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H-ficolin binds *Aspergillus fumigatus* leading to activation of the lectin complement pathway and modulation of lung epithelial immune responses.

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Running title: H-ficolin modulates immunity to *A. fumigatus*.

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Abbreviations: Invasive aspergillosis, IA; Mannose-binding lectin (MBL); Surfactant proteins, SP; Fibrinogen-like, FBG; *N*-acetylglucosamine, GlcNAc; MBL-associated serine protease, MASP; Bronchoalveolar lavage, BAL; High resolution computed tomography, HRCT; Galactomannan, GM; European Organization for Research and Treatment of Cancer/Invasive Fungal Infection Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group, EORTC/MSG; Ficolin-binding buffer, FBB; Fluorescein isothiocyanate, FITC; Room temperature, RT; *N*-acetylgalactosamine, GalNAc; *N*-acetylcysteine, CysNAc; Forward scatter, FSC; Side scatter, SSC; Cytometric bead array, CBA; Barbital buffered saline, BBS; p-nitrophenyl-phosphate, pNPP; Receiver operating characteristics, ROC; Area under the curve, AUC; Collectin-11, CL-11.

Summary

Aspergillus fumigatus is an opportunistic fungal pathogen that typically infects the lungs of immunocompromised patients leading to a high mortality. H-ficolin, an innate immune opsonin, is produced by type II alveolar epithelial cells and could participate in lung defences against infections.

Here, we utilised the human type II alveolar epithelial cell line, A549, to determine the involvement of H-ficolin in fungal defence. Additionally, we investigated the presence of H-ficolin in bronchoalveolar lavage fluid (BAL) from transplant patients during pneumonia.

H-ficolin exhibited demonstrable binding to *A. fumigatus* conidia via L-fucose, D-mannose and N-acetylglucosamine residues in a calcium- and pH-dependent manner. Moreover, recognition led to lectin complement pathway activation and enhanced fungal association with A549 cells. Following recognition, H-ficolin opsonization manifested an increase in IL-8 production from A549 cells which involved activation of the intracellular signalling pathways MEK 1/2, p38 MAPK and JNK. Finally, H-ficolin concentrations were significantly higher in BAL of patients with lung infections compared to control subjects ($n=16$; $p=0.00726$). ROC curve analysis further highlighted the potential of H-ficolin as a diagnostic marker for lung infection ($AUC=0.77$; $p<0.0001$).

Thus, H-ficolin participates in *A. fumigatus* defence via activation of the lectin complement pathway, enhanced fungal-host interactions and modulated immune responses.

Introduction

Aspergillus fumigatus (*A. fumigatus*) is the most common mould pathogen in the developed world and is the primary causative species of the potentially fatal, invasive aspergillosis (IA) in immunocompromised patients. IA is an increasing problem due to the emergence of a number of antifungal resistant strains and even if treated effectively, harbours an infection associated mortality rate between 30-80% [1, 2]. Transplant patients are at particular risk and it is estimated that up to 1 in 4 lung or heart transplant patients will succumb to this infection [3]. IA is propagated via the inhalation of small hydrophobic spores (conidia) which penetrate deep into the alveolar space where they germinate, invading the vasculature and disseminate to other organs such as the brain and skin [4, 5]. *A. fumigatus* can be found ubiquitously and is mostly omnipresent, making inhalation difficult to avoid. Fortunately, a functional innate immune system can provide robust and effective responses to aid the removal of spores. The initial defence to the inhalation of *A. fumigatus* is mucociliary clearance but if this is evaded then an innate immune response comprising of type II pneumocytes, alveolar macrophages, neutrophils and serum pathogen recognising opsonins, can work synergistically to potentiate conidia recognition and removal [6]. One such family of opsonins are coined ficolins.

Ficolins are recently discovered serum opsonins with functions comparable to the widely studied collectins, mannose-binding lectin (MBL) and the surfactant proteins (SP). They are composed of two key domains; an N-terminal collagen-like domain and a C-terminal fibrinogen-like (FBG) domain with lectin activity highly specific for the acetylated carbohydrate, *N*-acetylglucosamine (GlcNAc), a key component of the *A. fumigatus* cell wall [7]. Humans possess three types of ficolin; the membrane-bound M-ficolin and the serum types, L- and H-ficolin. Ficolins primarily function as opsonins whereby they can enhance the

phagocytosis of pathogenic microorganisms but they are also capable of activating the lectin complement pathway via association with MBL associated serine protease (MASP)-2 [8, 9]. Novel immunomodulatory functions are also beginning to arise but mechanistic insights into these are in their infant stages [10-13].

Of the serum ficolins, H-ficolin is undoubtedly the most poorly understood, with the fewest pathogenic targets and a distinct lack of characterisation in comparison to L-ficolin. We have recently indicated that the other serum ficolins (L-ficolin and its rodent orthologue, ficolin-A) are capable of enhancing host-pathogen interactions within the fungal airway immunity [10, 14]. Whereas L-ficolin is not produced in the lung, H-ficolin can be secreted directly by resident type II epithelial cells [15]. In addition, previous observations have indicated that H-ficolin could recognise *A. fumigatus* conidia but this interaction was not characterised in any detail [16].

Therefore, we investigated whether opsonization of *A. fumigatus* by H-ficolin could potentiate the functions of A549 cells, a cell line with characteristics of type II epithelial cells [17]. Additionally, we investigated the role of H-ficolin in lectin complement pathway activation, cytokine modulation and we measured the H-ficolin concentrations in the bronchoalveolar lavage (BAL) of lung transplant patients with, or without post-transplant infection.

Materials and Methods

Ethical approval and patient consent

Bronchoalveolar lavage sampling of lung transplant patients from the Royal Brompton and Harefield NHS Foundation Trust was performed under Biomedical Research Unit ethics approval (RBH/AS1).

Fungal pathogens

A clinical isolate of *A. fumigatus*, obtained from an invasive aspergillosis patient at the Norfolk and Norwich University NHS Foundation Trust (as used in [14, 18]), was subcultured on Sabouraud dextrose agar at 37°C for 7 days, and conidia were harvested using sterile physiological saline (Oxoid). Resting live conidia were used immediately or fixed in 4% phosphate-buffered saline (PBS)–formaldehyde for 10 min at room temperature (RT), washed, and resuspended in PBS. Fixed *A. fumigatus*. conidia were stored at 4°C for up to 1 month until further use.

Cells and reagents

All experiments were conducted using the A549 adenocarcinomic human alveolar basal epithelial cell line as a model for type II alveolar epithelial cells. A549 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated foetal calf serum and 50 I.U mL^{-1} penicillin and 50 µg mL^{-1} streptomycin. Experiments were all performed in serum-free conditions. Recombinant H-ficolin was purchased from R&D Systems. FITC was purchased from Sigma-Aldrich. The MEK 1/2 inhibitor (U0126), p38 MAPK inhibitor (SB202190) and the JNK inhibitor (SP600125) were purchased from Tocris Biosciences.

Detection of infection and H-ficolin in bronchoalveolar lavage

BAL fluid was collected from lung transplant recipients at Royal Brompton and Harefield NHS Foundation Trust by instilling 200 mL sterile saline into distal airway segments under flexible bronchoscopy. BAL return was centrifuged at 200 g for 10 min. Supernatant was subsequently analyzed via the lateral-flow device for *Aspergillus* antigens, indicative of invasive aspergillosis, as previously described [19] and/or via detection of galactomannan (GM) using a Platelia™ *Aspergillus* antigen kit (Bio-Rad). For BAL samples, an index of < 0.5 was considered negative, an index of ≥ 0.5 was considered positive for GM [20]. Samples were tested for a panel of respiratory viruses (multiplex PCR) and bacteria by culture (B57, UK standard for microbiology investigations) [21]. High resolution computed tomography (HRCT) chest imaging was reviewed for evidence of findings consistent with fungal infection [21]. The presence of H-ficolin in the BAL fluid of lung transplant patients was detected using a ficolin-3 human ELISA kit (Hycult). Patients were categorised for possible, probable and proven invasive fungal infection according to revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria [22].

Fluorescent microscopy

To detect the presence of intracellular H-ficolin in A549 cells, A549 cells were grown in 8-well chamber Permanox slides (Lab-Tek). Following growth, cells were permeabilised with PBS containing 0.1% Tween-20 prior to staining with primary monoclonal anti-H-ficolin antibodies (Hycult) and secondary Alexa Fluor 594 antibodies (Invitrogen). Cells were visualised using a Zeiss Axioplan II microscope at x40 objective (Zeiss).

Ficolin binding assays

Fixed *A. fumigatus* conidia were washed twice in ficolin binding buffer (FBB) (5.0 mM $CaCl_2$, 155.0 mM NaCl, 25.0 mM HEPES, 0.5% BSA, 0.1% Triton X-100). *A. fumigatus* was then incubated with 18.4 μg mL^{-1} H-ficolin for 1 h at 37°C. Fungi were washed as described above, prior to antibody-labelling of the *Aspergillus*-H-ficolin complex with monoclonal mouse anti-H-ficolin antibodies (1:100; R&D Systems) for 30 min at 4°C. Following washing, the secondary antibody (goat anti-mouse Ig-fluorescein isothiocyanate [FITC]; Beckman Coulter, United Kingdom) at a 1:200 dilution was incubated with the complex for 30 min at room temperature (RT). Following washing, the *Aspergillus*-ficolin complex was fixed with 4% PBS–formaldehyde for 10 min at RT, washed, and resuspended in sterile PBS. Calcium-dependent binding was investigated in FBB without calcium and in the presence of 5 mM EGTA. For testing the influence of the pH on H-ficolin binding, the pH of FBB was adjusted with concentrated HCl or NaOH to generate a range of pH from pH 3.7 to 10.7.

The carbohydrate-recognition characteristics of H-ficolin were determined using a ligand inhibition assay prior to *Aspergillus* binding. Concentrations (0.75, 3.1, 12.5, 25, 50, 100 mM) of various carbohydrates (glucose, GlcNAc, galactose, *N*-acetylgalactosamine [GalNAc], L-fucose, D-mannose and *N*-acetylcysteine [CysNAc]) were incubated with 18.4 μg mL^{-1} of H-ficolin for 2 h at RT. Following the ligand inhibition, the ficolin/sugar complex was then added to the *Aspergillus* spp. and analyzed for binding as described above.

H-ficolin binding to conidia or hyphae was analyzed by flow cytometry (Detection was on the FL1-A detector channel: laser excitation, 488 nm; emission detection, 533/30 nm) using a BD Accuri C6 flow cytometer with BD CFlow software (BD Accuri Cytometers).

Fungal association assays

Human A549 type II alveolar adenocarcinoma cells were seeded on 48-well plates (Nunc, United Kingdom) in supplemented RPMI 1640 and grown to semi-confluence at 37°C in a 5% CO_2 atmosphere. FITC-labelled fixed *A. fumigatus* conidia were opsonized with 18.4 μg mL^{-1} BSA or H-ficolin for 1 h at 37°C in FBB lacking Triton X-100. H-ficolin-opsonized *A. fumigatus* conidia (5×10^5) were incubated for 16 h with adherent A549 cells (ratio of conidia to cells of 5:1) at pH 5.7 or pH 7.4 at 37°C in a 5% CO_2 atmosphere. Following incubation, non-adherent conidia and A549 cells were removed and the adherent cells were washed with warm supplemented RPMI 1640. Adherent cells were subsequently removed by the use of trypsin-EDTA and gentle trituration. Cells were pooled and fixed in 4% PBS–formaldehyde for 10 min at RT and analyzed by flow cytometry using a BD Accuri C6 flow cytometer with BD CFlow software. The percentage of A549 cells associated with FITC-positive *A. fumigatus* conidia was determined by gating on the A549 cell population (forward scatter channel/side scatter channel [FSC/SSC]) and calculating the percentage of A549 cells staining positive for fluorescence (FL1-A).

Cytokine determination

Supernatants were collected 24 h following A549 challenge with live *A. fumigatus* conidia. Cytokine production from supernatants was quantitated using a BD cytometric bead array (CBA) Human Inflammatory Cytokines kit (BD Biosciences). Assays were conducted as outlined in the protocol. In brief, capture beads for the measurement of IL-8, IL-1β, IL-6, IL-10 and TNF-α were mixed together prior to their addition to the sample and standard tubes. Following the addition of capture beads to the samples, Human Inflammatory Cytokine PE Detection Reagent was added to all tubes and incubated for 3 h in the absence of light.

Following incubation, samples were washed in wash buffer for 5 min at 200 g prior to aspiration of the supernatant, re-suspension in wash buffer and flow cytometry (Exλ 488 nm, Emλ 585/40nm) and (Exλ 633 nm, Emλ 780/30 nm) on a BD Accuri C6 flow cytometer with BD CFlow® Software, collecting 1500 events as outlined in the protocol.

To investigate the role of MAPK signalling in IL-8 production, inhibitors of MEK 1/2 (U0126), p38 MAPK (SB202190) and JNK (SP600125) were added to cells at a concentration of 10 μM for 1 h prior to and during *A. fumigatus* challenge.

Measurement of C3b deposition

Endogenous ficolins were removed from donor serum by passing 5 mL through a 1 mL *N*-acetyl-D-glucosamine column at 4°C overnight. Removal of ficolins from the serum was confirmed using a ficolin-3 human ELISA kit (Hycult). Ficolin-depleted serum was stored at -80°C until use.

To measure lectin-pathway C3 deposition, 96-well Maxisorb plates (Nunc) were coated with fixed *A. fumigatus* conidial suspensions (OD_{550} of 0.5) or 5 μg mL^{-1} *N*-acetyl BSA in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Non-specific proteins were then blocked with 1% (wt/vol) BSA-TBS for 2 h at RT. Once blocked, plates were washed three times with wash buffer (TBS, 0.05% Tween-20, 5 mM $CaCl_2$, pH 7.4). To quantify C3 deposition, 100 μL of ficolin-sufficient/deficient serum was added to the wells in barbital buffered saline (BBS; 4 mM barbital, 145 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4). Ficolin-deficient serum was either reconstituted with recombinant H-ficolin (18.4 μg mL^{-1}) or left ficolin-deficient for 1.5 h at 37°C. Plates were washed again three

times prior to the addition of primary antibody rabbit anti-human C3c (1:500; Dako) and incubated for 1.5 h at 37°C. Following three washes, the secondary antibody anti-rabbit alkaline phosphatase (1:5000; Sigma-Aldrich) was added and incubated for 1 h at RT. The assay was developed using p-nitrophenyl-phosphate (pNPP; Sigma-Aldrich) and absorbance was measured at 405 nm. *N*-acetyl BSA was used as a positive control.

Statistical analysis

Results were expressed as mean ± SD. Descriptive and 2-tailed Students *t*-test analyses were performed using GraphPad prism software (version 5). One-way ANOVA's were performed using SigmaStat software (version 3.5). A value of $p<0.05$ was considered statistically significant. Receiver operating characteristics (ROC) curve analysis was conducted using MedCalc (version 13.1.1).

Results

H-ficolin binds to A. fumigatus conidia.

It has been acknowledged that H-ficolin is capable of binding *A. fumigatus*, albeit with low affinity [16]. We further characterised this binding and showed that the recognition of *A. fumigatus* by H-ficolin occurred in a pH- and calcium-dependent manner and was inhibited by L-fucose, D-mannose and GlcNAc.

H-ficolin is produced by type II alveolar epithelial cells[15]. However, it wasn't established whether A549 cells; a widely used model for the type II alveolar epithelium, could also

produce H-ficolin. We made the observation that H-ficolin could be found within A549 cells (data not shown).

We also demonstrated H-ficolin binding to *A. fumigatus* conidia. Following opsonization of fixed *A. fumigatus* conidia (5×10^5) with H-ficolin (18.4 µg mL^{-1}), we observed significant binding (median fluorescence intensity of 632.5 ±21.0 versus 207.7 ±1.5 and 215.0 ±1.0 for H-ficolin opsonized conidia versus un-opsonized and BSA opsonized conidia, respectively; $p=4.01 \times 10^{-6}$, Fig. 1A and Fig. 1C).

A characteristic of the related collectin family is the requirement of calcium to bind to their ligands [23]. Therefore we investigated H-ficolin binding to conidia in the absence of extracellular calcium. When calcium was removed from the binding buffer and excess calcium was chelated by EGTA, H-ficolin binding was observed to significantly decrease to levels observed for the BSA control (median fluorescence intensity of 294.7 ±13.8 versus 632.5 ±21.0 and 206.3 ±3.5 for H-ficolin opsonized conidia versus un-opsonized and BSA opsonized conidia, respectively; $p=2.02 \times 10^{-5}$, Fig. 1A and Fig. 1C).

The pH is an important aspect of infection as it decreases at the local site of infection to as low as pH 5.5 [24]. Therefore, it is vital that the functions of innate recognition proteins are enhanced during host infection. We had previously observed that binding of L-ficolin and its rodent orthologue, ficolin-A, to *A. fumigatus* was pH-dependent, occurring optimally at pH 5.7 (Bidula et al., 2013; Bidula et al., 2015). Additionally, others had highlighted that pH could alter the confirmation of the FBG domain (Garlatti et al., 2007; Garlatti et al., 2007) therefore binding studies were conducted in a range of from pH 3.7 to pH 10.7.

Here, binding occurred in a pH-dependent manner, with maximal binding observed in acidic (pH 5.7) conditions. Binding at pH 5.7 was significantly greater than binding at pH 7.4 (median fluorescence intensity of 632.5 ±21.0 versus 549.3 ±12.0 for H-ficolin opsonized conidia in pH 5.7 and pH 7.4 conditions, respectively; p=0.00398, Fig. 1D). However, binding at pH 7.4 was still significant (median fluorescence intensity of 549.3 ±12.0 versus 207.6 ±1.5 for H-ficolin opsonized conidia versus un-opsonized conidia; $p=1.09 \times 10^{-6}$; Fig. 1D).

Ficolins recognise pathogens via carbohydrate moieties on their cell surface. Therefore, to investigate H-ficolin carbohydrate-binding specificity, H-ficolin was pre-incubated with a range of carbohydrates (glucose, galactose, GlcNAc, L-fucose and D-mannose) prior to incubation with *A. fumigatus* conidia. L-fucose, GlcNAc and D-mannose exhibited the greatest binding inhibition (% total inhibitions of 70.0% ±1.6%, 88.7% ±7.0% and 93.4% ±7.0% at maximal carbohydrate concentration of 100 mM and IC_{50} of <0.75 mM, IC_{50} of <3.25 mM and IC_{50} of <3.25 mM for L-fucose, GlcNAc and D-mannose, respectively; Fig. 1E). Conversely, pre-incubation with glucose and galactose led to significantly lower percentage inhibitions (% inhibitions of 15.5% ±1.5% and 24.8% ±10.5% at maximal carbohydrate concentration of 100 mM for glucose and galactose, respectively; Fig. 1E).

H-ficolin opsonization enhances association of A. fumigatus conidia with A549 cells.

H-ficolin is secreted from type II pneumocytes [15] and can be found within A549 cells, which suggests it could be important in potentiating immune responses within the lung. Additionally, patients homozygous for a truncated form of H-ficolin exhibit a recurrence of

respiratory infections[25]. We were, therefore, interested in whether H-ficolin could modulate the association of *A. fumigatus* with the A549 airway epithelial cell line.

To investigate the interaction of H-ficolin opsonised conidia with the alveolar epithelium, FITC-labelled, fixed *A. fumigatus* conidia were opsonized with H-ficolin prior to incubation with A549 cells for 16 hours in pH 5.7 or pH 7.4 conditions. A549 cells were gated (Fig. 2A) and the percentage of FITC negative and positive were used to identify associated cells (Fig. 2B). There was a uniform shift in the fluorescence of the A549 population meaning that the majority of A549 cells were associated with conidia. However, in the presence of H-ficolin, the number of conidia associated per A549 cell was significantly increased (based upon the median fluorescence intensity) but only in inflammatory (pH 5.7) conditions (median fluorescence intensity of 313,045 ±77,970 versus 596,893 ±18,846 for un-opsonized and H-ficolin opsonized conidia, respectively; p=0.00359, Fig. 2C). Furthermore, *A. fumigatus* were still capable of germinating within A549 cells regardless of H-ficolin opsonization (data not shown).

H-ficolin opsonisation leads to lectin complement pathway C3b deposition on A. fumigatus conidia.

Following the observation that H-ficolin could enhance host-fungal interactions, we explored potential mechanisms to explain this augmentation. One way in which ficolins could enhance this interaction was via the activation of the lectin complement pathway and deposition of C3b on the pathogen surface. C3b itself can contribute to enhanced phagocytosis of *Aspergillus* by functioning as an opsonin[26]. However, whether H-ficolin could activate the lectin complement pathway on *A. fumigatus* and contribute to C3 deposition was unknown [8, 27].

To investigate H-ficolin activation of the lectin pathway on *A. fumigatus* conidia, human serum was either run through a GlcNAc-sepharose column to remove endogenous ficolins or left ficolin-sufficient. Following the removal of endogenous ficolins, there was significantly diminished C3 deposition on *A. fumigatus* ($p<0.05$) but ficolin-sufficient serum maintained maximal levels of C3 deposition (Fig. 3). Here we observed an EC_{50} of 1.15% serum versus an EC_{50} of 0.32% serum for ficolin-deficient and ficolin-sufficient serum, respectively (not shown). Moreover, reconstitution of the ficolin-deficient serum with recombinant H-ficolin was capable of significantly restoring the level of C3 deposition to normal levels (EC_{50} of 0.34% serum; $p<0.05$; Fig. 3).

Opsonization by H-ficolin increases IL-8 production from A549 cells after A. fumigatus challenge and involves the activation of the intracellular signalling pathways MEK 1/2, p38 MAPK and JNK.

Previously, we have observed that another serum ficolin, ficolin-A is capable of modulating IL-8 production [10]. Therefore, we utilised cytometric bead arrays to measure the concentration of IL-8, IL-1β, IL-6, IL-10 and TNF-α following challenge by H-ficolin opsonized live *A. fumigatus* conidia.

From the cytokine panel tested, H-ficolin opsonization induced a significant increase in the secretion of pro-inflammatory IL-8 compared to challenge with un-opsonized conidia after 24 h (IL-8 concentrations of 611.5 pg mL^{-1} ±56.1 versus 1893.3 pg mL^{-1} ±263 for un-opsonized and H-ficolin opsonized conidia, respectively; $p=0.00117$, Fig. 4A). H-ficolin in the absence of conidia induced an increase in IL-8 but this increase wasn't statistically significant

(p=0.06725, Fig. 4A). All other measured cytokines were not significantly affected by H-ficolin opsonization (data not shown).

Based upon previous studies that showed that IL-8 secretion from respiratory cells following *A. fumigatus* challenge was governed by PI3K, p38 MAPK and ERK 1/2, we chose to inhibit key members within the MAPK signalling pathways [28]. In order to investigate the intracellular signalling pathways involved in the increase of IL-8 secretion from A549 cells following challenge by H-ficolin opsonized *A. fumigatus*, we used the MEK 1/2 inhibitor U0126, the p38 MAPK inhibitor SB202190 and the JNK inhibitor SP600125 prior to the quantification of IL-8 by bead array. It was observed that MEK 1/2 inhibition completely abrogated IL-8 production following challenge with un-opsonized or H-ficolin-opsonized conidia ($p<0.001$, Fig. 4B). Inhibition of p38 MAPK and JNK also significantly reduced IL-8 production both in the presence and absence of H-ficolin opsonization ($p<0.001$; Fig. 4B).

The incremental increase of IL-8 from A549 cells that was induced by H-ficolin alone in the absence of *A. fumigatus* also appeared to be significantly reduced via inhibition of MEK 1/2 and JNK but less so by the p38 MAPK inhibitor SB202190 ($p<0.001$, Fig. 4B).

Raised H-ficolin concentrations in the bronchoalveolar lavage fluid of lung transplant recipients displaying signs of post-transplant pneumonia.

H-ficolin can be secreted into the BAL fluid from bronchial epithelial cells and alveolar type II epithelial cells; therefore, we chose to investigate the concentration of H-ficolin in the BAL fluid from transplant patients (a high risk group for invasive aspergillosis) with or without signs of lung infection.

Here, we utilised an H-ficolin-specific ELISA to detect the presence of H-ficolin in the BAL samples of lung transplant recipients. H-ficolin was observed to be present in both the lungs of healthy and infected transplant recipients. However, in patients who were diagnosed with invasive pulmonary fungal infection based on EORTC/MSG criteria and/or positive GM/lateral-flow, bacterial or viral infection, H-ficolin BAL concentration was significantly higher compared to healthy recipients ($p=0.00726$; Fig. 5A). Out of the 16 infected lung transplant patients, one was infected with *Penicillium* spp., one with *Staphylococcus aureus*, one with rhinovirus, three with *Pseudomonas aeruginosa*, four with *A. fumigatus* and six without detectable cultures but with radiology suggestive of fungal infection or positive GM and/or lateral flow test. Within these groups, patients infected with *A. fumigatus* had consistently higher concentrations of H-ficolin in the BAL (average concentration of 19.4 ng mL^{-1}; Fig. 5B). The one patient with rhinovirus had a higher BAL H-ficolin concentration (average concentration of 24.1 ng mL^{-1}) but this was only data from one patient (Fig. 5B). An ROC curve analysis was conducted to investigate whether the detection of H-ficolin could be used as a potential biomarker/diagnostic tool for lung infection. The area under the curve (AUC) was calculated to be 0.77 which suggested there was an 77% chance that infected transplant patients would have H-ficolin present in their BAL fluid ($p<0.0001$; Fig. 5C).

Discussion

In this study, we focused on the role of H-ficolin in the innate immune recognition of *A. fumigatus* and its role in host-fungal interactions. This work led to a number of new observations. Firstly, H-ficolin was capable of recognising *A. fumigatus* conidia in a calcium-dependent and pH-dependent manner, in an interaction inhibited by D-mannose, L-fucose and GlcNAc. This recognition also led to enhanced host-microbe interactions. Secondly, H-

ficolin opsonization led to activation of complement on *A. fumigatus*. Thirdly, in the presence of H-ficolin, we observed an increase in the production of IL-8 which was abrogated following inhibition of MEK 1/2, p38 MAPK and JNK. Finally, H-ficolin concentration in the BAL fluid was greater in transplant recipients with post-transplant lung infections compared to uninfected control patients.

This work has, therefore, led us to postulate that as part of an anti-fungal inflammatory response, H-ficolin concentrations are increased in the lung environment. Moreover, H-ficolin recognizes conidia via its cell surface glycoproteins and enhances the immune function of lung epithelial cells in the defence against *A. fumigatus*.

To date, all of the serum ficolins had been observed to be capable of binding to *A. fumigatus* conidia but of these serum ficolins, H-ficolin binding was least characterized[10, 16, 29]. We therefore proceeded to characterize the manner in which H-ficolin recognised *A. fumigatus* conidia. H-ficolin was observed to significantly bind to conidia, albeit much less avidly than serum L-ficolin or its rodent orthologue, ficolin-A. This could be explained by H-ficolin's much lower affinity for GlcNAc compared to L-ficolin. Similarly to L-ficolin and the related collectins, MBL and the surfactant proteins, H-ficolin binding to *A. fumigatus* also required the presence of calcium[30, 31].

Ficolins are capable of distinguishing between self and non-self carbohydrates on pathogenic microorganisms via their FBGs [32] and we can identify their specificity for carbohydrates in the pathogen cell wall via competitive inhibition of their FBG. Few studies to date have

highlighted the carbohydrate recognition properties of H-ficolin, with binding previously only observed to a polysaccharide of *Aerococcus viridians,* acetylated carbohydrates and very few pathogens [16, 33, 34]. The cell wall of *A. fumigatus* is predominantly composed of glucan, chitin and galactomannan and it is these cell wall glycoproteins that constitute the *Aspergillus* virulence factors [35]. When further deconstructed, the wall is composed of glucose, glucosamine, galactose and mannose [7, 36]. In the present study, we indicate novel binding targets for H-ficolin in the shape of D-mannose and L-fucose while re-establishing the preference of ficolin binding to GlcNAc. Interestingly, neither D-mannose nor L-fucose was able to inhibit binding of H-ficolin in previous studies but these inhibition studies were not conducted at varying pH [34]. This data implies that H-ficolin recognises its targets via multiple binding sites on the fibrinogen-like domain with additional sites for mannose and/or fucose as well as separate sites for GlcNAc. All in all, the binding of H-ficolin to a range of carbohydrate structures on the conidial surface is essential for efficient pathogen recognition. This could have the potential to influence *Aspergillus* infection via restricting fungal adhesion and masking inflammation induced by surface virulence factors. However, those fungi evading immune recognition can provoke an inflammatory response and lead to tissue necrosis, acidifying the local pH.

Based upon our previous observations whereby ficolin-A binding to *A. fumigatus* was dependent upon the pH in addition to studies characterising H-ficolin [10, 34], we have shown that the affinity of H-ficolin for *A. fumigatus* is also significantly enhanced in an acidic pH. The pH is an important aspect of infection as it decreases at the local site of infection to as low as pH 5.5 [24]. Our findings support that the acidic conditions found in inflammatory sites could be capable of potentiating the opsonization of *A. fumigatus* by H-ficolin.

One of the key biological consequences of opsonization is the enhancement of uptake by host cells. Conidia are very small structures (2-4 μm in size) and because of this, they can penetrate deep into the alveolar space where they encounter the first line of defence in the shape of type II pneumocytes [37]. The important role of ficolins in the enhancement of host-microbe interactions is well documented. We have previously shown that L-ficolin and ficolin-A can function as opsonins and enhance association of *A. fumigatus* and the clinically relevant yeast, *Cryptococcus neoformans* with host cells[10, 38]. Our current study indicates that H-ficolin is also capable of significantly increasing the association of conidia with A549 cells in comparison to un-opsonized or BSA-opsonized controls, suggesting an important role for H-ficolin in immobilising the fungus following inhalation.

One such immune mechanism that can lead to enhanced interactions is the activation of complement. Complement activation can lead to the deposition of C3b, which, like H-ficolin, also functions as an opsonin [26]. Ficolins are able to activate the lectin complement pathway following the association with MASPs. Therefore, we investigated whether H-ficolin could contribute to complement activation on *A. fumigatus*. Following the depletion of ficolins from the serum we still observed some C3b deposition. This is likely due to complement activation by the residual MBL and/or collectin-11 (CL-11) that was not removed by the depletion method. However, following reconstitution of ficolin depleted human serum with recombinant H-ficolin, we observed a restoration of C3b deposition to normal levels. Previous observations by Ma *et al* [29] demonstrated a lack of H-ficolin binding to a clinical sample of *A. fumigatus* and, thus, no complement activation. However, recognition and lectin-pathway activation may be species and strain specific, as highlighted for *Pseudomonas aeruginosa* [39]. Therefore, future studies with a range of established strains may be necessary to further illuminate the role of H-ficolin in lectin complement pathway activation.

Recent advances in the field have highlighted novel functions of ficolin-A, L-ficolin and M-ficolin in the immunomodulation of cytokines [10-12, 14]. Whether this could also be achieved following H-ficolin opsonization was previously unknown. Indeed, as identified previously for ficolin-A [10], we observed potentiation of IL-8 production following A549 cell challenge with H-ficolin opsonized conidia. IL-8 is crucial in the recruitment of neutrophils during aspergillosis, which play a key role in *A. fumigatus* infection [40]. *A. fumigatus* itself is not a strong stimulator of IL-8 production from the A549 cell line and dampening of the immune response could be a potential immune evasion mechanism [41]. Therefore, we postulated that H-ficolin performs an important role in the enhancement of the immune response and could be important in immune responses, such as neutrophil recruitment. Unlike its serum counterparts, ficolin-A and L-ficolin, H-ficolin in the absence of *A. fumigatus* did not significantly enhance IL-8 production. As H-ficolin is produced directly in the lung, it would not be beneficial for it to increase inflammation in the absence of pathogen challenge; as excess inflammation is detrimental to normal lung function [10].

The mechanisms underlying this increase were, and still are, poorly understood, as cell surface receptors for the serum ficolins and signalling pathways involved are unknown. Therefore, we aimed to delineate some of the signalling pathways that may be involved. Following inhibition of the ERK 1/2 activator, MEK 1/2, by the highly potent inhibitor U0126, inhibition of p38 MAPK by SB202190 and inhibition of JNK by SP600125, we observed almost complete abrogation of IL-8 production following A549 cell challenge with H-ficolin opsonized conidia. Of these, MEK 1/2 inhibition appeared to lead to the greatest inhibition. The ERK 1/2 pathway has been demonstrated to be important for IL-8 synthesis following challenge by pathogens and elucidating how H-ficolin activates this pathway could prove important in the understanding of its immunomodulatory function [28, 42, 43]. Due to their

importance in fungal immunity, potential cell surface receptor targets include TLRs and C-type lectin-like receptors of the dectin-1 cluster [44-48], but the signalling mechanisms involved require much greater investigation.

In addition to the *in vitro* studies, we were interested if *in vivo* observations could highlight an important role for H-ficolin during lung infections. To investigate this, we took lung transplant patients (a high risk group for invasive aspergillosis) and measured the concentration of H-ficolin in their BAL fluid. H-ficolin is normally secreted into the BAL fluid of healthy lungs regardless of infection but there were significantly higher concentrations of H-ficolin in the BAL of lung transplant recipients diagnosed with post-transplant pneumonia compared to healthy recipients. Interestingly, the average BAL H-ficolin concentration was highest in the patients who were infected with *A. fumigatus* but the absolute concentration of H-ficolin in the lung is difficult to quantify due to the variability of dilution factors during bronchoscopy. Recent *in vivo* observations have acknowledged that serum ficolins could play an important role in lung defence against pathogenic microorganisms, with serum L-ficolin present in the lung only during fungal infection [14] and truncated forms of H-ficolin increasing susceptibility to respiratory infections [25]. Although the current sample size is small (32 patients), ROC analysis has indicated that the presence of H-ficolin in the lungs of transplant patients could be a potential human diagnostic biomarker for infection and in combination with fungal specific markers such as galactomannan, may be used in the diagnosis of fungal infection. However, this diagnostic potential will need to be further investigated in larger clinical trials.

In conclusion, H-ficolin may play a significant role in the defence against *A. fumigatus* via the activation of the lectin complement pathway, enhanced host-microbe interactions and modulation of the immune response. Yet, the use of the A549 cell line is a limitation to the current study and further investigation using primary airway epithelial cells could yield important observations.

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Conflict of interest

All authors disclose no conflicts of interest.

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Figures legends

Figure 1. Binding characteristics of H-ficolin to *A. fumigatus* conidia. H-ficolin (18.4 µg mL^{-1}) was incubated with 5 x 10^5 conidia in the presence or absence of calcium, a range of pHs from pH 3.7-10.7 or following pre-incubation with a variety of carbohydrates. (**A**) Representative histogram of H-ficolin binding to *A. fumigatus* conidia in the presence of

calcium. (**B**) Representative histogram of H-ficolin binding to *A. fumigatus* conidia in the absence of calcium. (**C**) Binding of H-ficolin to *A. fumigatus* conidia in the presence and absence of calcium indicating the median fluorescence intensity (MFI). (**D**) Binding of H-ficolin to *A. fumigatus* conidia in a range of pHs from pH 3.7- pH 10.7. (**E)** Carbohydrate binding specificity of H-ficolin to *A. fumigatus* conidia. H-ficolin was pre-incubated with a range of carbohydrates (glucose, galactose, L-fucose, D-mannose and GlcNAc) at various concentrations (0-100 mM) prior to conidial binding at pH 5.7. Results are representative of the average of all the data points gained from three independent experiments. Error bars represent the SD and significance was determined via two-tailed Students *t*-test. For carbohyrdrate inhibitions, significance was determined via one-way ANOVA and post-hoc analysis was conducted using the Tukey test. An asterisk indicates a significant difference ($p<0.05$).

Figure 2. H-ficolin opsonization enhances association of *A. fumigatus* conidia with A549 epithelial cells. FITC-labelled *A. fumigatus* conidia (5×10^5) were opsonized with 18.4 μg mL^{-1} H-ficolin prior to incubation with A549 cells (conidia:A549 ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 16 h. (**A**) P1 gate on the A549 population. (**B**) Representative histogram depicting the fluorescence (FL1-A) of the A549 population following challenge with un-opsonized or H-ficolin opsonized *A. fumigatus* conidia. (**C**) The relative number of associated FITC-labelled conidia (based upon the median fluorescence intensity (MFI) either un-opsonized (- H-ficolin), BSA-opsonized (+ BSA) or H-ficolin opsonized (+ H-ficolin). Results are representative of the average of all the data points gained from three independent experiments. Error bars represent the SD and significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant difference ($*p<0.05$).

Figure 3. H-ficolin activates the lectin complement pathway on *A. fumigatus* conidia. The restoration of lectin complement pathway C3 deposition was observed on *A. fumigatus* following the reconstitution of ficolin-depleted human serum with recombinant H-ficolin. Serum dilutions of serum were incubated in Maxisorb microtiter 96-well coated plates and deposited C3 was detected by using rabbit anti-human C3c complement antibodies and goat anti-rabbit IgG-alkaline phosphatase conjugate. *N*-acetyl BSA was used as a positive control for C3 deposition (*p<0.05). **HS** = human serum; **HF** = H-ficolin; **AF** = *A. fumigatus*; **NBSA** = *N*-acetyl BSA; **rHF** = recombinant H-ficolin. All data points are represented as mean absorbance measured at 405 nm in the presence of 1.25% serum and are representative of the data obtained from three independent experiments. Significance was determined via one-way ANOVA and post-hoc analysis was determined using the Tukey test.

Figure 4. IL-8 secretion from A549 epithelial cells is enhanced following challenge with H-ficolin opsonized *A. fumigatus* conidia and is reliant upon MEK 1/2, p38 MAPK and JNK activation. (A) Supernatants from A549 cells were collected 24 h after challenge with H-ficolin (18.4 µg mL^{-1}) alone, conidia (1 x 10^6) alone or H-ficolin (18.4 µg mL^{-1})-opsonized *A. fumigatus* (1 x 10^6 conidia), prior to cytometric bead array. **(B)** To investigate the cell signalling pathways involved, A549 cells were pre-incubated with 10 µM of the MEK 1/2 inhibitor (U0126), the p38 MAPK inhibitor (SB202190) or the JNK inhibitor (SP600125) prior to *A. fumigatus* challenge. Media from A549 cells without stimulus and LPS (100 ng mL^{-1}; grey bar) were used as negative and positive controls, respectively. DMSO was used as a vehicle control for MAPK inhibitors (white bar). All data are represented as the average concentration of IL-8 produced (pg mL^{-1}) and are representative of the averages of all the data points gained from three independent experiments. Error bars represent the SD.

Significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant difference (*$p<0.05$).

Figure 5. BAL H-ficolin concentrations from infected lung transplant patients are higher than those found in non-infected patients. BAL fluid was collected following bronchoscopies on lung transplant patients prior to the measurement of H-ficolin concentrations by ELISA. (**A**) The concentration of H-ficolin (ng mL^{-1}) in BAL samples. BAL samples were considered **positive** (n=16) or **negative** (n=16) for infection dependent upon patients classification according to EORTC/MSG criteria, *Aspergillus* antigen detection, radiology and bacterial, viral or fungal cultures. (**B**) H-ficolin concentration in the BAL fluid of patients testing culture positive for *Penicillium* (Pen; n=1), *P. aeuriginosa* (PsA; n=3), *A. fumigatus* (AsF; n=4), *S. aureus* (StA; n=1), rhinovirus (Rhi; n=1) or culture negative (Nil; n=6). (**C**) ROC curve analysis for H-ficolin detection in infected transplant patients compared to non-infected transplant patients. Results are representative of the data points gained from three independent experiments (16 positive and 16 negative patients). Bars represent the median and significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant difference (*$p<0.05$).