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Treatment with rhDNase in patients with cystic fibrosis alters *in-vitro* CHIT-1 activity of isolated leucocytes

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Summary

Recent data suggest a possible relationship between cystic fibrosis (CF) pharmacotherapy, *Aspergillus fumigatus* colonization (AC) and/or allergic bronchopulmonary aspergillosis (ABPA). The aim of this study was to determine if anti-fungal defence mechanisms are influenced by CF pharmacotherapy, i.e. if (1) neutrophils form CF and non-CF donors differ in their ability to produce chitotriosidase (CHIT-1); (2) if incubation of isolated neutrophils with azithromycin, salbutamol, prednisolone or rhDNase might influence the CHIT-1 activity; and (3) if NETosis and neutrophil killing efficiency is influenced by rhDNase. Neutrophils were isolated from the blood of CF patients (n = 19; mean age 26·8 years or healthy, non-CF donors (n = 20; 38·7 years) and stimulated with phorbol-12-myristate-13-acetate (PMA), azithromycin, salbutamol, prednisolone or rhDNase. CHIT-1 enzyme activity was measured with a fluorescent substrate. NETosis was induced by PMA and neutrophil killing efficiency was assessed by a hyphae recovery assay. Neutrophil CHIT-1 activity was comparable in the presence or absence of PMA stimulation in both CF and non-CF donors. PMA stimulation and preincubation with rhDNase increased CHIT-1 activity in culture supernatants from non-CF and CF donors. However, this increase was significant in non-CF donors but not in CF patients ($P < 0·05$). RhDNase reduced the number of NETs in PMA-stimulated neutrophils and decreased the killing efficiency of leucocytes in our *in-vitro* model. Azithromycin, salbutamol or prednisolone had no effect on CHIT-1 activity. Stimulation of isolated leucocytes with PMA and treatment with rhDNase interfered with anti-fungal defence mechanisms. However, the impact of our findings for treatment in CF patients needs to be proved in a clinical cohort.

Keywords: fungal, human, inflammation, lung, neutrophils

Introduction

Patients with cystic fibrosis (CF) are susceptible to a number of respiratory infections. This study focuses on *Aspergillus fumigatus*, a ubiquitous saprophyte growing on decaying organic material in both indoor and outdoor environments [1]. Infection can lead to *A. fumigatus* colonization (AC) within the CF lung tissue, as well as *Aspergillus* sensitization (AS), which can lead to allergic bronchopulmonary aspergillosis (ABPA). The latter is characterized by symptoms including wheezing, productive cough, lung function decline, immediate skin reactivity to *A. fumigatus*, elevated total and specific serum immunoglobulin (Ig)E level and increased infiltrates or central bronchiectasis on chest X-rays [2]. Both the prevalence of AC and ABPA has been reported to be increased in patients with CF compared to non-CF donors, with 50% of adult CF patients presenting with AC and 2–15% with ABPA [3].

Neutrophils are known to play a crucial role in the host defence against *Aspergillus* infections; however, the mechanisms by which they are able to recognize and kill this fungi remain poorly understood [4]. Neutrophils possess several anti-fungal properties, including the production of reactive oxygen species (ROS), the release of granular content and the formation of neutrophil extracellular traps (NETs)

Table 1. Patient characteristics.

Patient ID	Age (years)	Microbiology of the sputum	FEV_1% predicted	CFTR gene mutation	Colonization with *Pseudomonas aeruginosa*	Past history of ABPA
1	16	*A. fumigatus*	109	F508del/ 1524 + 1G>A	Intermittent	–
2	34	*Pseudomonas aeruginosa*	40	F508del/2183AA-G	Chronic	+
3	20	*P. aeruginosa, Staphylococcus aureus*	46	F508del/F508del	Chronic	+
4	36	*P. aeruginosa, A. fumigatus*	72	F508del/cftr dele2,3	Chronic	–
5	18	*Proteus mirabilis, Candida glabrata*	51	F508/2143delT	None	+
6	35	*P. aeruginosa*	119*	F508del/F508del	Intermittent	–
7	23	*P. aeruginosa*	25	F508del/F508del	Chronic	–
8	34	*S. aureus*	55	F508del/2nd mutation not identified	Intermittent	–
9	20	*C. albicans, A. fumigatus*	61	F08del/F508del	Chronic	–
10	27	Coagulase-negative *Staphylococci*	81	F08del/C3849T	None	–
11	23	*Physiological flora*	73	F08del/F508del	Chronic	–
12	40	*Physiological flora*	35	F508del/ c.2657 + 5G>A	Chronic	+
13	31	*P. aeruginosa*	52	F508del/N1303K	Intermittent	–
14	20	*P. aeruginosa, C. albicans*	43	F508del/N1303K	Chronic	–
15	20	*C. dubliniens*	106	F508del/F508del	None	–
16	20	*Physiological flora*	85	F508del/F508del	Chronic	–
17	24	*Physiological flora*	65	F508del/F508del	None	–
18	34	*C. albicans*	30	F508del/F508del	Chronic	–
19	33	*Physiological flora*	62	G542X/pArg334Trp	Intermittent	–

Indicated are: no = number of patient (serial); age at time of sample taking (years); microbiological result of the last sputum prior to sample taking (name of major microbes); data from last lung function testing prior to sample taking (%predicted forced expiratory volume in 1 s = FEV_1%); CFTR mutation: molecular genetically proven mutation in the CFTR gene (name of mutation); no/intermittently/chronically colonization status with pseudomonas in sputum; sensitization to a. Fumigatus in skin or blood testing (+ = positive; – = negative; ? = unknown). ABPA = allergic bronchopulmonary aspergillosis.

[5–7]. One class of the enzymes stored in release granules are chitinases, a family of enzymes with the capability of hydrolyzing chitin, which is the second most abundant biopolymer on the planet found, for example, in the outer skeleton of insects, fungi, yeasts, algae, crabs, shrimps and lobsters [8]. Two enzymes with endochitinase activity are described in humans: acidic mammalian chitinase (AMCase) and chitotriosidase (CHIT-1) [9,10], the latter of which has been suggested to be the main functional chitinase in the human lung [11]. CHIT-1 exists as two isoforms, the inactive, mature form and active form of a 50 kDa mature protein, and a second 39 kDa fully chitinolytic isoform which results from proteolytic cleavage of the parent molecule [12]. A mutation in the *CHIT1* gene leads to an inability to produce CHIT-1; individuals with this mutation make up approximately 6% of the population and are referred to as non-secretors. Homozygosity for this mutation has been estimated to be approximately 6% [13,14].

Neutrophils are able to form NETs, which are web-like structures composed of chromatin, granular and cytoplasmic proteins [15]. Neutrophils release NETs in response to large pathogens, such as fungal hyphae and extracellular aggregates of *Mycobacterium bovis*, but not in response to individual bacterial units [16–19]. NET production is a final act of defence by dying neutrophils, and has been termed NETosis [20]. However, recent studies demonstrated that neutrophils, eosinophils and basophils can form apoptosis-independent extracellular traps by expelling their mitochondrial DNA [21–23].

NETosis has been reported in CF patients. Papayannopoulos *et al.* and Marcos *et al.* were the first to show that the extracellular DNA present in the sputum of CF patients originates predominantly from NET formation [24,25]. Additionally, the elevated levels of lung interleukin (IL)−8 and granulocyte–monocyte colony stimulating factor (GM-CSF) have been debated controversially as potential inducer of NETosis in CF [26,27].

Recently, Jubin and colleagues demonstrated a possible relationship between CF pharmacotherapy and AC/ABPA in children with CF [28]. In univariate analysis, ABPA was associated significantly with rhDNase mucolytic therapy,

and multivariate analysis revealed an independent association between long-term azithromycin use and AC. While the authors speculated that the latter association might be explained by the inhibitory effect of azithromycin on both the recruitment and activation of neutrophils [28], the study did not investigate the possible pathophysiological mechanisms behind these epidemiological findings, and these warrant further investigation.

This study sought to investigate whether current CF medications (macrolide antibiotic, beta-mimetic, corticosteroid and rhDNase) might interact with the neutrophil anti-fungal defence mechanisms. Therefore, we recruited a population of CF patients and healthy controls to elucidate the release of CHIT-1 and NET formation in supernatants of isolated leucocytes *in vitro*.

Methods

Patients and study population

We collected samples of 19 CF patients (74% male, mean age = 26.8 years, range = 16–40) and 20 non-CF donors with no other respiratory disease (80% male; mean age = 38.7 years, range 21 = 66).

Consenting CF patients were recruited from the outpatient department at the University Hospital Schleswig Holstein (UKSH), Campus Luebeck and Kiel when blood sampling was taking place routinely. Into this study we accepted CF patients receiving one or more routine CF treatments. All of them received medication typical for CF treatment (pancreatic enzymes $n = 19$; 100%; inhaled rhDNase; $n = 11$; 57·9%; inhaled salbutamol; $n = 16$; 84·2%; inhaled corticosteroids; $n = 12$; 63·2%; oral macrolide antibiotics; $n = 7$; 36·8%; other oral antibiotics $n = 10$; 52·6%).

Pseudomonas aeruginosa, *Candida albicans*, *Staphylococcus aureus*, *A. fumigatus* and physiological flora were the main results of sputum testing in the patient cohort. Only four (21·1%) patients never experienced colonization with *P. aeruginosa*, while five (26·3%) patients had intermittent and 10 (52·6%) patients had chronic proof of *P. aeruginosa*. Mean relative forced expiratory volume in 1 s was 64% (range = 0–119%). Different mutations in the CFTR gene were found in our population. Most common was F508del, found in nearly all our CF patients. There was one patient in our population (patient 8) without identification of his second mutation. Sensitization to *A. fumigatus* was unknown for most of our patients. Only three (15·8%) had a proven sensitization. While acute ABPA was an exclusion criteria for our study, past history of ABPA was not. Four (21·1%) patients had experienced at least one episode of ABPA prior to our testing. Details of the population characteristics are given in Table 1.

Consenting healthy non-CF donors with no current respiratory infection or history of respiratory disease were recruited from the blood bank of the UKSH, Campus Luebeck. Current infection was excluded by questionnaire and temperature measurement. Written informed consent was obtained from all participants and the study design was approved by the local Ethics Committees of the University of Luebeck, Germany (AZ 11-044).

Neutrophil isolation and culture

Venous blood samples were obtained by cubital venipuncture. All blood samples were stored at room temperature and neutrophil isolation was performed within 1 h of collection. Preparation of the blood samples was performed immediately. Buffy coat was used from non-CF donors and whole blood from CF patients. Both were treated with lithium–heparin and all following steps performed identically. Granulocytes were isolated by density gradient centrifugation using Percoll® (Sigma-Aldrich, Steinheim, Germany), at 400 g for 30 min at 8°C using a centrifuge (Eppendorf AG, Hamburg, Germany). The Percoll solution contained phosphate-buffered saline (PBS), sterile aqua bidest and HCl (hydrochloric acid), and resulted in a final density of 1·082 g/cm^3 at a pH of 7·4. Centrifugation was performed with 400 g for 30 min at 8°C (centrifuge 5810R; Eppendorf AG, Hamburg, Germany). Erythrocytes were lysed with sterile H_2O for 45 s using 10 × PBS to stop lysis, before being centrifuged at 300 g for 10 min at 8°C. The final cell-pellet was diluted in culture medium with RPMI-1640 (Biochrom AG, Berlin, Germany) and 0·5% bovine serum albumin (BSA; Sigma-Aldrich, Munich, Germany).

Sample quality was assessed by microscopic examination (EVOS fl; AMG, Mill Creek, WA, USA) using propidium iodide to determine cell viability, and CD62L (PN IM1231U; Beckman Coulter, Krefeld, Germany) stain inclusion to determine degree of neutrophil activation. Sample purity was determined by the counting the number of autofluorescent eosinophilic granulocytes and non-fluorescent neutrophilic granulocytes (neutrophil samples with > 95% purity were used).

Premature cell activation was excluded by labelling with CD62L antibody (PN IM1231U; Beckman Coulter). CD62L-negative staining was considered as an indicator of premature activation and cell fraction was not included for further examination.

Cell culture was performed in 96-well U-bottomed culture plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) with a cell concentration of 4×10^6/well. Neutrophils (4×10^6 cells/well) were preincubated for 1 h with either 100 μg/ml azithromycin (PZ0007-5MG; Geyer, Hamburg, Germany), 10^{-6} M salbutamol (Salbutamol-ratiopharm® 5 mg; Ulm, Germany), 10^{-6} M prednisolone (Prednisolut® 10 mg L, methylprednisolone 21-succinate sodium salt; mibe GmbH, Brehna, Germany) or 0·04, 0·4 or 4 μg/ml rhDNase (Pulmozyme®; Roche Pharma AG, Grenzach-Wyhlen, Germany) for 1 h at 37°C and 5% CO_2

followed by a 3-h stimulation was performed with 10 nM phorphol-12-myristate-13-acetate (PMA). Neutrophils were then pelleted at 500 g for 15 min at 8°C and supernatants harvested for enzyme activity measurement.

Enzyme activity assay

CHIT-1 enzyme activity was measured in the cell-free culture supernatants of preincubated and stimulated neutrophils using the fluorescent substrate 4-methyl umbelliferyl β-d-N,N',N"-triacetylchitotrioside hydrate (M5639; Sigma-Aldrich) 0·196 mg/ml in a buffer solution [0·2 M disodium hydrogen phosphate (Na_2HPO_4); 0·1 M citric acid] pH 4·4. Neutrophil supernatant was diluted 1 : 10 with substrate solution and added to the wells of a flat-bottomed 96-well plate (Nunc^{TM}; Nunc, Roskilde, Denmark) for 30 min (37°C, 5% CO_2). Activity was measured using a top-optic enzyme-linked immunosorbent assay (ELISA) reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany) at a wavelength of 360 nm, reference wavelength 450 nm. Enzyme activities were calculated as nmol/ml/h.

NETosis screening assay

Isolated neutrophils were adjusted to a concentration of 1×10^6/ml in medium (0·5% BSA in RPMI) and a standard curve was prepared in 5-plets for each concentration. Final concentrations (multiplied by 10^3) were 75, 50, 25, 12·5 and 6·25 neutrophils per well (sterile 96-well plate, clear bottom, black rims; Corning, Sigma-Aldrich, Germany). For stimulation each well contained 35 000 neutrophils in 100 µl 0·5% BSA/RPMI-1640 (Biochrom AG, Berlin, Germany) and 10 nM PMA [in dimethylsulphoxide (DMSO) vehicle] was used for stimulation. Neutrophils were preincubated with medications for 1 h at 37°C, 5% CO_2 before 10 nM PMA was added, followed by a final incubation for 2·5 h under conditions mentioned previously. The 96-well plate was then centrifuged for 5 min at 500 g 2 µg/ml propidium iodide was added to each well (incubated for 15 min) and the plate was measured (ex544 nm, em 590nm) with spectrometer (FLUOStar Omega; BMG). The standard curve was then used to convert the fluorescence signal to NETs numbers. All measurements were performed as replicates of 5.

A. fumigatus killing assay

A. fumigatus was obtained from the clinical microbiology group at the University Hospital in Luebeck. *A. fumigatus* was grown until sporulation was visible and plates were flushed with Ringer solution. Recovered conidia were counted and adjusted to 50 000 per 100 µl in 0·5% BSA/RPMI-1640 (Biochrom AG) with 1 mM HEPES. Prior to killing, conidia were given 6 h to grow and form hyphae. Residual supernatant was removed and with it any non-attached conidia/hyphae. Isolated neutrophils (5×10^5 cells/ml in to 0·5% BSA/RPMI1-640) were added to the *A. fumigatus*-coated wells in the presence of absence of rhDNase and incubated for 3 h at 37°C. Wells were then treated with rhDNase to remove any NETs and recover all *A. fumigatus*, and mixed vigorously with a pipette. Fifty microlitres of well supernatant was then plated onto an MAE-agar plate and examined for fungal growth after 24 h. To calculate fungal recovery/outgrowth, plates were photographed (Lumix Panasonic digital camera) and images analysed using ImageJ (available from http://imagej.nih.gov/ij/). Images were converted into an 8-bit grey-scale image and an ellipsoid background reduction step was applied. A black/white threshold adjustment was then used to identify all the areas overgrown by *A. fumigatus* and the percentage of total plate area was calculated. The effect of neutrophils on fungal growth was compared and corrected to *A. fumigatus* plates, which had no neutrophils added ('medium alone control').

Statistical analysis

Calculations were performed by one-way analysis of variance (ANOVA) using the Kruskal–Wallis test with Dunn's post-test or as indicated. Raw data were used for statistical analysis. The level of statistically significant difference was defined as $P < 0{\cdot}05$.

Results

CHIT-1 activity upon stimulation and identification of non-secretors

First, we were interested whether to determine or not neutrophils secreted CHIT-1 upon stimulation with IL-8, GM-CSF, opsonized zymosan and PMA, therefore we measured CHIT-1 activity in healthy controls. At the highest concentration, GM-CSF (100 ng/ml) opsonized zymosan and PMA all increased CHIT-1 activity compared significantly with unstimulated neutrophils (Fig. 1). However, neither IL-8 nor the lower concentrations of GM-CSF (1 ng/ml; 10 ng/ml) induced increased CHIT-1 activity from neutrophils compared to media alone. PMA induced the greatest increase in CHIT-1 activity and was thus chosen as the neutrophil stimulus for the remainder of this study.

Prior to the subsequent analysis, all samples were investigated regarding their CHIT-1 secretion to identify possible non-secretors. To assign a sample to the non-secreting group, the following criteria had to be fulfilled: low basal CHIT-1 activity and inability of PMA stimulation to increase CHIT-1 activity by at least 15% increase compared to unstimulated neutrophils. We identified five non-secretors (two females); median age = 33·0 years (± 10·68), a rate of 12·5% in our non-CF group. Smoking was reported with 40% in both groups. Body mass index (BMI) was slightly different, although insignificant, with 24·9 (± 3·39) kg/m^2 for non-secretors and 27·89

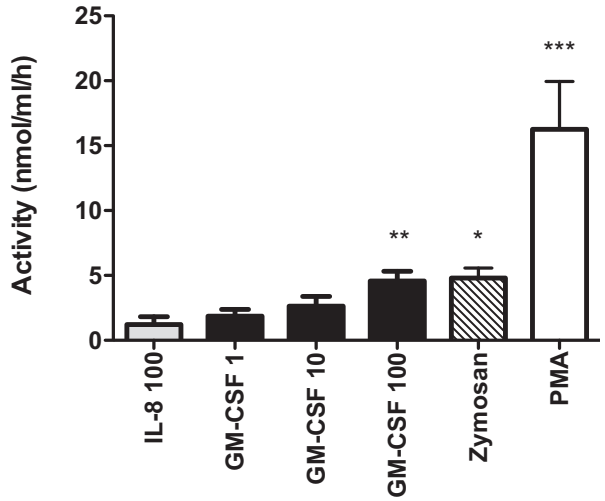

Fig. 1. Chitotriosidase (CHIT)-1 is secreted after granulocye–macrophage colony-stimulating factor (GM-CSF) and zymosan, but not interkeukin (IL)-8 stimulation. Neutrophils from non-cystic fibrosis (CF) donors were seeded at 4×10^6 cells/well in round-bottomed wells (4×10^6 per well) and stimulated with IL-8 (100 ng/ml), GM-CSF (1, 10, 100 ng/ml), opsonized zymosan, or 10 nM phorbol myristate acetate (PMA) for 2·5h, 37°C. Activity of CHIT-1 following stimulation was measured using 4-methylumbelliferyl-beta-D-N,N',N"-triacetylchitotriose (4-MU-TACT) in a Fluorstar Omega (BMG Labtech, Ortenberg, Germany) and controlled for basal activity in unstimulated wells; $n = 6$ $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

($\pm$ 1·79) kg/m^2 for secretors. All non-secretors were omitted from further analysis.

Comparable CHIT-1 activity in non-CF donors and CF patients

PMA-induced secretory activity of CHIT-1 was measured in CF and non-CF donors and compared to unstimulated ('media') and PMA vehicle (DMSO)-induced CHIT-1 activity levels in neutrophils from these two groups. PMA stimulation led to a significant, ~fourfold increase in CHIT-1 activity in the supernatant of non-CF donors (Fig. 2a); 22·92 nmol/ml/h) compared with the vehicle control (6·08 nmol/ml/h; $P < 0{\cdot}001$). Similarly, PMA stimulated an approximately fivefold increase in CHIT-1 activity in CF neutrophil cultures (Fig. 2b; 22·69 nmol/ml/h) compared with the vehicle control (4·53 nmol/ml/h; $P < 0{\cdot}001$).

Influence of drugs on CHIT-1 activity

Next, we elucidated our hypothesis that specific medications may alter the neutrophils' ability to secrete CHIT-1. To do so, CHIT-1 activity was measured from the supernatants of non-CF and CF neutrophils pretreated with PMA (as above), and incubated for 1 h with common CF treatments: $127{\cdot}4 \times 10^{-6}$ M azithromycin, 10^{-6} M salbutamol, 10^{-6} M prednisolone or $0{\cdot}108 \times 10^{-6}$ M rhDNase. Drug concentrations were based on approximated sputum or plasma physiological levels in patients undergoing treatment with these drugs [29–31,48]. We observed no significant change in CHIT-1 activity in the supernatants of PMA pretreated neutrophils after incubation with azithromycin (Supporting information, Fig. S1a), salbutamol (Supporting information, Fig. S1b) or prednisolone (Supporting information, Fig. S1c) compared with their respective vehicle controls. This was the case in both CF patients and non-CF donors. However, rhDNase induced a significant increase of CHIT-1 activity in PMA-stimulated neutrophils ($45{\cdot}37 \pm 11{\cdot}22$) compared to controls in non-CF donors (Fig. 3; $16{\cdot}50 \pm 4{\cdot}11$; $P < 0{\cdot}001$). We also observed a slight increase of CHIT-1 activity in CF donors in rhDNase-treated, PMA-stimulated neutrophils ($29{\cdot}31 \pm 5{\cdot}49$), although this increase was not statistically significant compared with the vehicle controls ($17{\cdot}99 \pm 8{\cdot}67$; $P = 0{\cdot}147$).

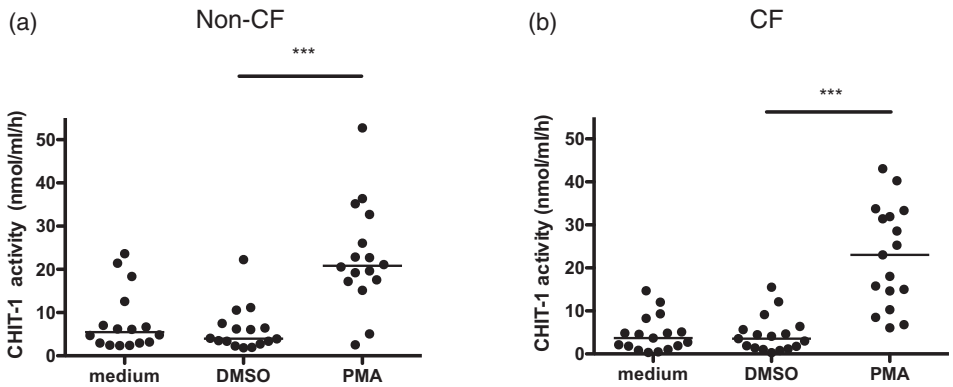

Fig. 2. Chitotriosidase (CHIT)-1 secretion in response to phorbol myristate acetate (PMA) is comparable in cystic fibrosis (CF) and non-CF donors. Neutrophils from (a) non-CF donors and (b) CF patients were seeded in round-bottomed wells (4×10^6 cells/well) and stimulated with 10 nM PMA for 2·5 h, 37°C. Activity of CHIT-1 was measured using 4-methylumbelliferyl-beta-D-N,N',N"-triacetylchitotriose (4-MU-TACT) in a Fluorstar Omega (BMG Labtech, Ortenberg, Germany); $n = 16$, $^{***}P < 0{\cdot}001$.

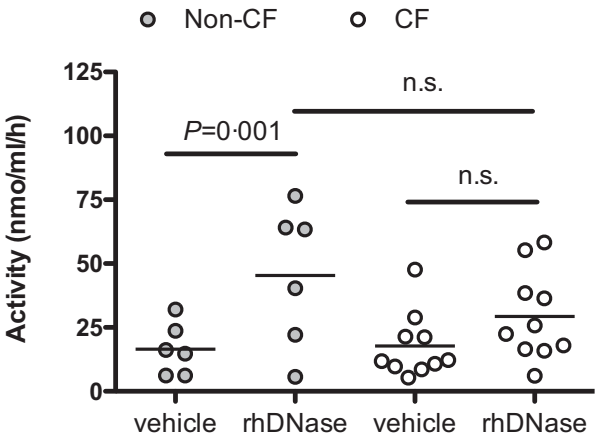

Fig. 3. Chitotriosidase (CHIT)-1 secretion increases significantly in non-cystic fibrosis (CF) donors in response to rhDNase and phorbol myristate acetate (PMA) but not in CF patients. Neutrophils (4×10^6 cells/well) from non-CF donors ($n = 6$) or CF patients ($n = 10$) were stimulated with 10 nM PMA for 2·5h at 37°C in the presence of 0.11M rhDNase or vehicle (milliQ water). Activity of CHIT-1 in supernatants was measured using 4-methylumbelliferyl-beta-D-N,N',N"-triacetylchitotriose (4-MU-TACT) in a Fluorstar Omega (BMG Labtech, Ortenberg, Germany); $^*P < 0{\cdot}05$.

We then tested rhDNase at a range of concentrations ($0{\cdot}11 \times 10^{-6}$ M, $0{\cdot}11 \times 10^{-7}$ M and $0{\cdot}11 \times 10^{-8}$ M) in a subsample of non-CF donor, PMA-pretreated neutrophils. Here we observed that the increase of CHIT-1 activity was independent from the rhDNase concentration used, with rhDNase pretreatment inducing significantly greater CHIT-1 activity at $0{\cdot}11 \times 10^{-8}$ M (Fig. 4; $61{\cdot}33 \pm 10{\cdot}50$ compared with vehicle control, $25{\cdot}08 \pm 3{\cdot}19$; $P < 0{\cdot}05$), $0{\cdot}11 \times 10^{-7}$ M ($59{\cdot}15 \pm 10{\cdot}31$ compared with vehicle control, $35{\cdot}80 \pm 7{\cdot}70$; $P < 0{\cdot}05$) and $0{\cdot}11 \times 10^{-6}$ M ($52{\cdot}15 \pm 10.46$ compared with vehicle control, $25{\cdot}11 \pm 5.38$; $P < 0{\cdot}05$).

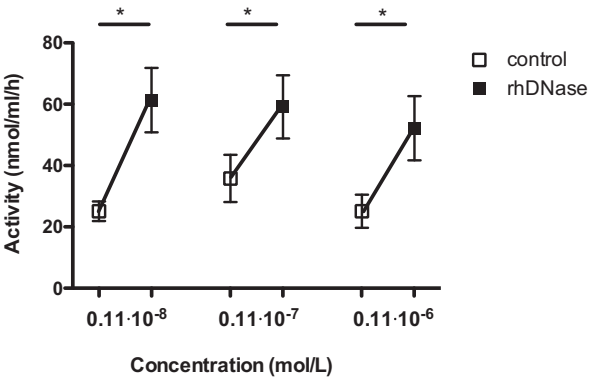

Fig. 4. Chitotriosidase (CHIT)-1 increase in activity is not dose-related to rhDNase *in vitro*. Non-cystic fibrosis (CF) neutrophils ($4{\times}10^6$ cells/well) were stimulated with 10 M phorbol myristate acetate (PMA) for 3 h at 37°C in the presence or absence of $0{\cdot}11{\times}10^{-6}$ M, $0{\cdot}11 \times 10^{-7}$ M or $0{\cdot}11 \times 10^{-8}$ M rhDNase or vehicle (Ringer solution), and CHIT-1 activity was measured using 4-methylumbelliferyl-beta-D-N,N',N"-triacetylchitotriose (4-MU-TACT) in a Fluorstar Omega (BMG Labtech, Ortenberg, Germany); $n = 5$, $^*P < 0{\cdot}05$.

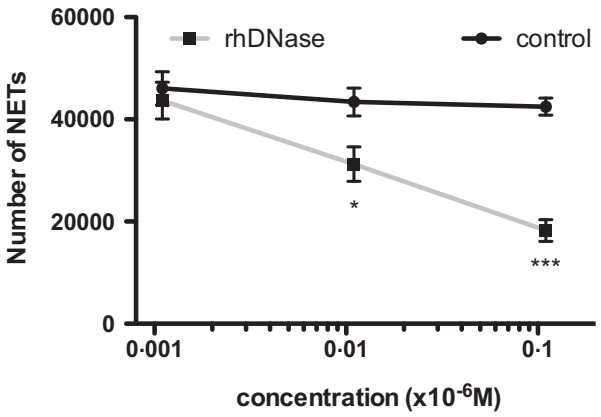

Fig. 5. Number of neutrophil extracellular traps (NETs) is reduced in response to rhDNase. Neutrophils from non-cystic fibrosis (CF) donors (5×10^4 cells/well) were stimulated with 10nM phorbol myristate acetate (PMA) and increasing concentrations (0·04, 0·4 and 4 μg/ml) of rhDNase or vehicle (0·1, 1 and 10% Ringer solution) were added for 2.5h at 37°C. Propidium iodide was added and fluorescence measured at ex544nm/em590 nm using a FluoStar Omega plate reader (BMG Labtech, Ortenberg, Germany). Standard curves were used to determine NET-numbers; $n = 11$; $^*P < 0{\cdot}05$, $^{***}P < 0{\cdot}001$.

rhDNase reduces NETosis and neutrophil killing of *A. fumigatus* conidia

Next we looked at additional effects of rhDNase on anti-fungal defence mechanisms such as NETosis. Number of NETs formed by PMA-treated, non-CF donor neutrophils were counted following treatment with rhDNase or unstimulated in media alone (Ringer solution). We showed that rhDNase was able to suppress NET formation, and this was dependent upon rhDNase concentration (Fig. 5).

Next we incubated pregrown hyphae from *A. fumigatus* with unstimulated neutrophils with or without rhDNase. Adding neutrophils to the hyphae resulted in reduced growth of fungal colonies compared to untouched *A. fumigatus* plates (Fig. 6). However, addition of rhDNase *A. fumigatus* colony growth increased significantly back to that seen on untouched *A. fumigatus* plates. These results are in line with previously reported results that DNases limit anti-fungal neutrophil capacity via NETs breakdown [32,33].

Discussion

The primary goal of our study was to clarify the clinical findings of Jubin *et al.*, who observed a possible relationship between pharmacotherapy, i.e. rhDNase and azithromycin, and AC/ABPA in children with CF [28]. The innate anti-fungal defence depends highly upon chitinases, and

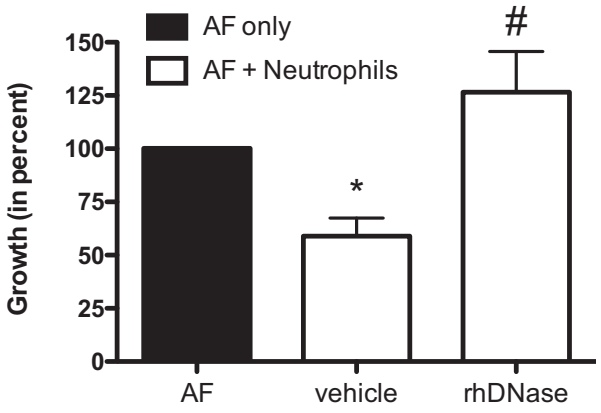

Fig. 6. RhDNase reduces neutrophil killing efficiency. *Aspergillus fumigatus* conidia (5×10^5/well) were left for 6 h at 37°C to form hyphae before non-cystic fibrosis (CF) neutrophils (1×10^6 cells/well) were added. Neutrophils were given 3 h at 37°C to form neutrophil extracellular traps (NETs) before the addition of rhDNase or vehicle control (10% Ringer solution). After 15 min, supernatant was collected and added to MAE-agar to examine *A. fumigatus* growth after 24 h at 37°C. *A. fumigatus* growth was determined as a percentage of growth in the absence of neutrophils; $n = 9$. *AF *versus* AF + neutrophils and vehicle, $P < 0{\cdot}05$; # AF + neutrophils + rhDNase, $P < 0{\cdot}05$.

CHIT-1 is the major functional chitinase in the lungs. This paper examined the significance of CHIT-1 in CF. Our study eliminated the hypothesis that CHIT-1 activity would be dysfunctional in CF, showing PMA-stimulated CF neutrophils releasing CHIT-1 with similar levels of activity to non-CF neutrophils. This paper proved that CHIT-1 activity could be induced sufficiently by rhDNase, but no other common CF drugs. RhDNase induced some neutrophil anti-fungal activities by increasing CHIT-1 activity, but also limiting the ability of neutrophils to form NETs needed to kill fungal colonies.

We showed that basal secretion and activity of CHIT-1 is not dysfunctional in CF. Basal secretion levels and resulting CHIT-1 activities *in vitro* are comparable between CF and non-CF patients after PMA stimulation. This suggests that the secretory mechanism via specific granule *per se* is not affected in CF patients with regard to CHIT-1. However, CHIT-1 levels in supernatants of neutrophils from CF patients (compared to non-CF donors) did not show a significant increase after incubation with rhDNase and stimulation with PMA, which could be considered as a possible explanation or at least a contribution to the association of rhDNase treatment and AC/ABPA.

The anti-fungal effect of rhDNase has been speculated in the past, and the findings from this paper support some potential limitations of this treatment option. While CHIT-1 activity is increased, the killing activity of neutrophils towards *Aspergillus* hyphae is reduced after addition of rhDNase. This confirmed previous reported results that DNases limit anti-fungal neutrophil capacity via NETs breakdown [15,33,34]. Reduction of viable, colony-forming hyphae has been attributed to the rich anti-microbial content of NETs [15,32,33]. Engulfment of fungal hyphae by NETs would increase the local concentration of anti-fungal enzymes, i.e. myeloperoxidase and calprotectin, leading to damage of the chitin skeleton of *A. fumigatus* and limiting or stalling its growth rate [32,33]. Recently, however, a group of researchers has found that NETs may, in fact, only entrap the microbes, rather than killing them [35], raising questions concerning the role of NETs as an anti-fungal defence mechanism. Another study examined the innate anti-fungal response by which human neutrophils recognize and kill *Aspergillus* directly [4]. They observed that neutrophil recognition of conidia in an early period involves integrin CD11b/CD18, which triggers a PI3K-dependent non-oxidative intracellular mechanism of killing. The extracellular destruction of the *Aspergillus* hyphae needs opsonization by antibodies and predominantly involves recognition via Fcγ receptors, signalling via Syk, PI3K and protein kinase C to trigger the production of toxic reactive oxygen metabolites by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase. *A. fumigatus* induces NET formation; however, NETs *per se* did not contribute to *A. fumigatus* killing.

The inhibitory effect of rhDNase on neutrophil NET formation and consequential fungal killing ability raises questions of the potential detrimental effect of this treatment to CF patients. Treatment with rhDNase has not been associated with invasive pulmonary aspergillosis in CF. Our experiments may suggest an intrinsic and therapeutic induced deficiency of CF neutrophils to handle larger fungal agglomerations. However, clinical consequences, such as an invasive pulmonary aspergillosis, are possibly counteracted by several non-specific anti-fungal mechanisms (including reactive oxygen species) and large numbers of recruited neutrophils in the lung in CF.

Interestingly, Marcos and colleagues showed recently that DNA levels in airways correlate with pulmonary obstruction in CF patients and mice [36]. They speculated that in milder stages of CF lung disease, NET formation may act beneficially in providing extracellular anti-bacterial and anti-fungal host defence, while at severe stages of CF the amount of mucus and DNA accumulation causes airway obstruction. In accordance with this hypothesis, the authors differentiated their recommendations for rhDNase therapy in CF patients, suggesting cautious use of rhDNase in the early stage of CF, as bacteria or fungi not captured by NETs might be freed. Conversely, in severe stages, the effect of rhDNase in cleaving DNA traps might outweigh the anti-microbial actions of NET formation.

The observed reduction of CHIT-1 activity in stimulated leucocytes of CF donors might not explain sufficiently the clinical observation of Jubin *et al.* An alternative hypothesis for the findings of Jubin *et al.* is that CHIT-1 is less or even

not secreted by leucocytes in the CF patients included in their study population. We identified five non-CF donors, but no CF patients, to be CHIT-1 non-secretors, who were excluded from the subsequent analyses. This equals a percentage of 12·5%, twice as high as reported in the literature [13]. This might be explained primarily by the fact that our study cohort is small and therefore not representative of the general population. Moreover, it is of note that the individuals in both groups showed a broad distribution of CHIT-1 activity. This might suggest that some individuals may produce or secrete lower levels of CHIT-1. It is tentative to speculate that this may lead to a slightly higher predisposition for fungal infections *in vivo*, which has also been reported for several other human enzymes [11,13,14]. Single nucleotide polymorphisms or a described 24 base pairs (bp) insertion in the gene encoding for CHIT-1 and influencing secretion or activity is one possible explanation for our findings; however, this hypothesis must be proved in a prospective clinical trial in large CF cohorts [37].

It was interesting to see that the other commonly used CF treatments had little effect on CHIT-1 activity, with macrolide antibiotics, beta-mimetics and corticosteroids failing to increase CHIT-1 activity significantly to greater than baseline, because these mediations possess anti-inflammatory properties [38]. In contrast to our findings, other groups have reported changes to the anti-microbial activity of neutrophils in response to corticosteroids; however, it is important to note that most of these studies utilized drug at concentrations much higher than that found *in vivo* in patients receiving these treatments [39–41]. Insensitivity of neutrophils to salbutamol for other parameters (chemotaxis, viability and apoptosis) has been shown by Perkins *et al.* [42]. It may be argued that peripheral granulocytes differ from alveolar neutrophils; however, this can only be clarified in additional experiments, which was outside the scope of this study. Interestingly, prophylactic antibiotic therapy has been associated with an increased prevalence of AC in adult CF patients [43]. Low-dose long-term therapy with the macrolide azithromycin was associated in particular with AC in children [28]. Macrolide antibiotics have been described to accumulate intracellular in polymorphonuclear cells (including neutrophils) [44]. This may lead to an inhibition of the production of proinflammatory cytokines, e.g. IL-1, IL-8 and TNF-α, as well as the formation of leukotriene B4 [45,46]. It is also reported that macrolides can lead to degranulation of azurophilic granules from neutrophils; however, CHIT-1 is found solely in the so-called 'specific' granules [10,47].

It is important to acknowledge that our study has a number of limitations. The use of peripheral blood neutrophils is convenient, although we acknowledge that these differ from pulmonary neutrophils in their degree of deformability, cell adhesion molecule expression and activation and release of soluble mediators [25,35]. Peripheral neutrophils might react differently to stimuli. It is ethically challenging to obtain neutrophils from lavages from CF patients and it stands to question if these neutrophils are of adequate quality, as they have already been in contact with potential pathogens and stimuli. Moreover, our non-CF controls were older compared to the CF patients, which might be a source of bias of our results. Another limitation refers to the translational ability of this research to the clinic setting. Many CF patients are prescribed combination therapy for effective maintenance of their condition. It would be valuable for future research to investigate the effect of drug combinations.

Finally, we were not able to present a comprehensive explanation for our detected connection of decreased CHIT-1 release in CF patients. As we observed no difference in CHIT-1 activity in PMA-stimulated leucocytes in CF and non-CF leucocytes, we speculate that NETs might interact with and bind CHIT-1 similarly to other previously reported enzymes calprotectin and myeloperoxidase [32,33]. Concentrated CHIT-1 activity in NETs close to the fungal hyphae target would facilitate damage to the fungus cell wall and enhance its destruction. To confirm this hypothesis, we stimulated neutrophils with PMA and removed excessive supernatant and cellular debris. After degradation of the deposited NETs with rhDNase, we observed that a significant amount of endo-chitinase activity was retained (Supporting information, Fig. S2).

If CF neutrophils have impaired CHIT-1 activity and CHIT-1 is associated partially with NETs, then it is crucial to elucidate if CF neutrophils have impaired NET formation. Unfortunately, we do not have any information in favour of such a mechanism in our population.

In summary, there are several indirect indications that rhDNase might interact with anti-fungal defence mechanisms *in vitro*, i.e. changes in CHIT-1 activity and NET formations.

It is evident that CF neutrophils did not exhibit dysfunctional CHIT-1 secretion, eliminating intrinsic CHIT-1 secretion defects as a main cause for fungal colonization or ABPA. However, the lack of a significant increase of CHIT-1 activity after rhDNase in CF patients, combined with the reduced fungal killing efficiency, provides vital clues of a possible mechanism for an increased risk of fungal colonization or ABPA after rhDNase treatment. This study may help to facilitate investigations and prospective clinical trials to tailor therapeutic strategies to combat fungal infection and neutrophil inflammation in chronic lung diseases such as CF.

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Author contributions

M. W. conceived, performed and analysed experiments. C. S. recruited CF blood donors, performed and analysed experiments. A. H. performed experiments. I. B. recruited CF blood donors. J. R. and M. W. designed the *A. fumigatus* experiments. M. K. designed and analysed experiments. M. W., M. K. and C. S. wrote the manuscript.

Disclosure

The authors confirm that there are no disclosures to declare.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. No impact of standard medications on chitotriosidase (CHIT)-1 activity from neutrophils in cystic fibrosis (CF) and non-CF donors CHIT-1 secretion in response to standard medication and phorbol myristate acetate (PMA) is not different in non-CF donors compared to CF patients. Neutrophils (4×10^6 per well) were stimulated with 10 nM PMA for 3 h at 37°C in the presence or absence of 100 μg/ml azithromycin [vehicle = dimethylsulphoxide (DMSO)], 10^{-6} M salbutamol (vehicle = milliQ water) or 10^{-6} M prednisolone (vehicle = DMSO); activity of CHIT-1 in supernatant was measured using 4-methylumbelliferyl-beta-D-N,N',N"-triacetylchitotriose (4-MU-TACT) in a Fluorstar Omega (BMG Labtech, Ortenberg, Germany).

Fig. S2. Endochitinase activity is retained in neutrophil extracellular traps (NETs) endochitinase activity is rhDNase-sensitive. Neutrophils were isolated and 50 000 cells were seeded per flat-bottomed well. Cells were then stimulated for 3 h at 37°C with 10 nM phorbol myristate acetate (PMA) (control: dimethylsulphoxide). Supernatant was removed and wells washed extensively to remove remaining traces of secreted CHIT-1. RhDNase was added to remnants (NETs) for 15 min. Recovered digest was then analysed using 4-methylumbelliferyl-beta-D-N,N',N"-triacetylchitotriose (4-MU-TACT) substrate and measured in FlouStar Omega plate reader (ex365 nm, em460 nm); $n = 3$; $^{**}P < 0{\cdot}01$.