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The serum opsonin L-ficolin is detected in lungs of human transplant recipients following fungal infections and modulates inflammation and killing of Aspergillus fumigatus.

Serum opsonin, L-ficolin, is detected in human lungs of transplant patients following fungal infections and modulates inflammation and killing of *A. fumigatus*

Running title: L-ficolin and immunity to *A. fumigatus*

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FOOTNOTES

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ABSTRACT

Background. Invasive aspergillosis (IA) is a life threatening systemic fungal infection in the immunocompromised caused by *Aspergillus fumigatus*. The human serum opsonin, L-ficolin has been observed to recognise *A. fumigatus* and could participate in fungal defence.

Methods. Using lung epithelial cells, primary human monocyte-derived macrophages (MDM) and neutrophils from healthy donors, we assessed phagocytosis and killing of L-ficolin opsonized *A. fumigatus* live conidia by flow cytometry and microscopy. Additionally, cytokines were measured by cytometric bead array and L-ficolin was measured in bronchoalveolar lavage (BAL) fluid from lung transplant recipients by ELISA.

Results. L-ficolin opsonization increased conidial uptake and enhanced killing of *A. fumigatus* by MDM and neutrophils. Opsonization was also shown to manifest an increase in IL-8 release from A549 lung epithelial cells but decrease IL-1β, IL-6, IL-8, 1L-10 and TNF-α release from MDM and neutrophils 24 h post-infection. The concentration of L-ficolin was significantly higher in BAL of patients with fungal infection than in control subjects (p=0.00087) and ROC curve analysis highlighted the diagnostic potential of L-ficolin for lung infection (AUC=0.842; p<0.0001).

Conclusions. L-ficolin modulates the immune response to *A. fumigatus.* Additionally, for the first time, L-ficolin has been demonstrated to be present in human lungs.

Keywords. L-ficolin, *Aspergillus fumigatus*, macrophage, neutrophil, epithelial, phagocytosis, cytokines, lung transplant

Introduction

Aspergillus fumigatus (*A. fumigatus*) is a major worldwide prevalent pathogenic mold and the primary cause of invasive pulmonary aspergillosis (IA) in immunocompromised hosts. [1]. In those at risk such as leukaemia, solid-organ and haematopoietic transplant patients or those with neutropenia, IA is associated with a mortality rate of up to 30% if treated and 100% in untreated patients [2-6]. Infection is initiated following the inhalation of small hydrophobic conidia from the environment which have the propensity to germinate into filamental (hyphal) structures. These invade local tissues causing thrombosis, necrosis and dissemination of the fungus to other organs such as the skin and brain, ultimately leading to death [7-9].

Alveolar macrophages, neutrophils, complement and pattern recognition proteins; such as the ficolins and collectins, all work synergistically to remove *Aspergillus*. The process of phagocytosis by macrophages is an integral aspect in innate host defence against *A. fumigatus* conidia [10, 11]. Neutrophils have also been observed to be important in the early stages of conidial removal, but are essential in the destruction of the large hyphal structures following degranulation and the production of neutrophil extracellular traps (NETs) [12-14].

Ficolins are a family of proteins composed of an N-terminal collagen-like domain and a C-terminal fibrinogen-like domain with lectin activity (highly specific for *N*-acetylglucosamine (GlcNAc)). Human serum L-ficolin has the potential to enhance phagocytosis via direct binding to pathogens [15] but the protective roles of ficolins in *Aspergillus* defence are still poorly characterised.

We have recently demonstrated that L-ficolin is able to enhance the binding of *Aspergillus* conidia to the lung epithelium, but little is known about the functional consequences following ficolin opsonization [16]. We therefore utilised L-ficolin to investigate its roles in phagocytosis and killing of *A. fumigatus* by phagocytes in addition to its role in modulating cytokine production. In this study we have also shown for the first time that L-ficolin is present in BAL from lung transplant patients suffering from fungal pneumonia compared to uninfected controls. Additionally, we highlight the potential of L-ficolin as a tool for the diagnosis of fungal infections following lung transplants.

Materials and Methods

Patients and Ethical approval

Evidence of fungal infection was based on clinical European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria [17]. BAL sampling of lung transplant patients from the Royal Brompton and Harefield NHS Trust was performed under Biomedical Research Unit ethics approval (RBH/AS1).

Ethical approval for blood donation by healthy participants was obtained from the Faculty of Health Research Ethics Committee (Ref. Mechanisms of airway diseases – 2008042). Blood was acquired through venepuncture of healthy participants who gave informed consent at the time of collection. All donors were not on medication at the time of collection.

Informed consent was obtained from patients and human experimentation guidelines of the United States Department of Health and Human Services were adhered to in the conduct of clinical research.

Cells and reagents

All experiments were conducted using the A549 adenocarcinomic human alveolar basal epithelial cell line, human monocyte-derived macrophages (MDM) or peripheral blood neutrophils. MDM and neutrophils were isolated from healthy donor blood via a 68% percoll gradient modified from Walsh et al (1999) [18]. Monocytes were selected for by adherence to tissue culture plastic ware for 1h and differentiated in RPMI-1640 supplemented with 10% autologous serum and 50 I.U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin over 5-9 days. A549 cells and neutrophils were briefly 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. Polymorphonuclear preparations containing greater than 90% neutrophils and exhibiting

>98% viability (as determined by trypan blue staining) were placed in culture. Experiments were all performed in serum-free conditions. Recombinant L-ficolin was purchased from R&D Systems. FITC was purchased from Sigma-Aldrich. A clinical *A. fumigatus* strain isolated from a respiratory specimen was used in all experiments and maintained/harvested as previously described [16].

Detection of infection and L-ficolin in bronchoalveolar lavage

BAL fluid was collected from lung transplant recipients at Royal Brompton and Harefield NHS Trust by instilling 200 mL sterile saline into distal airway segments under flexible bronchoscopy. BAL return was centrifuged at 1500 rpm for 10 minutes. *Aspergillus* antigens, indicative of invasive aspergillosis, were detected via the lateral-flow device 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 L-ficolin in the BAL fluid of lung transplant patients was detected using a ficolin-2 human ELISA kit (Hycult). Patients were categorised for possible, probable and proven invasive fungal infection according to revised EORTC/MSG criteria [17].

Phagocytosis assays

FITC-labelled live *A. fumigatus* conidia were opsonized with 5 μg ml^{-1} L-ficolin as previously described [16]. MDM or human neutrophils were seeded in 24-well plates (Nunc) prior to challenge with ficolin-opsonized FITC-labelled *A. fumigatus* conidia (5 x 10^5; conidia:cell ratio of 5:1) for 2 h at 37°C. Adherent cells were subsequently removed by the use of trypsin/EDTA, gentle trituration and scraping. Neutrophils in suspension were pelleted at 300 g for 5 mins. Cells were fixed in 4% PBS/formaldehyde for 10 min at RT before re-suspension in PBS. Phagocytosis was analysed by flow cytometry (Exλ 488 nm, Emλ 533/30 nm) on a BD Accuri C6 flow cytometer with BD CFlow® Software (BD Biosciences) collecting 5000 events. To yield quantitative counts, positively phagocytic cells were identified and expressed as a percentage of all phagocytes present and the relative abundance of conidia contained within the positively ingesting phagocytes was determined by the relative fluorescence intensity (FL1-A) of the positively phagocytic cells.

Ficolin binding, visualising fungal growth and fungal killing assays

L-ficolin binding assays were conducted as previously described [16] and data was collected by flow cytometry as described above.

MDM or human neutrophils were seeded in 24-well plates prior to challenge with ficolin-opsonized live *A. fumigatus* conidia (5 x 10^5; conidia:cell ratio of 5:1) for 24 h at 37°C as above. Growth was observed using an Axiovert 40 CFL microscope (Zeiss) at 10x objective for neutrophils and 20x objective for MDM.

Fungal killing was measured using a LIVE/DEAD® Fungal Viability Kit (Invitrogen). In brief, fungi were stained with 15 μM FUN-1 prior to the measurement of green fluorescence (FL1-A, Exλ 488 nm, Emλ 585/40nm), an increase in which, represents a reduction in fungal viability. Fluorescence was quantified by flow cytometry as above.

Cytokine determination

Cytokine protein concentrations from the supernatants of *A. fumigatus* challenged A549, MDM and neutrophils were determined using a BD cytometric bead array (CBA) Human Inflammatory Cytokines kit (BD Biosciences). Data was gained by 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 1800 events as outlined in the protocol.

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

L-ficolin opsonization enhances phagocytosis and killing of A. fumigatus by human monocyte-derived macrophages

We, and others, have previously acknowledged that L-ficolin is capable of binding to *A.fumigatus* [16,22]. Here we verify that L-ficolin can recognize *A.fumigatus* live conidia ($p=2.7 \times 10^{-5}$; Figure 1A) and we demonstrate enhanced binding in acidic pH (5.7) ($p=0.00089$; Figure 1B).

We have shown that the phagocytosis of conidia by the airway epithelial cell line, A549, is enhanced following L-ficolin opsonization [16]. Another integral cell type involved in the early defence against *Aspergillus* conidia are macrophages.

Initially, the ability of L-ficolin to enhance phagocytosis was investigated using FITC-labelled *A. fumigatus* conidia opsonized with L-ficolin prior to incubation with adherent human MDM for 2 hours. MDM were gated (Figure 2A) and the percentage of FITC negative and positive MDM were used to identify phagocytic cells (Figure 2B and Figure 2C). The proportion of phagocytic MDM was unaffected in physiological (pH 7.4) or acidic conditions (pH 5.7) (Figure 2D), however, the number of FITC labelled L-ficolin opsonized conidia ingested per MDM (based upon the median fluorescence intensity of phagocytic MDM) was significantly enhanced in inflammatory (pH 5.7) conditions but not at pH 7.4 (Figure 2E and Figure 2F) ($p=6.6 \times 10^{-5}$).

Additionally, light microscopy demonstrated that MDM inhibited conidial germination following opsonization by L-ficolin in inflammatory conditions (Figure 2G-J). L-ficolin in the absence of phagocytes had no effect on *A. fumigatus* growth (data not shown). Moreover, following gating (Figure 2K), fungal viability assays demonstrated a significant increase in fungal killing following opsonization by L-ficolin in these conditions; as quantitated by flow cytometry (p=0.00249) (Figure 2L and Figure 2M). When ingested A.fumigatus and free A.fumigatus populations were gated separately, death-associated green-yellow fluorescence emitted by A.fumigatus within MDM was observed to be significantly greater compared to the un-associated fungi, highlighting potent intracellular killing (Supplementary Figure 1).

L-ficolin opsonization enhances phagocytosis and killing of A. fumigatus by human neutrophils

Neutropenia poses a significant risk factor for developing aspergillosis which led us to investigate the importance of neutrophils in the recognition and removal of *A. fumigatus* conidia following ficolin opsonization.

The association of L-ficolin opsonized conidia with human neutrophils was investigated as per MDM-protocols. In this case, neutrophils were gated (Figure 3A) and the percentage of

FITC negative and positive neutrophils were used to identify phagocytic cells (Figure 3B and Figure 3C). Again, the percentage of cells phagocytosing was unaffected in physiological (pH 7.4) or acidic conditions (pH 5.7) (Figure 3D). However, as for the macrophages, flow cytometric analysis indicated a significant increase in the number of conidia phagocytosed per neutrophil following L-ficolin opsonization, but only in pH 5.7 conditions (p=0.01056) (Figure 3E and Figure 3F).

Light microscopy demonstrated that in the absence of L-ficolin opsonization in pH 5.7 and pH 7.4 or L-ficolin opsonization in pH 7.4 conditions, hyphal growth appeared very dense (Figure 3G, 3H and 3J). Following opsonization by L-ficolin at pH 5.7, hyphal growth appeared significantly less dense and clumping was observed (Figure 3I). Following gating (Figure 3K), the viability assays demonstrated a significant decrease in fungal viability following opsonization by L-ficolin in these conditions (p=0.04324) (Figure 3L and Figure 3M). As for MDM, death-associated green-yellow fluorescence emitted by *A.fumigatus* within neutrophils was observed to be significantly greater compared to the unassociated fungi (Supplementary Figure 2A). Conversely, the fluorescence of the free *A.fumigatus* in the presence of neutrophils was significantly greater when compared to *A.fumigatus* in the absence of neutrophils, suggesting augmentation of extracellular killing mechanisms (Supplementary Figure 2B)

L-ficolin opsonization modulates the secretion of inflammatory cytokines in response to A. fumigatus

We utilised cytometric bead arrays to investigate the concentration of IL-8, IL-1β, IL-6, IL-10 and TNF-α secreted from A549 type II alveolar cells, MDM and human neutrophils following challenge by L-ficolin-opsonized *A. fumigatus* conidia.

From the cytokine panel tested, IL-8 was the only cytokine significantly modulated in A549 cells in response to L-ficolin (Supplementary Figure 3). L-ficolin opsonization induced a significant increase in the secretion of pro-inflammatory IL-8 compared to challenge with un-opsonized conidia after 8 h and 24 h (Supplementary Figure 3). L-ficolin in the absence of conidia induced a significant spike in IL-8 secretion at 8 h which was maintained up to 24 h (Supplementary Figure 3).

L-ficolin opsonization also modulated cytokine secretion from MDM. Following MDM challenge with conidia opsonized by L-ficolin an anti-inflammatory effect was observed. The secretion of IL-8, IL-1β, IL-6, IL-10 and TNF-α from MDM cells 24 h post-infection were decreased (Figure 4A-E). Again L-ficolin alone appeared capable of significantly increasing the cytokine concentrations of all tested (Figure 4A-E).

Additionally, L-ficolin opsonization led to significantly decreased secretion of IL-8, IL-1β, IL-6 and TNF-α from neutrophils, compared to un-opsonized conidia (Figure 5A-E). We observed that IL-10 was only secreted at baseline levels regardless of any challenges (Figure 5D). L-ficolin was also observed to have the ability to increase the secretion of IL-8, IL-1β and TNF-α in the absence of *A. fumigatus* (Figure 5A, B and E).

L-ficolin is present in the bronchoalveolar lavage fluid of lung transplant recipients with fungal pneumonia

Based upon our recent observations [16], it was important to investigate whether L-ficolin was detectable in the lungs of patients with invasive fungal infections, particularly as L-ficolins have never formally been described to be present in lungs.

Here, we utilised an L-ficolin-specific ELISA to detect the presence of L-ficolin in the BAL samples of lung transplant recipients. In patients who were diagnosed with probable or proven invasive pulmonary fungal infection based on EORTC/MSG criteria and/or positive fungal biomarkers (GM/lateral-flow), L-ficolin was detected at significantly higher concentrations (p= 0.00087; Figure 6A) compared to uninfected control patients. L-ficolin was only detected once in the BAL samples that tested negative for fungal growth or fungal radiology features, albeit at a very low concentration (Figure 6A). An ROC curve analysis was conducted to investigate whether the detection of L-ficolin could be used as a potential biomarker/diagnostic tool for fungal infection in the lung. The area under the curve (AUC) was calculated to be 0.842 which suggested there was an 84.2% chance that fungal infected transplant patients would have L-ficolin present in their BAL fluid (p<0.0001; Figure 6B).

Discussion

Our study focused on the functional consequences of L-ficolin opsonization of *A. fumigatus*; in particular, its effect on *Aspergillus*-phagocyte interactions. In order to translate our in vitro findings to clinical infections, we also investigated whether L-ficolin is present in human lungs during fungal pneumonia. As a result a number of new observations have been made. Firstly, L-ficolin opsonization led to enhanced uptake of *A. fumigatus* conidia by MDM and neutrophils under inflammatory conditions. Secondly, this opsonization led to enhanced inhibition of hyphal formation and an increase in *A. fumigatus* killing by MDM and neutrophils. Thirdly, opsonization of *A. fumigatus* conidia by L-ficolin, evoked an anti-inflammatory cytokine response from MDM and neutrophils. Finally, for the first time we provide evidence that L-ficolin is present in the BAL fluid of lung transplant recipients diagnosed with fungal infections, which could potentially be used as a diagnostic tool for fungal infection in a clinical setting.

Initially, we showed that L-ficolin bound to *A. fumigatus* at low pH (5.7) which was similar to ficolin-A [16]. The ability of such pattern recognition molecules to function at decreased pH is important in the defence against microorganisms, with pH at the local site of infection being observed to drop as low as pH 5.5 during inflammation [23].

Another key participant during infection induced inflammation is the macrophage, which is the most prominent phagocyte in the lung in the early stages of *A. fumigatus* infection [24]. We have demonstrated here that L-ficolin enhances conidial uptake by primary MDM from healthy donors. Opsonophagocytosis was enhanced at inflammatory pH, which is also optimal for ficolin binding.

The other essential phagocyte in the defence against *Aspergillus* is the neutrophil which is known to prevent fungal growth, although the mechanism has not been fully elucidated [25]. We have shown here that L-ficolin enhances neutrophil function by increasing conidial uptake following opsonization.

Our observations are adding to the knowledge of previous reports on L-ficolin enhancing the opsonophagocytosis of not only bacteria such as *Salmonella typhimurium* and *Streptococcus agalactiae*, but also of fungi [26, 27]. It is likely that ficolins work together with other pattern recognition molecules (SP-A, SP-D and mannose-binding lectin (MBL)) and receptors (dectin-1 and Toll-like receptor 2), which have also been observed to bind *A. fumigatus* conidia and enhance phagocytic uptake [11, 16, 28-32]. Although binding and phagocytosis is important, ultimately, killing of the fungi is crucial in order to sterilize infected tissues.

Macrophages are usually able to kill conidia in their acidic phagolysosomes [33], but if conidia escape this process and germinate into hyphae, they become too large a structure to be phagocytosed. Neutrophils are then recruited to the site of infection (in response to IL-8) where they assist the inhibition of fungal invasion by degranulation and the production of fungistatic NETs following adherence to the hyphal cell wall [14, 24, 34].

In our study we observed that L-ficolin opsonization potentiated the ability of macrophages and neutrophils to significantly enhance fungal killing. Macrophages appeared more capable of inhibiting germination of conidia in comparison to neutrophils. Gating separately on the neutrophil/MDM populations containing *A. fumigatus* or the free *A. fumigatus* further illuminated the roles of these cells in killing. These observations were in keeping with previous reports that macrophages are involved in early conidial phagocytosis and killing whereas neutrophils are recruited for help at a later stage whereby extracellular killing mechanisms are integral [35].

This represents the first observation of the ability of ficolins to enhance killing of *A. fumigatus* by phagocytes which is supporting observations of others who have reported this as a characteristic of the related surfactant proteins [30]. The importance of surfactant proteins was further highlighted by their protective role against *A. fumigatus* in an in vivo model [31]. Additionally, MBL has been observed to be a key component in systemic *Aspergillus* infections, further emphasizing that humoral pattern recognition molecules play an important role in the defence against fungi [32, 36]. However, we are currently investigating the role of L-ficolin in the *in vivo* defence against aspergillosis.

As indicated earlier, we have previously observed that ficolin-A opsonization leads to an increase in IL-8 secretion from A549 cells, a cytokine that is crucial for the recruitment of neutrophils during *Aspergillus* infection. It is known that in response to *A. fumigatus,* a plethora of cytokines are secreted from various host cells, including; IL-2, IL-5, IL-6, IL-8, IL-13, IL-17A, IL-22, IFN-γ, TNF-α, GM-CSF and MCP-1 [24, 37-42].

In the present study, we found that L-ficolin opsonized conidia were also capable of inducing an increase in IL-8 as previously observed for ficolin-A opsonized conidia [16]. In contrast, opsonization of *A. fumigatus* by L-ficolin led to a significant decrease in IL-8, IL-1β, IL-6, IL-10 and TNF-α production from MDM and neutrophils. In support of our observations, it was recently observed that ficolin-A could act in an anti-inflammatory manner by binding to lipopolysaccharide (LPS) and inhibiting LPS-mediated pro-inflammatory responses on murine mast cells [43]. Additionally, the pattern recognition proteins SP-A and –D modulate an anti-inflammatory cytokine profile in response to viruses, LPS-induced cytokine and nitric oxide production, and allergens [44-46].

Our work represents the first observations that unbound ficolins may have the potential to increase cytokine secretion. The mechanisms of this interaction are still not fully understood but it most likely depends on the orientation of ficolin binding. Interestingly, both SP-A and -D have been observed to function in both an anti- and pro-inflammatory manner, dependent upon the interaction of their globular heads with SIRPα or their collagenous tails with calreticulin/CD91, respectively [47]. Some data suggests that L-ficolin binds to calreticulin but there has been no demonstrable binding to SIRPα [48]. Another caveat to be aware of is that in its native state, L-ficolin normally exists as quiescent polymers but the recombinant form used in this study is in a depolymerised state and may not be completely representative of normal *in vivo* function. This is an area of research that is currently ongoing in our laboratory.

The most important clinical observation of our study was the detection of the serum L-ficolin in BAL of patients lungs diagnosed with invasive A.fumigatus infection. Moreover, L-ficolin could also be detected in the lungs of recipient's infected with *A.flavus, Penicillium* spp.,*Acremonium* spp., *Scedoporium apiospermum* and at very low concentration in one incidence of *S.aureus* infection. This ficolin has, until now, not been reported to be present in the lung. We postulate that L-ficolin, which is normally produced by the liver, enters the alveolar space during infection from the blood stream similarly to the related acute phase protein, MBL (a serum collectin), which has also been found in the BAL fluid from infected lungs [49]. Although the current sample size is small (39 patients), ROC analysis has indicated that the presence of L-ficloin in the lungs of transplant patients could be linked with fungal infection, but this diagnostic potential will need to be further investigated in larger clinical trials.

In conclusion, L-ficolin is present in fungal infected lungs of transplant patients and has immunomodulatory properties that highlight an important role in the innate defence against *Aspergillus* through enhancing opsonophagocytosis by macrophages and neutrophils, increasing fungal killing and manifesting an anti-inflammatory cytokine profile post-infection. Future research will be concerned with understanding the signalling pathways involved in immune defence and utilizing ficolin-deficient transgenic animal models to elucidate the function of ficolins in the defence against *Aspergillus in vivo.*

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Figure legends

Figure 1. L-ficolin binding to live *A. fumigatus* conidia. Live *A.fumigatus* conidia (5 x10^5) were opsonized with 5 µg ml^{-1} L-ficolin in the presence or absence of Ca^{2+} and in a range of pH's from 3.7-10.7 prior to staining and flow cytometric analysis. (**A**) Binding of L-ficolin to *A. fumigatus* in the presence or absence of Ca^{2+}. **AF** represents *A. fumigatus* alone. **+ Abs** represents the antibody background fluorescence. **BSA** was used as a negative control for binding. (**B**) Binding of L-ficolin to *A. fumigatus* in pH 3.7-pH 10.7 conditions. Results are representative of the average of all data points gained from three independent experiments. Error bars represent SD and significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant difference: $p<0.05$. MFI, median fluorescence intensity; AF, *A. fumigatus*, Abs, antibodies.

Figure 2. Phagocytosis and fungal viability following incubation of L-ficolin-opsonized *A. fumigatus* conidia with monocyte-derived macrophages. FITC-labelled or live freshly harvested *A. fumigatus* conidia (5 x 10^5) were opsonized with 5 µg ml^{-1} L-ficolin prior to incubation with MDM (conidia:MDM ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 2 h or 24 h for phagocytosis and viability assays, respectively. **(A)** Gate P1 on MDM used to produce figures **B-F**. Some points were removed for clarity. (**B)** Representative flow data depicting % MDM phagocytosing in the absence of FITC-labelled *A. fumigatus* conidia (Q1). **(C)** or in the presence of FITC-labelled *A. fumigatus* conidia (Q1). **(D)** The percentage of MDM phagocytosing conidia in pH 5.7 or pH 7.4 conditions in the presence or absence of L-ficolin. (**E**) Representative histogram depicting the uptake of conidia in pH 5.7 conditions in the presence or absence of L-ficolin. (**F**) The relative number of phagocytosed FITC-labelled conidia (based upon the median fluorescence intensity; FL1-A) either un-opsonized (–L-

ficolin) or following opsonization by L-ficolin (+L-ficolin). (**G**) Hyphal germination following incubation of un-opsonized conidia in pH 5.7. The black arrows point to macrophages containing conidia which makes macrophages appear dark. The white arrow heads are used to trace single hyphae from MDM. Many hyphae are visible, some of which are blurry as they are growing in three-dimensions and are out of the focal plane. Or. (**H**) in pH 7.4. (**I**) Hyphal germination following incubation of L-ficolin opsonized conidia in pH 5.7. Hyphae are present although growth is much less dense . Or. (**J**) in pH 7.4. (**K**) Gate P2 on MDM and *A. fumigatus* used to produce figures **L** and **M**. (**L**) Representative histogram depicting the killing of conidia in pH 5.7 conditions in the presence or absence of L-ficolin. Increased FL1-A depicts enhanced killing. (**M**) Viability of un-opsonized or L-ficolin opsonized conidia after incubation with MDMs. Results are representative of the average of all data points gained from three independent experiments. Error bars represent SD and significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant difference: $p<0.05$. FITC, fluorescein isothiocyanate; MDM, monocyte-derived macrophage; MFI, median fluorescence intensity; AF, *A. fumigatus*; SSC-A, side scatter; FSC-A, forward scatter; FL1-A, fluorescence.

Figure 3. Phagocytosis and fungal viability following incubation of ficolin-opsonized *A. fumigatus* conidia with human neutrophils. FITC-labelled or live freshly harvested *A. fumigatus* conidia (5×10^5) were opsonized with 5 µg ml^{-1} L-ficolin prior to incubation with neutrophils (conidia:neutrophil ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 2 h or 24 h for phagocytosis and viability assays, respectively. **(A)** Gate P1 on neutrophils used to produce figures **B-F** **(B)** Representative flow data depicting % neutrophils phagocytosing in the absence of FITC-labelled *A. fumigatus* conidia (Q1). **(C)** or in the presence of FITC-labelled *A. fumigatus* conidia (Q1). **(D)** The percentage of neutrophils phagocytosing conidia

in pH 5.7 or pH 7.4 conditions in the presence or absence of L-ficolin. (**E**) Representative histogram depicting the uptake of conidia in pH 5.7 conditions in the presence or absence of L-ficolin. (**F**) The relative number of phagocytosed FITC-labelled conidia (based upon the median fluorescence intensity; FL1-A) either un-opsonized (–L-ficolin) or following opsonization by L-ficolin (+L-ficolin). (**G**) Hyphal germination following incubation of un-opsonized conidia in pH 5.7 or. (**H**) in pH 7.4. (**I**) Hyphal germination following incubation of L-ficolin opsonized conidia in pH 5.7 or. (**J**) in pH 7.4. (**K**) Gate P2 on neutrophils and *A. fumigatus* used to produce figures **L** and **M**. (**L**) Representative histogram depicting the killing of conidia in pH 5.7 conditions in the presence or absence of L-ficolin. Increased FL1-A depicts enhanced killing. (**M**) Viability of un-opsonized conidia or L-ficolin opsonized conidia after incubation with neutrophils. 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: $p<0.05$. FITC, fluorescein isothiocyanate; SSC-A, side scatter; FSC-A, forward scatter; FL1-A, fluorescence.

Figure 4. Inflammatory cytokine release from monocyte-derived macrophages following challenge by un-opsonized or L-ficolin-opsonized conidia. Supernatants were collected after 8h and 24 h time points during challenge with live *A. fumigatus* conidia (5×10^5) either un-opsonized or L-ficolin opsonized ($5\ \mu g\ ml^{-1}$) prior to the conduction of cytometric bead arrays. (**A)** The concentration of IL-8 secreted**.** (**B**) The concentration of IL-1β secreted**.** (**C**) The concentration of IL-6 secreted**.** (**D**) The concentration of IL-10 secreted**.** (**E**) The concentration of TNF-α secreted**.** Following *A. fumigatus* challenge. **MDM** is representative of MDM alone. **+L-ficolin** represents MDM in the presence of L-ficolin alone. **+AF** and **+AF+L-ficolin** are representative of un-opsonized *A. fumigatus* or L-ficolin opsonized

conidia, respectively. Results are representative of the average of all the data points gained from three independent experiments. Error bars represent the SD. Significance was determined via one-way ANOVA and pair-wise comparisons were conducted using the Student-Newman-Keuls method. An asterisk indicates a significant difference: $p<0.05$. MDM, monocyte-derived macrophage; IL, interleukin.

Figure 5. Inflammatory cytokine release from neutrophils following challenge by un-opsonized or L-ficolin opsonized conidia. Supernatants were collected after 8h and 24 h time points during challenge with live *A. fumigatus* conidia (5 x 10^5) either un-opsonized or L-ficolin opsonized (5 µg ml^{-1}) prior to the conduction of cytometric bead arrays. (**A)** The concentration of IL-8 secreted**.** (**B**) The concentration of IL-1β secreted**.** (**C**) The concentration of IL-6 secreted**.** (**D**) The concentration of IL-10 secreted**.** (**E**) The concentration of TNF-α secreted**.** Following *A. fumigatus* challenge. **Neutrophils** is representative of neutophils alone. **+L-ficolin** represents neutrophils in the presence of L-ficolin alone. **+AF** and +**AF+L-ficolin** are representative of un-opsonized *A. fumigatus* or L-ficolin opsonized conidia, respectively. Results are representative of the average of all the data points gained from three independent experiments. Error bars represent the SD. Significance was determined via one-way ANOVA and pair-wise comparisons were conducted using the Student-Newman-Keuls method. An asterisk indicates a significant difference: $p<0.05$. IL, interleukin.

Figure 6. L-ficolin is found in the bronchoalveolar lavage fluid of lung transplant recipients. BAL fluid was collected following bronchoscopies from lung transplant recipients. (**A**) BAL samples were considered **positive** or **negative** for invasive fungal

infection dependent upon patients classification according to EORTC/MSG criteria. All samples were tested for fungal infection via *Aspergillus* antigen detection, radiology and culture. (**B**) ROC curve analysis for L-ficolin detection in fungal-infected transplant patients compared to non-infected transplant patients. Results are representative of the data points gained from three independent experiments (19 positive and 20 negative patients). Bars represent the median and significance was determined via two-tailed Students *t*-test (p= 0.00087). Abbreviation: BAL, bronchoalveolar lavage.

Supplementary Figure 1. Intracellular and extracellular killing of L-ficolin opsonized conidia by MDM.

Live freshly harvested *A. fumigatus* conidia (5 x 10^5) were opsonized with 5 µg ml^{-1} L-ficolin prior to incubation with MDM (conidia:MDM ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 24 h. (**A**) The death-associated green-yellow fluorescence emitted by intracellular L-ficolin opsonized or un-opsonized *A. fumigatus*, after incubation with MDM. (**B**) The death-associated green-yellow fluorescence emitted by extracellular L-ficolin opsonized or un-opsonized *A. fumigatus*, after incubation with MDM. 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 asterisks indicated difference: $p<0.05$.

Supplementary Figure 2. Intracellular and extracellular killing of L-ficolin opsonized conidia by neutrophils.

Live freshly harvested *A. fumigatus* conidia (5 x 10^5) were opsonized with 5 µg ml^{-1} L-ficolin prior to incubation with neutrophils (conidia:neutrophil ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 24 h. (**A)** The death-associated green-yellow fluorescence emitted by intracellular L-ficolin opsonized or un-opsonized *A. fumigatus*, after incubation with neutrophils. (**B**) The death-associated green-yellow fluorescence emitted by extracellular L-ficolin opsonized or un-opsonized *A. fumigatus*, after incubation with neutrophils. 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 asterisks indicated difference: $p<0.05$.

Supplementary Figure 3. IL-8 production from A549 cells following challenge by unopsonized or L-ficolin opsonized conidia.

Supernatants were collected after 8h and 24 h 657 time points during challenge with live *A.fumigatus* conidia (5 x 10^5) either un-opsonized or 658 L-ficolin opsonized (5 µg ml^{-1} prior to the conduction of cytometric bead array for the measurement of IL-8. **A549** is representative of A549 cells alone. **+L-ficolin** represents A549 cells in the presence of L-ficolin alone. **+AF** and +**AF+L-ficolin** are representative of un-opsonized *A.fumigatus* or L-ficolin opsonized conidia, respectively. Results are representative of the average of all the data points gained from three independent experiments. Error bars represent the SD. Significance was determined via one-way ANOVA and pair-wise comparisons were conducted using the Student-Newman-Keuls method. An asterisk indicated a significant difference: $p<0.05$.