBMC and MNC showed a three- to four-fold increase in total ROS production over 2 h of stimulation compared to healthy cells ( $p=0.01$ ,  $p=0.01$  and  $p=0.038$ , respectively). Analysing the 2-hour ROS curves gives  $p=0.048$  for PMN,  $p=0.035$  for PBMC and  $p=0.372$  for MNC. Pre-incubation of phagocytes with cytochalasin B (Cyto-B) abolished ROS production by PMN and PBMC stimulated with *A. fumigatus*, demonstrating that ROS production is phagocytosis dependent (figures 3a,b). No reduction in ROS production was observed when TLR4 or Dectin-1 were blocked on PMN and PBMC before *A. fumigatus* stimulation. This was similar for healthy and CF phagocytes (supplementary figure S1). To determine if the increased ROS production by CF phagocytes was a compensation mechanism necessary for adequate killing of *A. fumigatus*, the production of ROS was blocked by diphenylethiodonium chloride (DPI) which resulted in impaired fungicidal activity. CF and healthy PBMC treated with DPI displayed reduced killing activity when compared to controls ( $70.4\% \pm 1.8\%$  versus  $80.1\% \pm 2.4\%$  ( $p=0.03$ ) and  $57.7\% \pm 2.8\%$  versus  $75\% \pm 2.3\%$  ( $p=0.01$ ), respectively) (figure 4a). Both CF and healthy PMN showed a >50% drop in fungicidal activity due to DPI but did not reach statistical significance for either healthy ( $18.7\% \pm 13.5\%$  versus  $47.4\% \pm 12.8\%$  ( $p=0.13$ )) or CF ( $34.9\% \pm 20.6\%$  versus  $76.7\% \pm 5.1\%$  ( $p=0.31$ )) cells (figure 4b). No differences were found in the relative decrease in fungicidal activity between healthy and CF phagocytes.

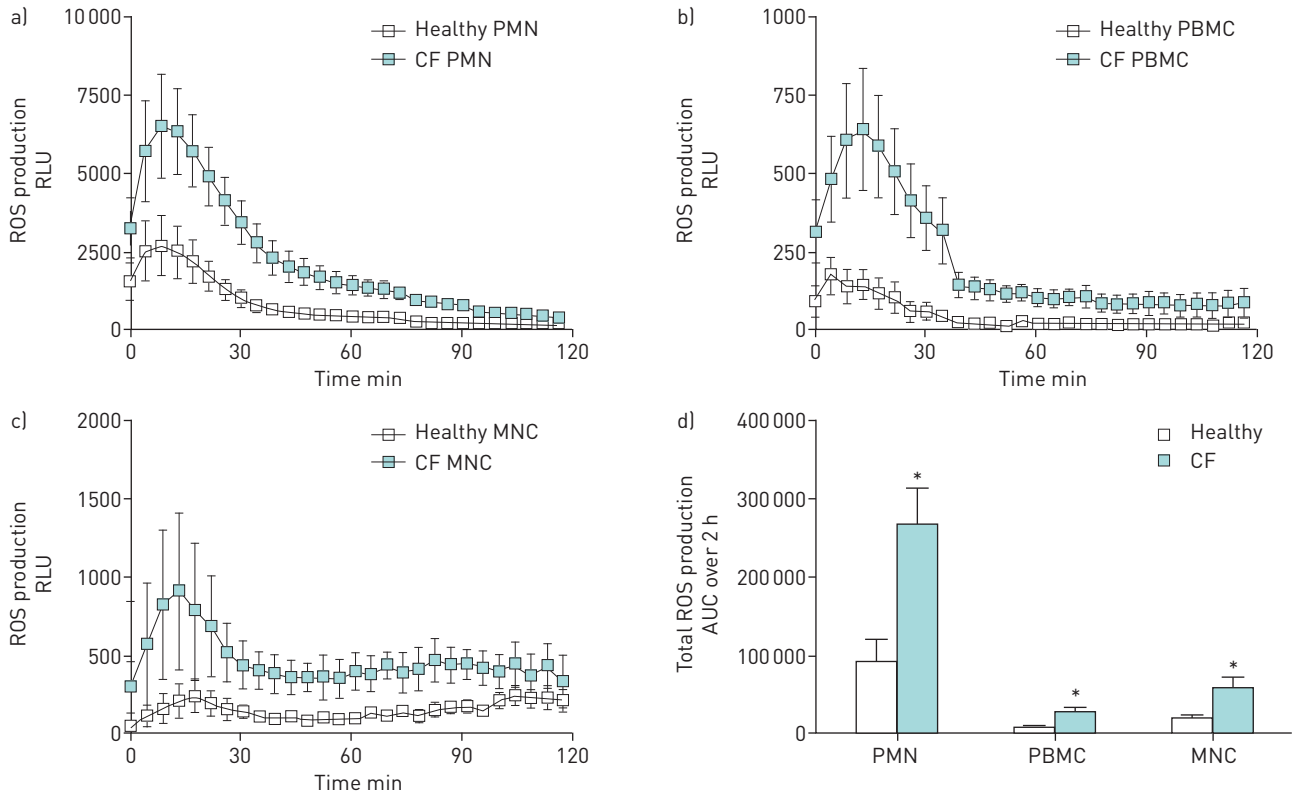
### **Increased respiratory burst in cystic fibrosis phagocytes is non-specific**

In order to assess if the increased ROS production by CF phagocytes was specific to *A. fumigatus*, cells were stimulated with latex beads of similar size to the *A. fumigatus* conidia, depleted zymosan or LPS.



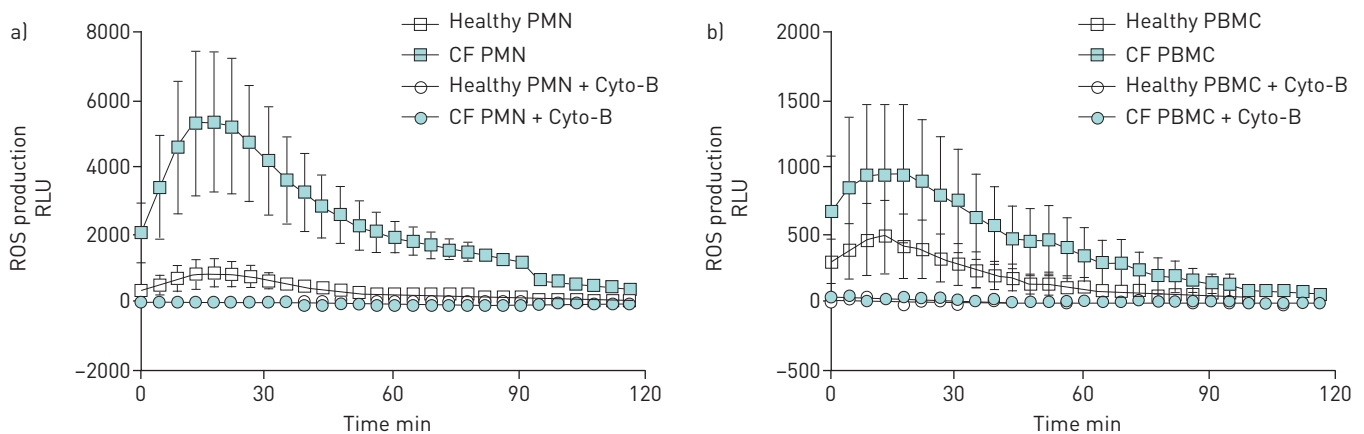
**FIGURE 1** Phagocytosis and antifungal activity for phagocytes taken from healthy and CF donors. For phagocytosis (a) and fungal germination (b), polymorphonuclear cells (PMN) and monocytes (MNC) were co-incubated with FLuorescent *Aspergillus* REporter (FLARE) conidia in a 1:3 ratio and imaged directly with confocal microscopy. The proportion of cells containing conidia was assessed 4 h post-inoculation. Error bars indicate SEM for three healthy and three CF donors. For fungicidal activity (c), PMN ( $5 \times 10^5$  per mL), peripheral blood mononuclear cells (PBMC) and MNC ( $2.5 \times 10^6$  per mL) were stimulated with *Aspergillus fumigatus* conidia ( $2.5 \times 10^6$  per mL) for 18 h and fungal metabolic activity was assessed with an XTT [2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] assay. Error bars indicate SEM for eight healthy and eight CF donors for PMN and PBMC and for four healthy and four CF donors for MNC. \*:  $p < 0.05$  (Mann-Whitney U-test).

A two-fold increase in ROS production by CF PMN was observed across all conditions and similarly for CF PBMC except for LPS stimulation (figures 5a,b). However, the relative difference in ROS production between healthy and CF phagocytes remained most pronounced upon stimulation with *A. fumigatus* conidia.

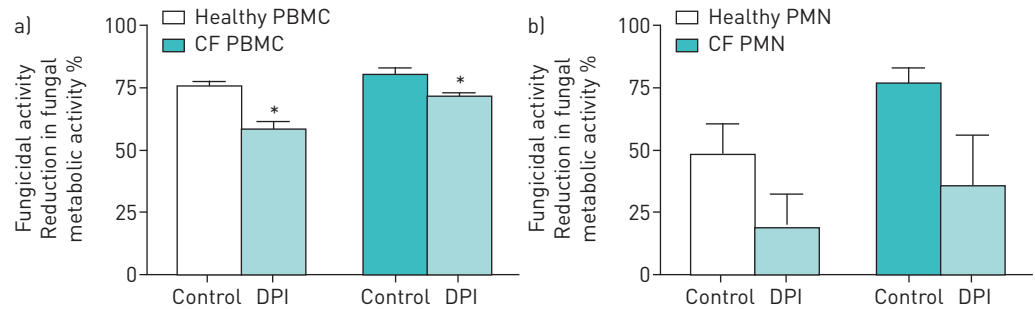


**FIGURE 2** Production of reactive oxygen species (ROS) by phagocytes from healthy and CF donors. Polymorphonuclear cells (PMN) (a), peripheral blood mononuclear cells (PBMC) (b) and monocytes (MNC) (c) [ $2.5 \times 10^6$  per mL] were stimulated with *Aspergillus fumigatus* conidia ( $5 \times 10^7$  per mL) and chemiluminescence was measured directly (as relative light units (RLUs)·s<sup>-1</sup> over a 2-h period). The total ROS production (d) was calculated as the area under the curve (AUC). Error bars indicate SEM for four-to-six healthy and four-to-eight CF donors. \*:  $p < 0.05$  (Mann-Whitney U-test).

To assess if peripheral CF blood phagocytes are at a higher state of activation due to soluble serum factors, cells were pre-incubated with either pooled healthy or CF serum or LPS before stimulation with *A. fumigatus*, beads or zymosan. Pre-incubation with LPS did not affect the ROS production by CF and healthy PMN upon stimulation with either *A. fumigatus*, beads or zymosan (figure 6a). In contrast, ROS production by both CF and healthy PBMC was increased approximately two-fold against depleted zymosan and three-fold against beads after pre-incubation with LPS ( $p < 0.05$ ) (figure 6b). Under all conditions tested the ROS production of healthy phagocytes was much less than that produced by CF



**FIGURE 3** Effect of inhibition of phagocytosis on the production of reactive oxygen species (ROS) against *Aspergillus fumigatus*. ROS production by polymorphonuclear cells (PMN) (a) and peripheral blood mononuclear cells (PBMC) (b) [ $2.5 \times 10^6$  per mL] is shown after pre-incubation with cytochalasin-B (Cyto-B) [ $5 \mu\text{g}\cdot\text{mL}^{-1}$ ] and stimulation with *Aspergillus fumigatus* conidia ( $5 \times 10^7$  per mL). Error bars indicate SEM for four healthy and four CF donors. RLU: relative light unit.



**FIGURE 4** Pre-treatment of phagocytes from healthy and CF donors with diphenyleneiodonium chloride (DPI). The effect of DPI pre-incubation on the fungicidal activity of peripheral blood mononuclear cells (PBMC) (a) [ $2.5 \times 10^6$  per mL] and polymorphonuclear cells (PMN) (b) [ $5 \times 10^5$  per mL] against *Aspergillus fumigatus* ( $2.5 \times 10^6$  per mL) was assessed by XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] colorimetric assay. Error bars indicate SEM for five healthy and five CF donors. \*:  $p < 0.05$  (Mann-Whitney U-test).

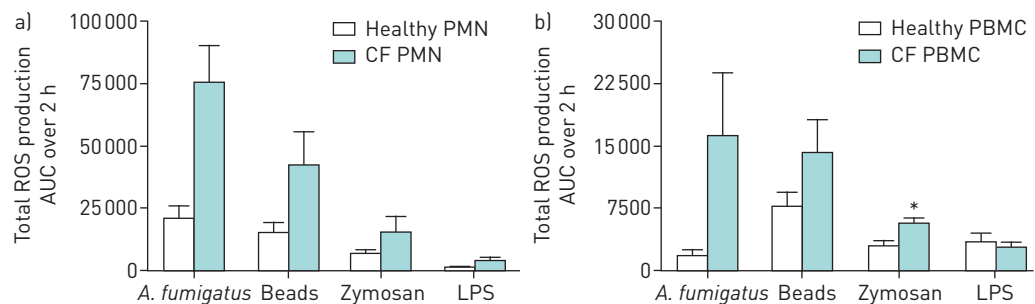
phagocytes. Pre-incubating healthy PMN and PBMC with CF serum did not affect the ROS production against *A. fumigatus* conidia and beads compared to pre-incubation with healthy serum (figures 6c and d).

#### **Cystic fibrosis polymorphonuclear cell production of reactive oxygen species against *Pseudomonas* is not affected**

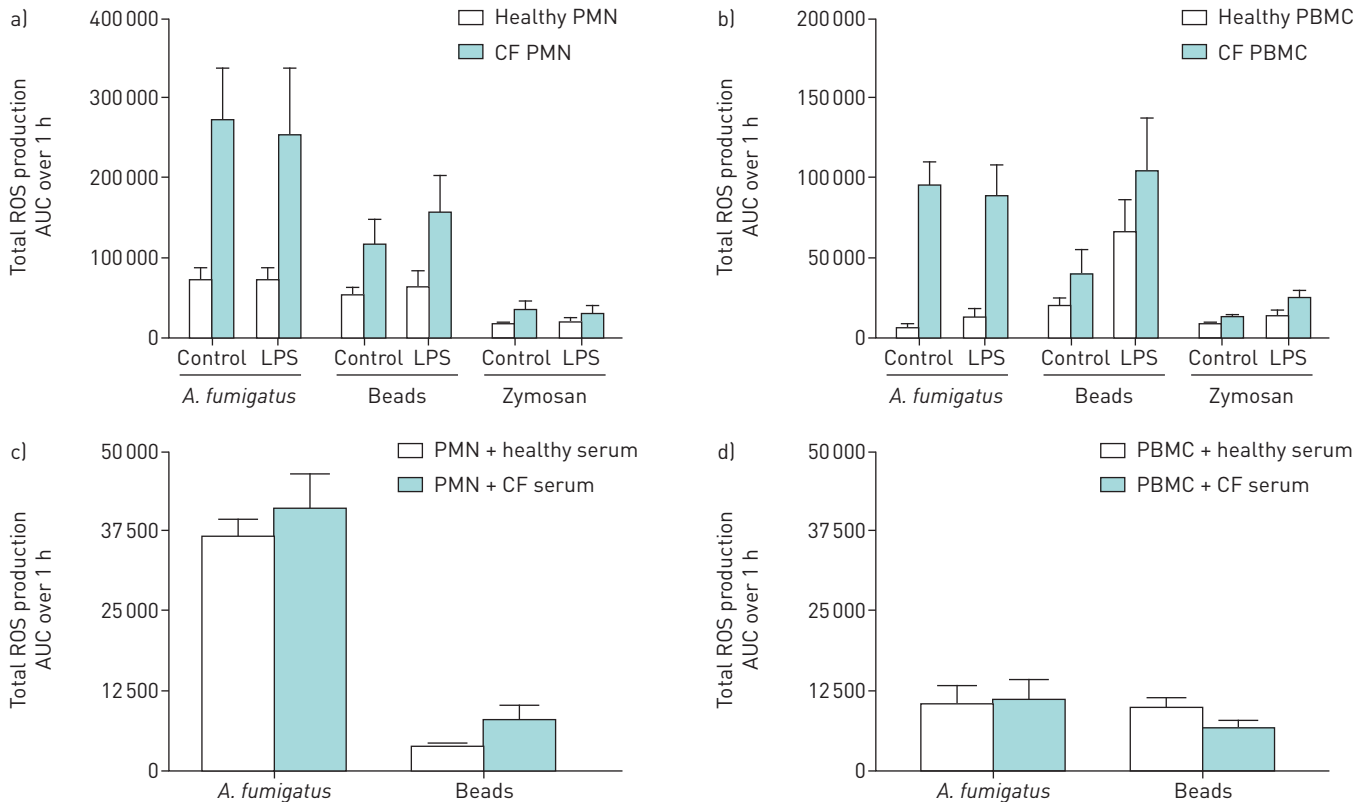
To better understand the hyperresponsive ROS production by CF PMN the cells were incubated with the exact same amount of *A. fumigatus* conidia, *P. aeruginosa* and beads of different sizes (figure 7). ROS production against *P. aeruginosa* was, remarkably, higher in absolute amounts but similar for CF and healthy PMN. A four-fold increase in ROS production was observed against *A. fumigatus* when comparing healthy and CF PMN ( $p = 0.004$ ). Furthermore, CF PMN displayed similar ROS production against 0.5  $\mu\text{m}$  beads compared to healthy controls; however, upon increasing bead size a strong relative increase in ROS production was observed by CF compared to healthy PMN, with a 3-fold increase against 6  $\mu\text{m}$  beads ( $p = 0.0012$ ).

#### **Polymorphonuclear cell reactive oxygen species induced by *A. fumigatus* and clinical metrics**

ROS production did not differ between homozygous and heterozygous dF508 CF donors (data not shown). Increased ROS production by CF PMN was highly and significantly correlated with the number of exacerbations in the previous year ( $\rho = 0.705$ ,  $p = 0.007$ ) and lung function. CF patients with  $\text{FEV}_1 \leq 50\%$  predicted exhibited higher PMN ROS production than those with a  $\text{FEV}_1 > 50\%$  ( $p = 0.014$ ) (figure 8). ROS responses were comparable for patients with and without *Aspergillus* sensitisation (supplementary figure S2). No associations were found with positive *Aspergillus* IgE or IgG levels. Remarkably, ROS responses were more pronounced in those patients without *Aspergillus* cultured from their sputum in the previous year (4.71-fold differences in ROS production (range 2.61–7.31),  $p = 0.03$ ).



**FIGURE 5** Specificity of production of reactive oxygen species (ROS) against *Aspergillus fumigatus* conidia by phagocytes from healthy and CF donors. Polymorphonuclear cells (PMN) (a) [ $2.5 \times 10^6$  per mL] and peripheral blood mononuclear cells (PBMC) (b) [ $2.5 \times 10^6$  per mL] were stimulated with *A. fumigatus* conidia [ $5 \times 10^7$  per mL], 2  $\mu\text{m}$  latex beads [ $5 \times 10^7$  per mL], depleted zymosan ( $200 \mu\text{g} \cdot \text{mL}^{-1}$ ) and lipopolysaccharide (LPS) ( $50 \text{ ng} \cdot \text{mL}^{-1}$ ). Total ROS production is shown as the area under the curve (AUC) over 2 h (mean  $\pm$  SEM) for five healthy and three CF donors. \*:  $p < 0.05$  (Mann-Whitney U-test).



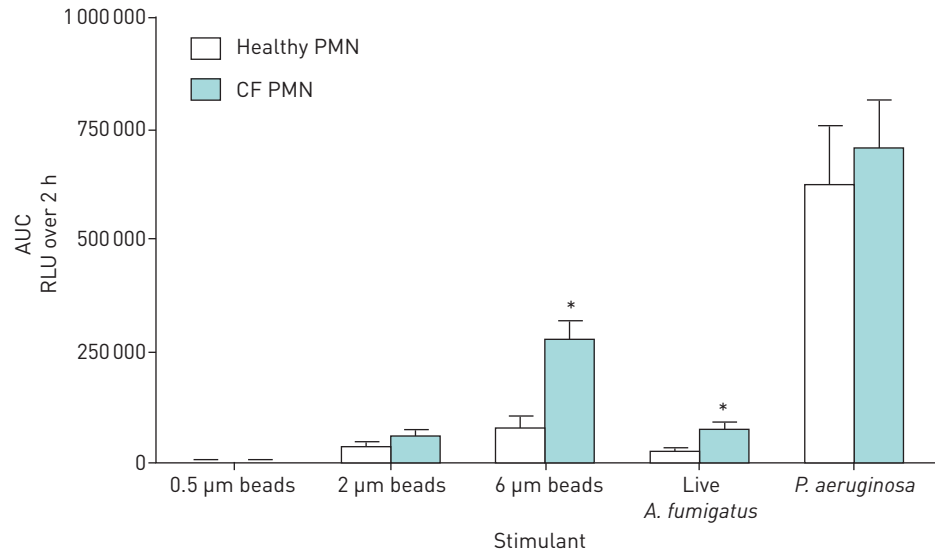
**FIGURE 6** Effect of pre-treatment with lipopolysaccharide (LPS) or cystic fibrosis (CF) serum on the production of reactive oxygen species (ROS) by phagocytes from healthy and CF donors. ROS production by LPS pre-treated healthy and CF polymorphonuclear cells (PMN) (a) ( $2.5 \times 10^6$  per mL) and peripheral blood mononuclear cells (PBMC) (b) ( $2.5 \times 10^6$  per mL) was measured upon stimulation with *Aspergillus fumigatus* conidia ( $5 \times 10^7$  per mL), depleted zymosan ( $200 \mu\text{g}\cdot\text{mL}^{-1}$ ) or  $2 \mu\text{m}$  latex beads ( $5 \times 10^7$  per mL). Total ROS production, as calculated by the area under the curve (AUC) of relative light units (RLUs) by chemiluminescence over 1 h, is shown compared to non-primed controls. Error bars indicate  $\pm\text{SEM}$  for five healthy and three CF donors. Total ROS production by healthy PMN (c) ( $2.5 \times 10^6$  per mL) and PBMC (d) ( $2.5 \times 10^6$  per mL) incubated with 20% healthy or CF serum and stimulated with *A. fumigatus* conidia ( $5 \times 10^7$  per mL) or  $2 \mu\text{m}$  latex beads ( $5 \times 10^7$  per mL) was also determined. Error bars indicate  $\pm\text{SEM}$  for five healthy donors.

## Discussion

The results from our study show that CF peripheral blood phagocytes are as effective as healthy cells in phagocytosis, killing and restricting germination of *A. fumigatus* conidia. However, CF phagocytes displayed exaggerated ROS production upon stimulation with *A. fumigatus* conidia compared to healthy controls. Abolishing ROS production by pre-treating the phagocytes with DPI significantly reduced fungal killing by both CF and healthy phagocytes similarly, demonstrating that the increased ROS production by CF phagocytes does not mask impaired fungal killing. ROS production was shown to be phagocytosis-dependent but not dependent on Dectin-1 and TLR4 signalling. We were able to show that the absence of a functional CFTR protein results in aberrant ROS production upon stimulation with *A. fumigatus* but that this is not observed for *P. aeruginosa*. Of clinical importance, the increased ROS production by CF PMN against *A. fumigatus* was highly correlated with the number of clinical exacerbations in the previous year and reduced lung function.

To our best knowledge, this is the first human study exploring the host–fungus interaction aimed at a better understanding of the role of a functional CFTR protein in innate antifungal immunity. Although associations between *A. fumigatus* and accelerated lung function decline in CF patients and increased hospitalisations have been reported, the clinical importance of *A. fumigatus* infections in CF remains less than clear and management strategies vary widely. Improving our insights into the pathophysiology of *Aspergillus* infections in the CF host will help to establish the clinical importance of this fungus in the progressive lung disease of CF and will assist in the development of targeted treatment modalities.

In this study peripheral blood phagocytes from CF patients were as effective as healthy controls in killing *A. fumigatus*, with even an increased fungicidal activity by CF PBMC compared to healthy cells. This was most likely due to an enhanced monocyte fraction within the CF PBMC population, as isolated CF MNC displayed no difference compared to healthy MNC. Fungicidal activity seems to be less influenced by a

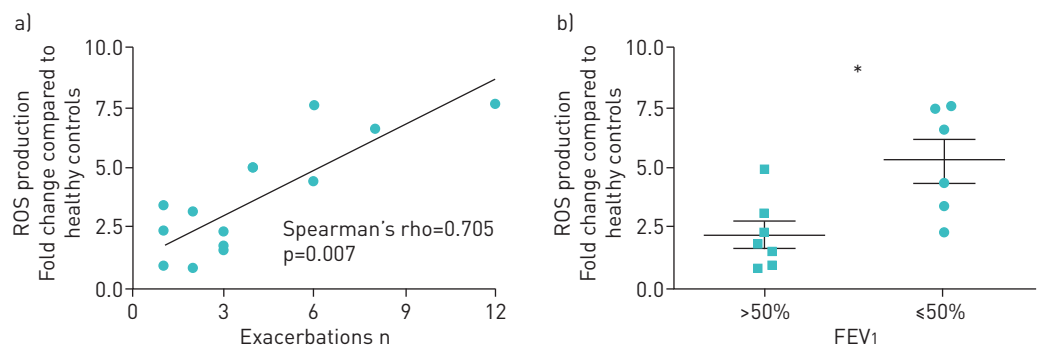


**FIGURE 7** Effect on production of reactive oxygen species (ROS) by polymorphonuclear cells (PMN) from healthy and CF donors on treatment with different sizes of bead and *Pseudomonas aeruginosa*. PMN ( $2.5 \times 10^6$  per mL) were stimulated with beads of different sizes (0.5–6 μm;  $5 \times 10^7$  per mL), *Aspergillus fumigatus* conidia and *P. aeruginosa*. Chemiluminescence was measured directly for 2 h and total ROS production was calculated as the area under the curve (AUC). Error bars indicate  $\pm$ SEM for nine healthy and 11 CF donors. RLU: relative light unit. \*:  $p < 0.05$  (Mann–Whitney U-test).

defective CFTR protein, as others have demonstrated that CF neutrophils display reduced bactericidal activity against *P. aeruginosa* [6].

Significantly increased ROS production was shown by CF PMN, PBMC and MNC upon stimulation with *A. fumigatus* conidia compared to healthy controls. Data from studies using specific phagocyte populations, such as CF neutrophils and monocyte-derived macrophages, have shown conflicting results regarding ROS production *in vitro* upon stimulation with phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (fMLP) or *P. aeruginosa* [17–20]. Interaction with fungal organisms has not been studied before and it seems likely that micro-organism-/stimulus-specific pathways do result in specific ROS responses. We did not observe a difference in ROS production against *P. aeruginosa* when comparing CF and healthy PMN although a four-fold increase in ROS was shown against *A. fumigatus*.

Dectin-1 and TLR4, which have been described as major pathogen recognition receptors (PRR) for *A. fumigatus*, are known to recognise conidia after uptake and binding and are associated with the initiation of an oxidative response [21–25]. However, blocking Dectin-1 and TLR4 recognition did not result in decreased ROS production by either CF or healthy phagocytes upon *A. fumigatus* stimulation. We



**FIGURE 8** Correlation of excessive reactive oxygen species (ROS) production to clinical disease severity. ROS production against *Aspergillus fumigatus* by polymorphonuclear cells (PMN) from 13 cystic fibrosis (CF) patients (compared to PMN from healthy patients) was correlated with the number of exacerbations in the previous year (a). Analysis was performed using Spearman's rank correlation coefficient (Spearman's rho). In addition, the 13 CF patients were divided into two groups based on percentage predicted forced expiratory volume in 1 s (FEV1) scores and their ROS production was compared. \*:  $p < 0.05$  (Mann–Whitney U-test).



questioned the specificity of the increased oxidative response observed for *A. fumigatus* and demonstrated that CF phagocytes elicited a much stronger oxidative burst compared to healthy phagocytes when stimulated with beads or with specific ligands for TLR4 and Dectin-1. These results support the hypothesis reported in the literature that CF immune cells are in a general state of hyperresponsiveness [26, 27]. Priming of healthy phagocytes with CF serum or LPS did not induce ROS responses comparable to those seen by CF phagocytes. This suggests either a specific CFTR-related form of intrinsic priming leading to a higher ROS response or a pathway of activation that takes a longer time to manifest, such as through epigenetic changes like those recently described in monocytes [28]. *Aspergillus* sensitisation seems not to be the explanation for the increased ROS responses as no association was found between the increased ROS responses and increased *Aspergillus* specific IgE or IgG levels.

It should be noted that *A. fumigatus* induced the highest relative increase in ROS production by CF phagocytes compared to healthy controls. Together with the observation that *P. aeruginosa* (a potent ROS inducer) induces similar amounts of ROS from both CF and healthy PMN, this supports the notion of a micro-organism specific induction of the oxidative response. *Aspergillus* conidia, at between 2 and 4  $\mu\text{m}$  in size, are larger than most bacteria and this amplifies dramatically once *Aspergillus* finds a niche and starts to germinate. The link between ROS and stimulus size has been demonstrated by PAPAYANNOPOULOS *et al.* [29], where pathogen size directly links to the amount of ROS produced, activation of inflammatory pathways and amount of neutrophils attracted to the site of infection. When combined with our findings, *e.g.* positive correlation with ROS production and diameter of the beads, one explanation would be that this programme of size-dependent ROS production is affected in CF PMN leading to excessive ROS production against *A. fumigatus*. ROS is an essential and potent stimulator of *NLRP3* and over activation of the *NLRP3* inflammasome in murine CF lungs upon *A. fumigatus* infection has recently been shown [30, 31]. Activation of *NLRP3* in turn leads to release of interleukin-1 $\beta$  (IL-1 $\beta$ ) and using *NLRP3* double knock-out mice or treatment with an IL-1 receptor antagonist has been shown to be protective against lung damage by *Aspergillus* [31]. The *A. fumigatus* induced release of excessive amounts of ROS by human CF phagocytes may well serve as a trigger for increased activation of the *NLRP3* inflammasome followed by an excessive hyperinflammatory response and subsequent tissue damage in the CF lung.

Increased ROS production against *A. fumigatus* conidia by CF phagocytes may provide an explanation for the observed correlation between *A. fumigatus* colonisation and increased lung damage in CF [14]. The CF lung is reported to be abundant in neutrophils from early life onwards even in the absence of infectious stimuli [32, 33]. In addition, it has been shown that bronchoalveolar lavage (BAL) fluids of CF patients contain higher levels of ROS and that these levels are correlated to the number of neutrophils present [34]. GERSUK *et al.* [35] showed that healthy monocyte-derived macrophages ingest and phagocytose *A. fumigatus* conidia effectively, while at the same time suppressing any significant ROS or pro-inflammatory cytokine production. The ability to suppress ROS production when it is not required is likely to be important because ROS are toxic compounds which attract neutrophils and mediate a multitude of inflammatory pathways [36, 37]. It is estimated that each person inhales at least 100 *A. fumigatus* conidia a day [38] and as such sustained and persistent ROS production can be expected in the lungs of CF patients. The significant positive correlation found in our study between *A. fumigatus* induced ROS production, the number of clinical exacerbations and impaired lung function support the hypothesis that *Aspergillus* colonisation and infection in the CF lung have an adverse impact on disease progression.

In conclusion, CF peripheral blood phagocytes are as effective as those from healthy controls regarding phagocytosis and killing or controlling the growth of *A. fumigatus* conidia but show hyperresponsive ROS production. The excessive amounts of ROS induced by *A. fumigatus* in CF neutrophils is significantly correlated to disease severity in terms of clinical exacerbations and lung function. Our data suggest that a hyperresponsive state in CF phagocytes plays a crucial role in the hyperinflammatory response upon exposure to *A. fumigatus*. New interventions to prevent fungal induced ROS production need to be explored, such as early treatment of *Aspergillus* infection or targeting the defective molecular pathway underlying these aberrant responses.

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