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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*.**

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### Article

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

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

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45 **ABSTRACT**

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

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

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

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

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

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## 67 **Introduction**

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

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

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

88 We have recently demonstrated that L-ficolin is able to enhance the binding of *Aspergillus*  
89 conidia to the lung epithelium, but little is known about the functional consequences  
90 following ficolin opsonization [16]. We therefore utilised L-ficolin to investigate its roles in

91 phagocytosis and killing of *A. fumigatus* by phagocytes in addition to its role in modulating  
92 cytokine production. In this study we have also shown for the first time that L-ficolin is  
93 present in BAL from lung transplant patients suffering from fungal pneumonia compared to  
94 uninfected controls. Additionally, we highlight the potential of L-ficolin as a tool for the  
95 diagnosis of fungal infections following lung transplants.

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## 110 **Materials and Methods**

### 111 *Patients and Ethical approval*

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

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

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

### 123 *Cells and reagents*

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



133 >98% viability (as determined by trypan blue staining) were placed in culture. Experiments  
134 were all performed in serum-free conditions. Recombinant L-ficolin was purchased from  
135 R&D Systems. FITC was purchased from Sigma-Aldrich. A clinical *A. fumigatus* strain  
136 isolated from a respiratory specimen was used in all experiments and maintained/harvested as  
137 previously described [16].

### 138 ***Detection of infection and L-ficolin in bronchoalveolar lavage***

139 BAL fluid was collected from lung transplant recipients at Royal Brompton and Harefield  
140 NHS Trust by instilling 200 mL sterile saline into distal airway segments under flexible  
141 bronchoscopy. BAL return was centrifuged at 1500 rpm for 10 minutes. *Aspergillus* antigens,  
142 indicative of invasive aspergillosis, were detected via the lateral-flow device as previously  
143 described [19] and/or via detection of galactomannan (GM) using a Platelia™ *Aspergillus*  
144 antigen kit (Bio-Rad). For BAL samples, an index of < 0.5 was considered negative, an index  
145 of  $\geq 0.5$  was considered positive for GM [20]. Samples were tested for a panel of respiratory  
146 viruses (multiplex PCR) and bacteria by culture (B57, UK standard for microbiology  
147 investigations) [21]. High resolution computed tomography (HRCT) chest imaging was  
148 reviewed for evidence of findings consistent with fungal infection [21]. The presence of L-  
149 ficolin in the BAL fluid of lung transplant patients was detected using a ficolin-2 human  
150 ELISA kit (Hycult). Patients were categorised for possible, probable and proven invasive  
151 fungal infection according to revised EORTC/MSG criteria [17].

### 152 ***Phagocytosis assays***

153 FITC-labelled live *A. fumigatus* conidia were opsonized with  $5 \mu\text{g ml}^{-1}$  L-ficolin as  
154 previously described [16]. MDM or human neutrophils were seeded in 24-well plates (Nunc)  
155 prior to challenge with ficolin-opsonized FITC-labelled *A. fumigatus* conidia ( $5 \times 10^5$ ;  
156 conidia:cell ratio of 5:1) for 2 h at 37°C. Adherent cells were subsequently removed by the

157 use of trypsin/EDTA, gentle trituration and scraping. Neutrophils in suspension were pelleted  
158 at 300 g for 5 mins. Cells were fixed in 4% PBS/formaldehyde for 10 min at RT before re-  
159 suspension in PBS. Phagocytosis was analysed by flow cytometry (Exλ 488 nm, Emλ 533/30  
160 nm) on a BD Accuri C6 flow cytometer with BD CFlow<sup>®</sup> Software (BD Biosciences)  
161 collecting 5000 events. To yield quantitative counts, positively phagocytic cells were  
162 identified and expressed as a percentage of all phagocytes present and the relative abundance  
163 of conidia contained within the positively ingesting phagocytes was determined by the  
164 relative fluorescence intensity (FL1-A) of the positively phagocytic cells.

#### 165 ***Ficolin binding, visualising fungal growth and fungal killing assays***

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

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

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

#### 176 ***Cytokine determination***

177 Cytokine protein concentrations from the supernatants of *A. fumigatus* challenged A549,  
178 MDM and neutrophils were determined using a BD cytometric bead array (CBA) Human  
179 Inflammatory Cytokines kit (BD Biosciences). Data was gained by flow cytometry (Exλ 488

180 nm, Em $\lambda$  585/40nm) and (Ex $\lambda$  633 nm, Em $\lambda$  780/30 nm) on a BD Accuri C6 flow cytometer  
181 with BD CFlow<sup>®</sup> Software, collecting 1800 events as outlined in the protocol.

## 182 ***Statistical analysis***

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

188

## 189 **Results**

### 190 ***L-ficolin opsonization enhances phagocytosis and killing of *A. fumigatus* by human*** 191 ***monocyte-derived macrophages***

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

196

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

200 Initially, the ability of L-ficolin to enhance phagocytosis was investigated using FITC-  
201 labelled *A. fumigatus* conidia opsonized with L-ficolin prior to incubation with adherent  
202 human MDM for 2 hours. MDM were gated (Figure 2A) and the percentage of FITC negative

203 and positive MDM were used to identify phagocytic cells (Figure 2B and Figure 2C). The  
204 proportion of phagocytic MDM was unaffected in physiological (pH 7.4) or acidic conditions  
205 (pH 5.7) (Figure 2D), however, the number of FITC labelled L-ficolin opsonized conidia  
206 ingested per MDM (based upon the median fluorescence intensity of phagocytic MDM) was  
207 significantly enhanced in inflammatory (pH 5.7) conditions but not at pH 7.4 (Figure 2E and  
207 Figure 2F) ( $p=6.6 \times 10^{-5}$ ).

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

218

### 219 ***L-ficolin opsonization enhances phagocytosis and killing of A. fumigatus by human*** 220 ***neutrophils***

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

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

226 FITC negative and positive neutrophils were used to identify phagocytic cells (Figure 3B and  
227 Figure 3C). Again, the percentage of cells phagocytosing was unaffected in physiological (pH  
228 7.4) or acidic conditions (pH 5.7) (Figure 3D). However, as for the macrophages, flow  
229 cytometric analysis indicated a significant increase in the number of conidia phagocytosed  
230 per neutrophil following L-ficolin opsonization, but only in pH 5.7 conditions ( $p=0.01056$ )  
231 (Figure 3E and Figure 3F).

232 Light microscopy demonstrated that in the absence of L-ficolin opsonization in pH 5.7 and  
233 pH 7.4 or L-ficolin opsonization in pH 7.4 conditions, hyphal growth appeared very dense  
234 (Figure 3G, 3H and 3J). Following opsonization by L-ficolin at pH 5.7, hyphal growth  
235 appeared significantly less dense and clumping was observed (Figure 3I). Following gating  
236 (Figure 3K), the viability assays demonstrated a significant decrease in fungal viability  
237 following opsonization by L-ficolin in these conditions ( $p=0.04324$ ) (Figure 3L and Figure  
238 3M). As for MDM, death-associated green-yellow fluorescence emitted by *A.fumigatus* within  
239 neutrophils was observed to be significantly greater compared to the unassociated fungi  
240 (Supplementary Figure 2A). Conversely, the fluorescence of the free *A.fumigatus* in the  
241 presence of neutrophils was significantly greater when compared to *A.fumigatus* in the absence  
242 of neutrophils, suggesting augmentation of extracellular killing mechanisms (Supplementary  
243 Figure 2B)

244

### 245 ***L-ficolin opsonization modulates the secretion of inflammatory cytokines in response to A.*** 246 ***fumigatus***

247 We utilised cytometric bead arrays to investigate the concentration of IL-8, IL-1 $\beta$ , IL-6, IL-  
248 10 and TNF- $\alpha$  secreted from A549 type II alveolar cells, MDM and human neutrophils  
249 following challenge by L-ficolin-opsonized *A. fumigatus* conidia.

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

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

261 Additionally, L-ficolin opsonization led to significantly decreased secretion of IL-8, IL-1 $\beta$ ,  
262 IL-6 and TNF- $\alpha$  from neutrophils, compared to un-opsonized conidia (Figure 5A-E). We  
263 observed that IL-10 was only secreted at baseline levels regardless of any challenges (Figure  
264 5D). L-ficolin was also observed to have the ability to increase the secretion of IL-8, IL-1 $\beta$   
265 and TNF- $\alpha$  in the absence of *A. fumigatus* (Figure 5A, B and E).

266

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

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

272 Here, we utilised an L-ficolin-specific ELISA to detect the presence of L-ficolin in the BAL  
273 samples of lung transplant recipients. In patients who were diagnosed with probable or  
274 proven invasive pulmonary fungal infection based on EORTC/MSG criteria and/or positive

275 fungal biomarkers (GM/lateral-flow), L-ficolin was detected at significantly higher  
276 concentrations ( $p= 0.00087$ ; Figure 6A) compared to uninfected control patients. L-ficolin  
277 was only detected once in the BAL samples that tested negative for fungal growth or fungal  
278 radiology features, albeit at a very low concentration (Figure 6A). An ROC curve analysis  
279 was conducted to investigate whether the detection of L-ficolin could be used as a potential  
280 biomarker/diagnostic tool for fungal infection in the lung. The area under the curve (AUC)  
281 was calculated to be 0.842 which suggested there was an 84.2% chance that fungal infected  
282 transplant patients would have L-ficolin present in their BAL fluid ( $p<0.0001$ ; Figure 6B).

283

## 284 **Discussion**

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

297 Initially, we showed that L-ficolin bound to *A. fumigatus* at low pH (5.7) which was similar  
298 to ficolin-A [16]. The ability of such pattern recognition molecules to function at decreased

299 pH is important in the defence against microorganisms, with pH at the local site of infection  
300 being observed to drop as low as pH 5.5 during inflammation [23].

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

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

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

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



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

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

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

343 In the present study, we found that L-ficolin opsonized conidia were also capable of inducing  
344 an increase in IL-8 as previously observed for ficolin-A opsonized conidia [16]. In contrast,  
345 opsonization of *A. fumigatus* by L-ficolin led to a significant decrease in IL-8, IL-1 $\beta$ , IL-6,

346 IL-10 and TNF- $\alpha$  production from MDM and neutrophils. In support of our observations, it  
347 was recently observed that ficolin-A could act in an anti-inflammatory manner by binding to  
348 lipopolysaccharide (LPS) and inhibiting LPS-mediated pro-inflammatory responses on  
349 murine mast cells [43]. Additionally, the pattern recognition proteins SP-A and -D modulate  
350 an anti-inflammatory cytokine profile in response to viruses, LPS-induced cytokine and nitric  
351 oxide production, and allergens [44-46].

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

363 The most important clinical observation of our study was the detection of the serum L-ficolin  
364 in BAL of patients lungs diagnosed with invasive *A.fumigatus* infection. Moreover, L-ficolin  
365 could also be detected in the lungs of recipient's infected with *A.flavus*, *Penicillium*  
366 spp., *Acremonium* spp., *Scedoporium apiospermum* and at very low concentration in one  
367 incidence of *S.aureus* infection. This ficolin has, until now, not been reported to be present in  
368 the lung. We postulate that L-ficolin, which is normally produced by the liver, enters the  
369 alveolar space during infection from the blood stream similarly to the related acute phase

370 protein, MBL (a serum collectin), which has also been found in the BAL fluid from infected

371 lungs [49]. Although the current sample size is small (39 patients), ROC analysis has  
372 indicated that the presence of L-ficolin in the lungs of transplant patients could be linked with  
373 fungal infection, but this diagnostic potential will need to be further investigated in larger  
374 clinical trials.

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

382

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392

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528 **Figure legends**

529 **Figure 1. L-ficolin binding to live *A. fumigatus* conidia.** Live *A. fumigatus* conidia ( $5 \times 10^5$ )  
530 were opsonized with  $5 \mu\text{g ml}^{-1}$  L-ficolin in the presence or absence of  $\text{Ca}^{2+}$  and in a range of  
531 pH's from 3.7-10.7 prior to staining and flow cytometric analysis. **(A)** Binding of L-ficolin to  
532 *A. fumigatus* in the presence or absence of  $\text{Ca}^{2+}$ . **AF** represents *A. fumigatus* alone. **+ Abs**  
533 represents the antibody background fluorescence. **BSA** was used as a negative control for  
534 binding. **(B)** Binding of L-ficolin to *A. fumigatus* in pH 3.7-pH 10.7 conditions. Results are  
535 representative of the average of all data points gained from three independent experiments.  
536 Error bars represent SD and significance was determined via two-tailed Students *t*-test. An  
537 asterisk indicates a significant difference:  $p < 0.05$ . MFI, median fluorescence intensity; AF, *A.*  
538 *fumigatus*, Abs, antibodies.

539

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

552 ficolin) or following opsonization by L-ficolin (+L-ficolin). **(G)** Hyphal germination  
553 following incubation of un-opsonized conidia in pH 5.7. The black arrows point to  
554 macrophages containing conidia which makes macrophages appear dark. The white arrow  
555 heads are used to trace single hyphae from MDM. Many hyphae are visible, some of which  
556 are blurry as they are growing in three-dimensions and are out of the focal plane. Or. **(H)** in  
557 pH 7.4. **(I)** Hyphal germination following incubation of L-ficolin opsonized conidia in pH  
558 5.7. Hyphae are present although growth is much less dense. Or. **(J)** in pH 7.4. **(K)** Gate P2  
559 on MDM and *A. fumigatus* used to produce figures **L** and **M**. **(L)** Representative histogram  
560 depicting the killing of conidia in pH 5.7 conditions in the presence or absence of L-ficolin.  
561 Increased FL1-A depicts enhanced killing. **(M)** Viability of un-opsonized or L-ficolin  
562 opsonized conidia after incubation with MDMs. Results are representative of the average of  
563 all data points gained from three independent experiments. Error bars represent SD and  
564 significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant  
565 difference:  $p < 0.05$ . FITC, fluorescein isothiocyanate; MDM, monocyte-derived macrophage;  
566 MFI, median fluorescence intensity; AF, *A. fumigatus*; SSC-A, side scatter; FSC-A, forward  
567 scatter; FL1-A, fluorescence.

568

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

577 in pH 5.7 or pH 7.4 conditions in the presence or absence of L-ficolin. (E) Representative  
578 histogram depicting the uptake of conidia in pH 5.7 conditions in the presence or absence of  
579 L-ficolin. (F) The relative number of phagocytosed FITC-labelled conidia (based upon the  
580 median fluorescence intensity; FL1-A) either un-opsonized (-L-ficolin) or following  
581 opsonization by L-ficolin (+L-ficolin). (G) Hyphal germination following incubation of un-  
582 opsonized conidia in pH 5.7 or. (H) in pH 7.4. (I) Hyphal germination following incubation  
583 of L-ficolin opsonized conidia in pH 5.7 or. (J) in pH 7.4. (K) Gate P2 on neutrophils and *A.*  
584 *fumigatus* used to produce figures L and M. (L) Representative histogram depicting the  
585 killing of conidia in pH 5.7 conditions in the presence or absence of L-ficolin. Increased FL1-  
586 A depicts enhanced killing. (M) Viability of un-opsonized conidia or L-ficolin opsonized  
587 conidia after incubation with neutrophils. Results are representative of the average of all the  
588 data points gained from three independent experiments. Error bars represent the SD and  
589 significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant  
590 difference:  $p < 0.05$ . FITC, fluorescein isothiocyanate; SSC-A, side scatter; FSC-A, forward  
591 scatter; FL1-A, fluorescence.

592

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

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

607

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

622

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

626 infection dependent upon patients classification according to EORTC/MSG criteria. All  
627 samples were tested for fungal infection via *Aspergillus* antigen detection, radiology and  
628 culture. **(B)** ROC curve analysis for L-ficolin detection in fungal-infected transplant patients  
629 compared to non-infected transplant patients. Results are representative of the data points  
630 gained from three independent experiments (19 positive and 20 negative patients). Bars  
631 represent the median and significance was determined via two-tailed Students *t*-test ( $p=$   
632 0.00087). Abbreviation: BAL, bronchoalveolar lavage.

633

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

635 Live freshly harvested *A. fumigatus* conidia ( $5 \times 10^5$ ) were opsonized with  $5 \mu\text{g ml}^{-1}$  L-  
636 ficolin prior to incubation with MDM (conidia:MDM ratio of 5:1) in pH 5.7 and  
637 pH 7.4 conditions for 24 h. **(A)** The death-associated green-yellow fluorescence emitted by  
638 intracellular L-ficolin opsonized or un-opsonized *A. fumigatus*, after incubation with MDM.  
639 **(B)** The death-associated green-yellow fluorescence emitted by extracellular L-ficolin  
640 opsonized or un-opsonized *A. fumigatus*, after incubation with MDM. Results are  
641 representative of the average of all the data points gained from three independent  
642 experiments. Error bars represent the SD and significance was determined via two-tailed  
Students *t*-test. An asterisks indicated difference:  $p < 0.05$ .

643

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

645 Live freshly harvested *A. fumigatus* conidia ( $5 \times 10^5$ ) were 647 opsonized with  $5 \mu\text{g ml}^{-1}$   
646 L-ficolin prior to incubation with neutrophils (conidia:neutrophil ratio of 5:1) in pH 5.7 and

647 pH 7.4 conditions for 24 h. (A) The death-associated green-yellow fluorescence emitted by  
648 intracellular L-ficolin opsonized or un-opsonized *A. fumigatus*, after incubation with  
649 neutrophils. (B) The death-associated green-yellow fluorescence emitted by  
650 extracellular L-ficolin opsonized or un-opsonized *A. fumigatus*, after incubation with  
651 neutrophils. Results are representative of the average of all the data points gained from three  
652 independent experiments. Error bars represent the SD and significance was determined via  
653 two-tailed Students t-test. An asterisks indicated difference:  $p < 0.05$ .

654

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

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