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Original Article

Effect of *Aspergillus fumigatus* and *Candida albicans* on pro-inflammatory response in cystic fibrosis epithelium

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Abstract

Background: The identification of filamentous fungi and/or yeasts in the airway secretions of individuals with cystic fibrosis (CF) is becoming increasingly prevalent; yet the importance of these organisms in relation to underlying inflammation is poorly defined.
Methods: Cystic fibrosis bronchial epithelial cells (CFBE) and human bronchial epithelial cells (HBE) were co-incubated with *Candida albicans* whole cells or *Aspergillus fumigatus* conidia for 24 h prior to the measurement of pro-inflammatory cytokines IL-6 and IL-8 by ELISA.
Results: Treatment of HBE or CFBE with *C. albicans* whole cells did not alter cytokine secretion. However treatment of CFBE with *A. fumigatus* conidia resulted in a 1.45-fold increase in IL-6 and a 1.65-fold increase in IL-8 secretion in comparison to basal levels; in contrast there was far less secretion from HBE cells.
Conclusion: Our data indicate that *A. fumigatus* infection modulates a pro-inflammatory response in CF epithelial cells while *C. albicans* does not.

Keywords: Fungi; Epithelial cells; Inflammation

1. Introduction

Individuals with CF are susceptible to infection with a range of opportunistic pathogens identified in sputum culture in a time-ordered pattern [1]. Bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Burkholderia cepacia* complex and *Stenotrophomonas maltophilia* are typically associated with recurrent exacerbations in pulmonary disease underlying the irreversible airway damage and ultimate morbidity and mortality in CF [1]. In addition to these bacterial pathogens the sputum culture frequently isolates yeasts and/or filamentous fungi. *Aspergillus fumigatus*, a saprophytic fungal pathogen found worldwide has a very high sporulating capacity resulting in the ubiquitous presence of airborne conidia small enough in diameter (2–3 μM) to penetrate deeply into the airway [2]. Elimination of conidia by the innate immune response means healthy humans rarely have any adverse effects. However in immunocompromised patients *A. fumigatus* is of growing significance in relation to allergic bronchopulmonary aspergillosis (ABPA), chronic colonisation/infection (bronchitis) and post-transplant fungaemia. In terms of colonisation of the CF airways *A. fumigatus* is the most common filamentous fungal agent identified [3] and is positively associated with hospital admissions [4]. The frequency of *A. fumigatus* recovery from individuals with CF has been reported anywhere between 25 and 60% [5–8]. *Candida albicans,* ubiquitous commensal dimorphic yeast, is the most common fungal pathogen of humans and accounts for more than 50% of all fungal systemic infections [9] and is frequently isolated from the sputum of CF patients [10].

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Previous studies using non-CF derived epithelial cells have demonstrated *A. fumigatus* or *C. albicans* infection can elicit a pro-inflammatory response; implicated in the development of protective immunity against systemic infections [11–14]. However, despite the increasing prevalence of fungal identification in the respiratory secretions from CF patients the effect of fungal infection on the pro-inflammatory response in a CF setting is poorly defined. The aim of the present study was to investigate the hypothesis that CF epithelial cells will have an exaggerated pro-inflammatory response to stimulation with yeast (*C. albicans*) or filamentous fungi (*A. fumigatus*) commonly isolated from the airways of patients with CF.

2. Methods

2.1. Fungal isolates

The present study utilised wildtype isolates of *C. albicans* and *A. fumigatus* from adult CF patients and archived at the CF fungal repository at the Northern Ireland Public Health Laboratory. The *C. albicans* and *A. fumigatus* isolates were from a female F508del/F508del and a male F508del/F508del patient respectively. The identification of each isolate was confirmed through sequencing of the internal transcribed spacer (ITS) 1 region of the rRNA gene operon [15].

2.2. Generation of fungal preparations

C. albicans was cultured in Nagano's media (without selective substances) [16] for 6 h at 37 °C (corresponding to the early exponential phase of growth) prior to irradiation with 25 kGy ^{60}Co to ensure microbiological sterility. Whole yeast cells were isolated by centrifugation at 2000×*g* for 10 min at 4 °C, washed and resuspended in sterile PBS prior to enumeration with a haemocytometer. *A. fumigatus* was grown on Sabouraud's dextrose agar supplemented with chloramphenicol (Oxoid CM0041, Oxoid Ltd., Basingstoke) for 5 days at 37 °C. Conidia were then harvested from the plate by gentle scraping into a PBS/0.025% v/v Tween solution. The solution was filtered through a BD Falcon cell strainer (40 μM pore) to ensure a pure conidial preparation without hyphal contamination. Conidia were irradiated, washed and enumerated as described for the *C. albicans* preparation.

2.3. Epithelial cell culture

A human bronchial epithelial cell line (16HBE41o-) and a delta F508 homozygote bronchial epithelial cell line (CFBE410-) were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. All flasks and plates used for growing cells were pre-coated with a solution comprising LHC media (Invitrogen) (98%), fetal bovine serum (PAA laboratories) (1%) and PureCol collagen type I (Nutacon) (1%). Cells were maintained in Eagle's minimum essential medium (PAA laboratories) supplemented with 10% heat inactivated fetal bovine serum (PAA laboratories), 2 mM glutamine (PAA laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

2.4. THP1 cell culture

THP-1 cells (human acute monocytic leukaemia cell line) were maintained in RPMI 1640 medium (PAA laboratories) supplemented with 10% heat inactivated FBS (PAA laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.5. Simulation experiments

HBE and CFBE (8×10^4 cells in 0.5 ml) and THP-1 cells (2.5×10^5 cells in 0.25 ml) were seeded in 24-well plates prior to incubation in the presence or absence of various doses of fungal preparations. *A. fumigatus* conidia or *C. albicans* whole cells were added to epithelial or THP-1 cells at various ratios based on the cell seeding density. Cell culture supernatants were collected after 24 h then centrifuged at 1500×*g* for 10 min to remove any cell debris. Samples were stored at −70 °C until assayed.

2.6. ELISA

IL-6 and IL-8 levels were determined by ELISA kits supplied by R&D systems. Assays were performed in accordance with the manufacturer's instructions. For all ELISA assays a number of additional controls were included throughout the study. PBS (vehicle control) added to epithelial or THP-1 cells did not alter IL-6 or IL-8 secretion compared with basal (data not shown). Cell culture media, *A. fumigatus* conidia or *C. albicans* whole cells alone did not cause the release of any measurable level of IL-6 or IL-8 (<10 pg/ml) (data not shown). A positive control (denoted as cytomix) comprising 10 ng/ml IL-1β, 10 ng/ml TNFα and 1000 U/ml IFN-γ was also utilised throughout the study (data not shown).

2.7. Cytotoxicity assays

To ensure that all treatments utilised throughout this study were performed at non-toxic doses cytotoxicity was assessed by release of lactate dehydrogenase (LDH) employing the LDH-Cytotoxicity Assay Kit II (Bio Vision, California, USA) performed as described by the manufacturer.

2.8. Statistical analysis

All values are expressed as the mean ± SE. Statistical analysis was performed using ANOVA. $p<0.05$ was considered significant. Data analysis was carried out using Excel.

3. Results

3.1. Effect of C. albicans treatment on cytokine release from HBE and CFBE

IL-6 or IL-8 levels were unaffected in HBE or CFBE after challenge with *C. albicans* whole cells over a range of doses

(Fig. 1A and B). Similar results were found when experiments were performed using the alternative sequenced *C. albicans* isolates SC5314 [17] and WO-1 [18] (data not shown). It has previously been shown that exposure of THP-1 cells to live *C. albicans* results in an increase in IL-8 secretion from these cells [19]. We therefore employed the THP-1 cell line as a positive control for our *C. albicans* preparations. In contrast to epithelial cell lines THP-1 cells exposed to *C. albicans* did result in an increase in IL-8 secretion compared with basal levels ($p<0.05$) (Fig. 1C).

3.2. Effect of *A. fumigatus* treatment on cytokine release from HBE and CFBE

A. fumigatus conidia modulated IL-6 and IL-8 secretion in a dose-dependent fashion when added to CFBE (Fig. 2A and B). Exposure of CFBE to the lower range of doses caused a dose-dependent increase in cytokine secretion that was maximal at 50:1 conidia to epithelial cell ratio (IL-6 2441±305 pg/ml, $p<0.05$ and IL-8 1482±117 pg/ml, $p<0.01$) in comparison to basal levels (IL-6 1684±169 pg/ml and IL-8 907±64 pg/ml) (Fig. 2A and B). In contrast treatment of CFBE with higher doses of *A. fumigatus* (250:1 conidia to epithelial cell ratio) reduced cytokine release to below basal levels (IL-6: 1040±157 pg/ml, $p<0.05$) (IL-8: 726±74, $p=0.088$) (Fig. 2A and B). *A. fumigatus* conidia did not have any effect on cytokine release from HBE at the lower range of doses (1:1–50:1 conidia to epithelial cell ratio), however high dose treatment (250:1 conidia: epithelial cell ratio) did tend to reduce cytokine levels (IL-6: 751±108 pg/ml $p<0.05$, IL-8: 588±94 pg/ml $p=0.063$) in comparison to basal secretion (IL-6: 1013±97 pg/ml, IL-8: 878±102 pg/ml). The reduction in cytokine secretion seen at high-dose *A.* challenge is not attributable to a cytotoxic effect as LDH levels remained unaltered under these conditions (Fig. 2C). In experiments where THP1 cells were challenged with *A. fumigatus* conidia IL-8 secretion was unaffected (Fig. 1C).

4. Discussion

The central finding of our study is that *A. fumigatus* conidia stimulate a pro-inflammatory response in CF bronchial epithelial cells. Treatment of these cells with low doses of conidia increases cytokine secretion in a dose-dependent fashion (1–50:1 conidia to epithelial cell ratio); an effect that was not apparent in HBE cells (Fig. 2A and B). This finding is in agreement with the hypothesis that CFBE may be more pro-inflammatory in nature compared with non-CF epithelial cells upon fungal challenge.

In contrast incubation of HBE and CFBE with higher doses of *A. fumigatus* (>100:1 conidia to epithelial cell ratio) resulted in a significant reduction in IL-6 levels and a tendency where IL-8 levels were also diminished compared with spontaneous secretion (Fig. 2A and B). Although there is a paucity of data in this area there are a number of potential mechanisms whereby fungal pathogens can reduce cytokine secretion.

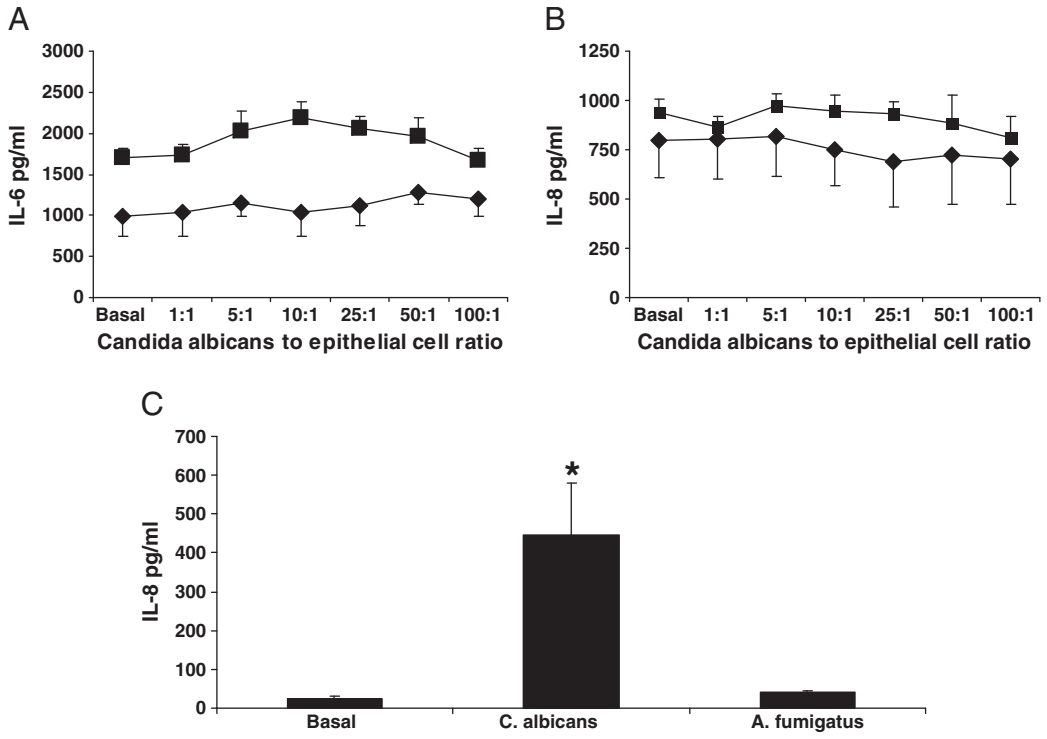

Fig. 1. Cytokine release from *Candida albicans*-treated HBE and CFBE. HBE (filled diamonds) and CFBE (filled squares) were treated with varying doses of *Candida albicans* whole cells as indicated for 24 h then IL-6 (A) and IL-8 (B) levels determined by ELISA (n=5). Treatment of cells with cytomix (positive control) gave the following values HBE IL-6 1695±305 pg/ml, IL-8 2013±117 pg/ml CFBE: IL-6 3050±322 pg/ml, IL-8 2297±236 pg/ml (data not shown) (C). THP-1 cells were treated with an equal ratio (1:1) of *Candida albicans* whole cells (n=3) or *Aspergillus fumigatus* conidia (n=3) for 24 h then IL-8 was assessed by ELISA. Treatment of THP-1 cells with cytomix gave the following: 1083±114.9 pg/ml. *$p<0.05$ compared with basal.

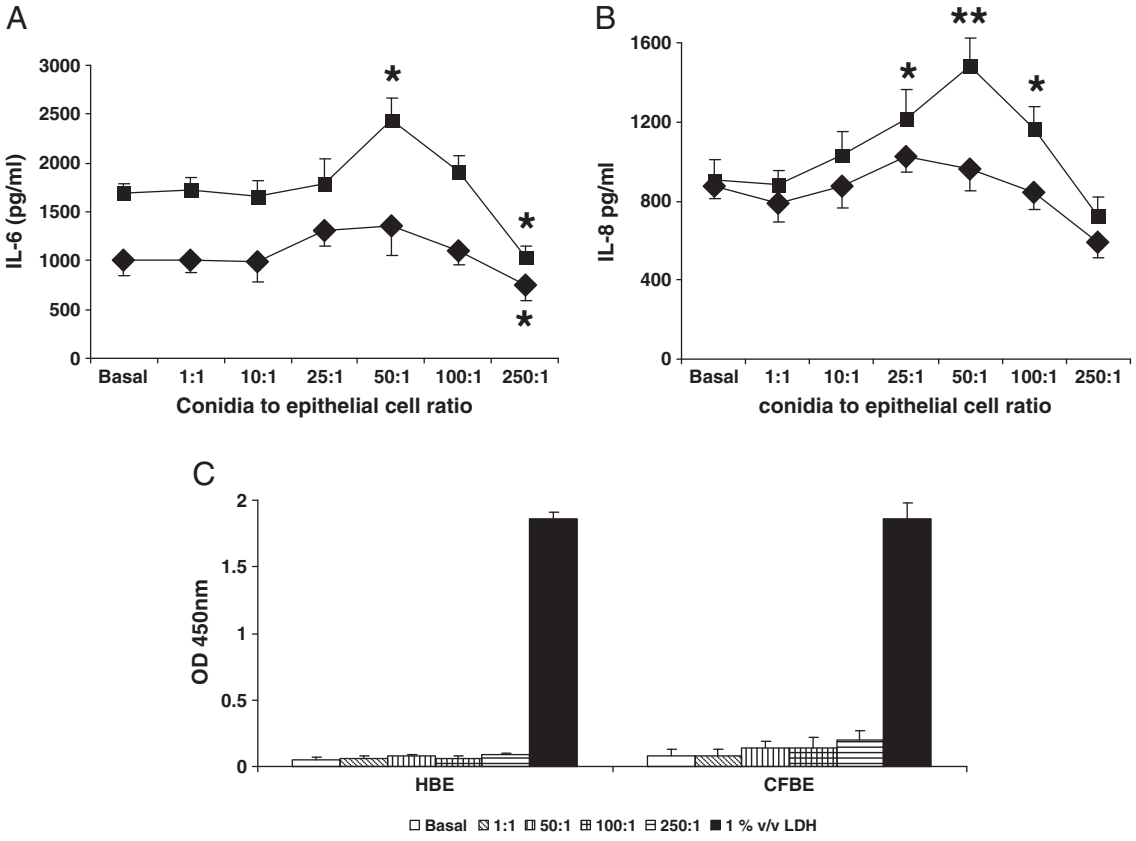

Fig. 2. Cytokine release from *Aspergillus fumigatus*-treated HBE and CFBE. HBE (filled diamonds) and CFBE (filled squares) were treated with varying doses of *Aspergillus fumigatus* conidia as indicated for 24 h then IL-6 (A) and IL-8 (B) levels determined by ELISA. Data shown in A and B is representative of at least 6 independent experiments. Treatment of cells with cytomix resulted in the following cytokine levels HBE IL-6 1795±236 pg/ml, IL-8 2022±116 pg/ml CFBE: IL-6 2983±335 pg/ml, IL-8 2320±310 pg/ml. (C) HBE or CFBE were treated with varying doses of *Aspergillus fumigatus* conidia then LDH levels were measured (n=3). A 1% v/v LDH solution in culture media served as a positive control. *$p<0.05$ **$p<0.01$ compared with basal.

Protease-protease activated receptor (PPAR) signalling pathways represent one mechanism whereby fungi can potentially dampen inflammation. Proteases from fungal extracts are known as potent inducers of epithelial cell desquamation and production of pro-inflammatory cytokines [20]. A report by Kauffman and colleagues has demonstrated that *A. fumigatus* protease extracts added to epithelial cells elicit a bell-shaped dose response for IL-6 and IL-8 release [21]. Cytokine production increased in a dose-dependent fashion (maximal at 10 μg/ml) which rapidly declined to below basal levels at higher concentrations (over 10 μg/ml) when added to A549 cells; with similar results found in primary nasal cells [21]. While these data are strikingly similar to our findings (Fig. 2A and B) the fact that our conidial preparations are inactivated by γ-irradiation diminishes the likelihood that PPAR signalling is responsible for our effects (though it is possible that some residual protease activity remains after γ-irradiation). It may be possible that the reduction in cytokine levels seen in our studies is mediated through changes in Toll-like receptor (TLR) expression. The expression of TLRs, which are pattern recognition receptors that recognise conserved molecular patterns, is important in the innate response against microbes in the epithelium. TLRs are known to be involved in the recognition of fungal pathogens including *A. fumigatus* and *C. albicans* generally leading to the production of cytokines, activation of leukocytes and subsequent resistance to infection. Fungi can however modulate TLR signalling pathways to induce an anti-inflammatory response. For example TLR4 signalling, regarded as important for the recognition of *A. fumigatus* conidia, is lost during germination of *A. fumigatus* conidia to hyphae [22]; whereas *C. albicans* infection has been demonstrated to promote a TLR2-mediated anti-inflammatory phenotype [23]. Although as in our studies the samples were irradiated thus not able to germinate, this exact mechanism cannot explain our findings.

It is tempting to speculate that in CF epithelial cells low doses of *A. fumigatus* conidia cause a normal inflammatory response; but above a certain threshold can modulate epithelial cell signalling pathways to dampen the pro-inflammatory response. This may represent a mechanism for *A. fumigatus* to escape host detection enabling airway colonisation.

Historically *C. albicans* has been regarded as a commensal organism of relatively low pathogenic potential compared with other bacterial pathogens and *A.* spp. [10]. Consistent with this notion, in our investigation *C. albicans* treatment did not alter cytokine release from HBE or CFBE suggesting that *C. albicans* has little pro-inflammatory effect on bronchial epithelial cells. However, a very recent paper has reported that *C. albicans* colonisation significantly predicts hospital-treated exacerbations and accelerates rates of decline for BMI and FEV1 [24]. The

underlying mechanisms for these effects remain unknown though they were best-predicted by pancreatic insufficiency, osteopenia and co-colonisation with *Pseudomonas* spp. [24].

Our findings that *C. albicans* stimulates IL-8 release from THP-1 cells and *A. fumigatus* conidia modulate IL-6 and IL-8 release from bronchial epithelial cells demonstrate that irradiation did not have a chronic effect on our preparations. The use of non-irradiated preparations may be of additional value as viability is a known virulence factor for *C. albicans* (and *A. fumigatus*) [13,14]. Thus it is feasible that live fungal preparations may evoke a more prominent response in bronchial epithelial cells.

Although initially not included in the scope of the present study some experiments were performed using THP-1 cell lines as a positive control for *C. albicans*-mediated IL-8 secretion. The effect of *C. albicans* challenge on IL-8 release from THP-1 cells highlights that fungi potentially exert effects on non-epithelial cells relevant to CF. Although *A. fumigatus* did not alter IL-8 levels in THP1 cells it has been shown to stimulate leukocyte adhesion molecules in endothelial cells during invasive pulmonary disease [25]. Additionally it should be noted that in our experiments only a 1:1 fungi (yeast cell or conidia) to THP-1 cell ratio was investigated; in contrast to the epithelial cell studies where a range of concentrations was studied. A more focused approach is required to better understand the role of fungal infection, in particular with relation to *A. fumigatus*, in this setting. Such work is of importance as yeast or filamentous fungi may modulate leukocytes (neutrophils) contributing to the amplified pro-inflammatory phenotype associated with CF.

Fungal pathogens in the CF lung must also be considered in the back-drop of polymicrobial infection. Bidirectional signalling between clinical isolates of *C. albicans* and *Pseudomonas aeruginosa* has previously been shown [26]. In addition to specific signalling between pathogens their presence in the same environment may be of significance. The formation of hyphae by *C. albicans* or *A. fumigatus* is important for the adherence of fungi to epithelium. Hyphae can penetrate epithelial tissue and as such may potentially provide unwanted access to other deleterious pathogens/molecules.

Overall we have demonstrated that *A. fumigatus* stimulates a pro-inflammatory response to a greater degree in CF bronchial epithelial cells compared with non-CF bronchial epithelial cells. *C. albicans* did not affect the pro-inflammatory response in our system. Additional investigation of the molecular mechanisms by which filamentous fungi and yeast modulate CF bronchial epithelial cells, and also their interaction with other cell types and pathogens present in the CF lung may provide novel insights into the merits and potential therapeutic targets for anti-fungal intervention.

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