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1 **Serum opsonin ficolin-A enhances host-fungal interactions and modulates**  
2 **cytokine expression from human monocyte-derived macrophages and**  
3 **neutrophils following *Aspergillus fumigatus* challenge**

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## 23 **Abstract**

24 Invasive aspergillosis is a devastating invasive fungal disease associated with a high mortality  
25 rate in the immunocompromised; such as leukaemia patients, transplant patients and those  
26 with HIV/AIDS. The rodent serum orthologue of human L-ficolin, ficolin-A, can bind to and  
27 opsonize *A. fumigatus*, the pathogen that causes invasive aspergillosis, and may participate in  
28 fungal defence. Using human monocyte-derived macrophages and neutrophils isolated from  
29 healthy donors, we investigated conidial association and fungal viability by flow cytometry  
30 and microscopy. Additionally, cytokine production was measured via cytometric bead arrays.  
31 Ficolin-A opsonization was observed to significantly enhance association of conidia, while  
32 also inhibiting hyphal growth and contributing to increased fungal killing following  
33 incubation with monocyte-derived macrophages and neutrophils. Additionally, ficolin-A  
34 opsonization was capable of manifesting a decrease in IL-8, IL-1 $\beta$ , IL-6, IL-10 and TNF- $\alpha$   
35 production from MDM and IL-1 $\beta$ , IL-6 and TNF- $\alpha$  from neutrophils 24 h post-infection. In  
36 conclusion, rodent ficolin-A is functionally comparable to human L-ficolin and is capable of  
37 modulating the innate immune response to *A. fumigatus*, down-regulating cytokine  
38 production and could play an important role in airway immunity.

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40 Key words; aspergillosis; macrophage; neutrophil; cytokines; innate immunity

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## 45 **Introduction**

46 *Aspergillus fumigatus* (*A. fumigatus*) is the most common invasive mold pathogen and is the  
47 primary causative species of the devastating disease, invasive pulmonary aspergillosis (IA) in  
48 immunocompromised hosts. IA is an ever increasing challenge in the developed world and is  
49 associated with a mortality rate of around 30-40% if treated and 100% if left untreated [1].

50 Those most at risk of disease include leukaemia sufferers, solid and haematopoietic transplant  
51 patients, neutropenic and diabetic patients or those undergoing corticosteroid therapies [2-4].

52 *A. fumigatus* infects via the propagation of its conidia (spores) in to the air, which are then  
53 inhaled by the host. Due to the small size of the conidia, those that evade mucocilliary  
54 clearance can invade the depths of the alveolar space where they can germinate into  
55 filamental (hyphal) structures. This invasive stage can lead to thrombosis, tissue necrosis and  
56 dissemination to other organs such as the skin and brain, leading to death [5,6].

57 Defence is initiated by the innate immune system and predominantly comprises of type II  
58 epithelial cells, alveolar macrophages and neutrophils. Macrophages are traditionally  
59 acknowledged to be essential in the phagocytosis and removal of conidia, whereas  
60 neutrophils are necessary for protection against the invasive hyphal stage whereby they  
61 produce fungistatic neutrophil extracellular traps (NETs) and degranulate, releasing  
62 antimicrobial compounds [7-10]. Serum opsonins such as ficolins, proteins similar to the  
63 collectins mannose-binding lectin (MBL) and the surfactant proteins (SP)-A and -D, can  
64 assist and enhance the functions of these host cells.

65 Ficolins are novel opsonins composed of an N-terminal collagen-like domain and a C-  
66 terminal fibrinogen-like (FBG) domain with lectin activity predominantly for the acetylated  
67 carbohydrate, *N*-acetylglucosamine (GlcNAc). However, ficolins do not bind exclusively to  
68 acetylated carbohydrates and can recognise pathogen specific structures such as (1,3)- $\beta$ -D-

69 glucan, lipotechoic acid and LPS, in addition to interacting with acute phase proteins such as  
70 C-reactive protein (CRP) and pentraxin-3 (PTX3) [11]. Humans possess three types of  
71 ficolin; the membrane-bound M-ficolin and the serum ficolins L-ficolin and H-ficolin. Only  
72 orthologues of human L-ficolin and M-ficolin can be found in rodents and are termed ficolin-  
73 A and ficolin-B, respectively.

74 Others have demonstrated that ficolin-A can recognise *A. fumigatus* conidia and we have  
75 recently demonstrated that ficolin-A is capable of recognizing *A. fumigatus* conidia,  
76 increasing the quantity of conidia associating with the A549 type II epithelial cell line and  
77 magnifying the production of pro-inflammatory IL-8 [12,13]. However, the protective roles  
78 of ficolins and whether opsonization can enhance phagocytosis by professional phagocytes  
79 during *Aspergillus* defence is poorly characterised. Therefore, we utilized ficolin-A and  
80 investigated if ficolin-A opsonization of *A. fumigatus* could enhance conidial associations,  
81 contribute to killing or modulate inflammatory cytokine production following incubation with  
82 human macrophages and neutrophils.

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## 90 **Materials and Methods**

### 91 *Fungal pathogens*

92 A clinical isolate of *A. fumigatus* was used as described previously [14]. Resting conidia  
93 were obtained after *A. fumigatus* was subcultured on Sabouraud dextrose agar (Oxoid,  
94 Basingstoke, UK) at 37 °C for 7 days, and conidia were harvested using sterile physiological  
95 saline (Oxoid, Basingstoke, UK). Resting live conidia were used immediately or fixed in 4%  
96 PBS–formaldehyde for 10 min at room temperature (RT), washed, and resuspended in PBS.  
97 Fixed *A. fumigatus* conidia were stored at 4 °C for up to 1 month until further use. To  
98 fluorescently label *A. fumigatus* conidia, up to 10<sup>9</sup> particles were incubated for 24 h at 4°C  
99 with 0.5 mg mL<sup>-1</sup> fluorescein isothiocyanate (FITC; Sigma) in FITC labelling buffer (500  
100 mM NaHCO<sub>3</sub>, 500 mM Na<sub>2</sub>CO<sub>3</sub>, 0.9% (w/v) NaCl), washed repeatedly with PBS and  
101 centrifuged at 775 g for 4 min until the supernatant was clear in colour.

102

### 103 *Ethical approval*

104 Ethical approval for blood donation by healthy participants was obtained from the Faculty of  
105 Health Research Ethics Committee (Ref. Mechanisms of airway diseases – 2008042). Blood  
106 was acquired through venepuncture of healthy participants who gave informed consent at the  
107 time of collection. All donors were not on medication at the time of collection. All  
108 procedures performed in studies involving human participants were in accordance with the  
109 ethical standards of the institutional and/or national research committee and with the 1964  
110 Helsinki declaration and its later amendments or comparable ethical standards.

111

### 112 *Cells and reagents*

113 All experiments were conducted using human monocyte-derived macrophages (MDM) or  
114 peripheral blood neutrophils. MDM and neutrophils were isolated from healthy donor blood  
115 via a 68% percoll gradient modified from Walsh et al (1999) [15]. Monocytes were selected  
116 for by adherence to tissue culture plastic ware for 1h and differentiated in RPMI-1640  
117 supplemented with 10% autologous human serum and 50 I.U ml<sup>-1</sup> penicillin and 50 µg ml<sup>-1</sup>  
118 streptomycin over 5-9 days. Granulocytes were removed from a separate Percoll layer and  
119 were stained with Kimura to determine the percentage of neutrophils in the population.  
120 Neutrophils were briefly maintained in RPMI-1640 supplemented with 10% heat-inactivated  
121 foetal calf serum and 50 I.U mL<sup>-1</sup> penicillin and 50 µg ml<sup>-1</sup> streptomycin (All purchased from  
122 Invitrogen, Paisley, UK). Granulocyte preparations containing greater than 90% neutrophils  
123 and exhibiting >98% viability (as determined by trypan blue staining) were placed in culture.  
124 Recombinant ficolin-A was produced from CHO-DXB11 cells containing ficolin-A cDNA  
125 and purified by affinity chromatography on *N*-acetylglucosamine (GlcNAc)-Sepharose  
126 columns as previously described [16]. Ficolin-A protein was eluted with 50 mM Tris-HCl  
127 (pH 7.5) containing 150 mM NaCl and 300 mM GlcNAc (Sigma-Aldrich, United Kingdom).  
128 Finally, GlcNAc was removed by dialysis and ficolin-A presence confirmed by SDS-PAGE.  
129 This was kindly provided by the laboratory of Prof. Russell Wallis (University of Leicester,  
130 UK). Experiments were all performed in serum-free conditions and data are representative of  
131 a minimum of three independent experiments.

132

### 133 *Association assays*

134 MDM or human neutrophils were seeded in 24-well plates (Thermo Scientific,  
135 Loughborough, UK) in supplemented RPMI-1640 at 37 °C in a 5% CO<sub>2</sub> atmosphere. FITC-  
136 labelled fixed *A. fumigatus* conidia (FL1-A) were opsonized with 5 µg mL<sup>-1</sup> ficolin-A for 1 h  
137 at 37°C. Ficolin-A-opsonized *A. fumigatus* conidia (5 x 10<sup>5</sup>) were incubated for 2 h with

138 adherent MDM or neutrophils in suspension (ratio of conidia to cells of 5:1) at pH 5.7 or pH  
139 7.4 at 37 °C in a 5% CO<sub>2</sub> atmosphere. Following incubation, non-adherent conidia and cells  
140 were removed and the adherent cells were washed with warm supplemented RPMI 1640.  
141 Adherent cells were subsequently removed by the use of trypsin-EDTA and gentle trituration  
142 prior to washing. Cells were fixed in 4% PBS–formaldehyde for 10 min at RT and analyzed  
143 by flow cytometry using a BD Accuri C6 flow cytometer with BD CFlow software. The  
144 percentage of MDMs and neutrophils associated with *A. fumigatus* conidia was determined  
145 by gating on the cell populations based upon their size and granularity (forward scatter  
146 channel/side scatter channel [FSC/SSC]). Additionally, the cells staining positive for conidia-  
147 derived FITC fluorescence were gated to quantify conidial association with cells (FL1-A  
148 [Exλ 488 nm, Emλ 530/33 nm]).

149

#### 150 *Fungal viability assays*

151 Fungal viability was measured using a LIVE/DEAD<sup>®</sup> viability kit (Invitrogen, Paisley, UK).  
152 Assays were conducted as outlined in the protocol. In brief, live resting conidia were  
153 opsonised with 5 µg mL<sup>-1</sup> prior to incubation with MDM or neutrophils for 6 h. Following  
154 cell challenge, conidia were stained with 15 µM FUN-1 cell stain and incubated in the  
155 absence of light for 30 min at 30 °C. FUN-1 stain is metabolised inside the fungus from  
156 yellow-green to orange-red fluorescence if the fungi are metabolically active and possess  
157 intact membranes. Dead fungi display diffuse bright green staining. Following incubation,  
158 supernatants were aspirated and the cells were washed with warm supplemented RPMI-1640.  
159 Adherent cells were subsequently removed by the use of trypsin/EDTA and gentle trituration.  
160 Cells and supernatants were fixed in 4% PBS/formaldehyde for 10 min at RT. Fungal  
161 viability was quantitated by flow cytometry using a BD Accuri C6 flow cytometer with BD  
162 CFlow<sup>®</sup> Software and measuring FL1-A (Exλ 488 nm, Emλ 530/33 nm). Microscopic



163 analysis was conducted after 24 h and growth was observed using an Axiovert 40 CFL  
164 microscope (Zeiss) at 10x objective.

165

#### 166 *Cytokine determination*

167 Cytokine production from supernatants was quantitated using a BD cytometric bead array  
168 (CBA) Human Inflammatory Cytokines kit (BD Biosciences, Oxford, UK). Assays were  
169 conducted as outlined in the protocol. In brief, live un-opsonized or ficolin-A opsonized  
170 conidia (ratio of conidia to cells of 5:1) were added to MDM or neutrophils for 24 h prior to  
171 collection of the supernatant and storage at -80°C. Capture beads for the measurement of IL-  
172 8, IL-1 $\beta$ , IL-6, IL-10 and TNF- $\alpha$  were mixed together prior to their addition to the  
173 supernatant sample and standard tubes. Following the addition of capture beads to the  
174 samples, Human Inflammatory Cytokine PE Detection Reagent was added to all tubes and  
175 incubated for 3 h in the absence of light. Following incubation, samples were washed in wash  
176 buffer for 5 min at 200 g prior to aspiration of the supernatant, re-suspension in wash buffer  
177 and flow cytometry (Ex $\lambda$  488 nm, Em $\lambda$  585/40nm) and (Ex $\lambda$  633 nm, Em $\lambda$  780/30 nm) on a  
178 BD Accuri C6 flow cytometer with BD CFlow<sup>®</sup> Software, collecting 1500 events as outlined  
179 in the protocol.

180

#### 181 *Statistical analysis*

182 Results were expressed as mean  $\pm$  SD. Descriptive and two-tailed Students *t*-test analyses  
183 were performed using GraphPad prism software (version 5). One-way ANOVA's were  
184 performed using SigmaStat software (version 3.5). A value of  $p < 0.05$  was considered  
185 statistically significant.

## 186 **Results**

187 *Ficolin-A opsonization enhances association of A. fumigatus with human monocyte-derived*  
188 *macrophages and contributes to enhancing fungal killing.*

189 Macrophages are the resident lung phagocytes responsible for the removal of *A. fumigatus*  
190 conidia. Therefore, we investigated whether ficolin-A opsonization of *A. fumigatus* could  
191 enhance association with macrophages and contribute to fungal killing. Experiments were  
192 conducted both at pH 5.7 conditions as well as pH 7.4, as we had previously observed acidic  
193 conditions to be optimal for ficolin binding [12].

194 MDM were gated based on size and granularity (FSC-A/SSC-A; Fig. 1A) and association  
195 was determined by the detection of FITC positive cells (Fig. 1B and C). We observed that the  
196 percentage of the MDM population staining positive for conidial association was not altered  
197 (Fig. 1D). However, we observed an increase in fluorescence within the gated cell population  
198 suggesting that the number of FITC-labelled conidia associated with the MDM was  
199 significantly enhanced following ficolin-A opsonization in acidic conditions (based upon the  
200 median fluorescence intensity) (Fig. 1E;  $p=0.00728$ ).

201 Next, we investigated the importance of ficolin-A opsonization to potentiate the ability of  
202 MDM to kill *A. fumigatus* conidia. Both the free and cell-associated *A. fumigatus* were gated  
203 based on size and granularity (Fig. 1F). Fungal viability assays demonstrated a significant  
204 reduction in fungal viability (demonstrated by an increase in green fluorescence) following  
205 opsonization by ficolin-A in acidic conditions (pH 5.7), as observed by flow cytometry (Fig.  
206 1G;  $p=2.2 \times 10^{-5}$ ).

207

208 *Ficolin-A opsonization enhances association of A. fumigatus with human neutrophils and*  
209 *contributes to reducing fungal viability*

210 Neutropenic patients are at significant risk for invasive aspergillosis, highlighting the  
211 importance of neutrophils in *Aspergillus* defence. In particular they are integral in the  
212 destruction of the invasive hyphal stage. Therefore, we also investigated whether ficolin-A  
213 opsonization of *A. fumigatus* could enhance phagocytosis and contribute to the reduction of  
214 fungal viability by human neutrophils that had been isolated from healthy donor blood.

215

216 Neutrophils were gated based on size and granularity (FSC-A/SSC-A; Fig. 2A) and  
217 association was determined by the detection of FITC positive cells (Fig. 2B and C). As for  
218 the macrophages, flow cytometry analysis indicated no difference in the percentage of the  
219 neutrophil population associated with conidia (Fig. 2D). Again however, a significant  
220 increase in the fluorescence indicated that the number of FITC-labelled conidia associated with  
221 the neutrophils (based upon the median fluorescence intensity) was observed following  
222 ficolin-A opsonization in acidic conditions (Fig. 2E;  $p=0.03215$ ).

223

224 As before, we were interested in investigating fungal viability following neutrophil challenge  
225 with un-opsonized or ficolin-A opsonized conidia. Light microscopy indicated that in the  
226 absence of ficolin-A in pH 5.7 or pH 7.4 conditions, hyphal growth was very dense (Fig. 2F,  
227 G). Conversely, following challenge with ficolin-A opsonized conidia in pH 5.7 conditions,  
228 hyphal growth was significantly reduced and branching much less dense (Fig. 2H). A  
229 minimal reduction in hyphal growth was also observed following ficolin-A opsonization in  
230 pH 7.4 conditions (Fig. 2I).

231

232 Additionally, the free and cell-associated *A. fumigatus* were gated based on size and  
233 granularity (Fig. 2J), fungal viability assays demonstrated a significant decrease in fungal  
234 viability following opsonization by ficolin-A in pH 5.7 conditions, as observed by flow  
235 cytometry (Fig. 2K;  $p=0.00262$ ).

236

237 *Ficolin-A opsonization modulates the secretion of inflammatory cytokines from MDM and*  
238 *neutrophils in response to A.fumigatus*

239 Previous observations demonstrated the ability of ficolin-A opsonization to elicit an increase  
240 in IL-8 production from the A549 cell line following live conidial challenge [12]. Whether  
241 ficolin-A could modulate cytokine production from other cell types involved in *Aspergillus*  
242 defence was poorly understood. Therefore, we utilised cytometric bead arrays and measured  
243 the concentration of IL-8, IL-1 $\beta$ , IL-6, IL-10 and TNF- $\alpha$  produced by MDM and neutrophils  
244 following challenge with un-opsonized or ficolin-A opsonized live *A. fumigatus* conidia.

245

246 Indeed, challenge of MDM with ficolin-A opsonized conidia led to a significant reduction in  
247 production of all of the cytokines tested (Fig. 3A-E). Ficolin-A itself in the absence of  
248 conidia was capable of stimulating an increase in IL-6, IL-10 and TNF- $\alpha$  production (Fig.  
249 3C-E). Additionally, following challenge of neutrophils with ficolin-A opsonized conidia we  
250 observed a significant reduction in the production of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (Fig. 4B, C, E).  
251 As with the MDM, unbound ficolin-A was capable of stimulating a significant increase in the  
252 production of IL-8, IL-1 $\beta$ , and TNF- $\alpha$  from neutrophils (Fig. 4A, B, E).

253

254

255

## 256 **Discussion**

257 Our study focused on whether opsonization of *A. fumigatus* by ficolin-A could significantly  
258 enhance *Aspergillus*-phagocyte interactions and modulate cytokine production. This led to a  
259 few new observations. Firstly, ficolin-A opsonization enhanced the quantity of *A. fumigatus*  
260 conidia associated with MDM and neutrophils. Next, ficolin-A opsonization significantly  
261 inhibited hyphal growth and contributed to the reduction of fungal viability in the presence of  
262 phagocytes. Finally, ficolin-A opsonization manifested a reduction in inflammatory cytokine  
263 production from MDM and neutrophils. This led us to postulate that ficolin-A could play an  
264 important role in airway immunity via efficient recognition and removal of *A. fumigatus* and  
265 modulation of cytokine production.

266 We recently demonstrated that in an acidic pH representative of infection/inflammation, the  
267 affinity of ficolin-A binding to *A. fumigatus* conidia was greatly increased and this increase in  
268 recognition subsequently led to enhanced conidia-epithelium interactions [12]. However, the  
269 interactions of ficolin-A opsonized *A. fumigatus* with professional phagocytes were unknown.

270 Following inhalation of *A. fumigatus*, macrophages are essential at ameliorating the early  
271 stages of infection whereby conidia are phagocytosed and destroyed in acidic  
272 phagolysosomes [17,18]. Conversely, neutrophils are traditionally recognised to be recruited  
273 to the site of infection during the later stages (in response to IL-8) where they assist the  
274 inhibition of fungal invasion by degranulation and the production of fungistatic NETs  
275 following adherence to the hyphal cell wall, albeit recently they have also been implicated in  
276 the early phagocytosis of conidia [10,19-21].

277 Initial observations indicated that ficolin-A was capable of enhancing conidial associations  
278 with the murine macrophage cell line Raw 264.7 (data not shown) which prompted the use of  
279 primary cells. Additionally, ficolin-A was also capable of enhancing the association of

280 conidia by both human MDM and neutrophils but only in acidic conditions. We have recently  
281 described that the binding of ficolin-A to *A. fumigatus* occurs with greatest affinity in acidic  
282 conditions, therefore functional enhancement in these conditions comes as no surprise [12].  
283 Potentiation of cell-microbe interactions is not uncommon amongst serum opsonins, as the  
284 human orthologue of ficolin-A, L-ficolin, in addition to the functionally and structurally  
285 similar molecules MBL, SP-A and SP-D, have all been observed to enhance phagocytosis of  
286 pathogenic microorganisms by macrophages and polymorphonuclear cells [22,23,12,24-27].

287 Although phagocytosis is important, conidial killing is even more crucial to sterilize infected  
288 tissues. Therefore we utilised fungal viability assays and investigated the contribution of  
289 ficolin-A to killing. In our study, we demonstrated that ficolin-A opsonization enhanced the  
290 ability of macrophages and neutrophils to significantly inhibit hyphal growth and reduce  
291 fungal viability. The most striking observation was how significantly ficolin-A opsonization  
292 reduced hyphal density following incubation with neutrophils. Conversely, the presence of  
293 ficolin-A almost completely inhibited germination when incubated with MDM.

294 Recent *in vitro* and patient evidence has highlighted that L-ficolin can also enhance  
295 opsonophagocytic killing by human MDM in addition to neutrophils and is present in the  
296 fungal infected lung [28]. Moreover, L-ficolin can interact with the acute phase protein,  
297 PTX3 to enhance recognition and complement deposition [29], indicating a role in antifungal  
298 immunity. However, to date, no *in vivo* studies have been conducted confirming the  
299 importance of ficolin-A in opsonophagocytosis and defence against *A. fumigatus*. However,  
300 there is a body of evidence describing the integral role of the related opsonins, SP-D and  
301 MBL, in airway immunity *in vivo* [25,27,30]. Deficiencies in human L-ficolin have also been  
302 observed to exacerbate susceptibility to recurrent respiratory infections, suggesting that its  
303 rodent orthologue, ficolin-A, could also potentially play a significant role in airway immunity

304 in a transgenic mouse model [31]. This is something we are currently investigating in our  
305 laboratory.

306 As indicated earlier, we have previously observed that ficolin-A opsonization leads to an  
307 increase in IL-8 secretion from A549 cells, a cytokine that is crucial for the recruitment of  
308 neutrophils during *Aspergillus* infection. Cytokines are key regulators of inflammation and  
309 play an essential role in the defence against fungal challenge. Conidial challenge can induce  
310 the production of a wide array of cytokines and chemokines including IL-1 $\beta$ , IL-2, IL-5, IL-6,  
311 IL-8, IL-10, IL-13, IL-17A, IL-22, IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF and MCP-1 [20,12,32-37]. In  
312 this study we highlighted that MDM challenged with ficolin-A opsonized conidia produced  
313 significantly less IL-8, IL-1 $\beta$ , IL-6, IL-10 and TNF- $\alpha$ , whereas only IL-1 $\beta$ , IL-6 and TNF- $\alpha$   
314 production was lower from neutrophils. Ficolins have been described to have the potential to  
315 be both pro- or anti-inflammatory, dependent upon the cell type involved [38,12,39,40]. We  
316 have previously observed that L-ficolin opsonization can modulate an anti-inflammatory  
317 response from MDM and neutrophils, while additionally, others have reported that ficolin-A  
318 can dampen LPS-induced inflammatory responses on mast cells [39,28]. In addition, we  
319 again show that unbound ficolin-A can increase cytokine production. The mechanisms by  
320 which these effects are achieved are currently unknown but preliminary studies in our lab  
321 have indicated the potential importance of the MAPK signalling cascades and TLRs.

322 In conclusion, we demonstrate that ficolin-A plays an important role in potentiating the  
323 functions of macrophages and neutrophils against *A. fumigatus* challenge *in vitro* and is  
324 functionally comparable to human L-ficolin. Additionally, we highlight that ficolin-A may be  
325 important in the down-regulation of cytokine production post-infection but relevant ficolin-A  
326 *in vivo* studies are lacking.

327

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## 334 **Conflict of interest**

335 The authors have no conflicts of interest.

336

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475 **Figure legends**

476 **Fig. 1.** Association and killing of ficolin-A opsonized *A. fumigatus* conidia with MDM.  
477 FITC-labelled *A. fumigatus* conidia ( $5 \times 10^5$ ) were opsonized with  $5 \mu\text{g mL}^{-1}$  ficolin-A prior  
478 to incubation with MDM (conidia:MDM ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 2 h.  
479 To measure killing, live unlabelled conidia were incubated with MDM for 24 h prior to  
480 LIVE/DEAD staining. **A** Gating strategy used to distinguish macrophages. **B** MDM in the  
481 absence of FITC-labelled conidia. **C** MDM in the presence of FITC-labelled conidia. **D** The  
482 percentage of MDM associated with conidia in pH 5.7 and pH 7.4 conditions in the presence  
483 or absence of ficolin-A. **E** The relative number of associated FITC-labelled conidia (based  
484 upon the median fluorescence intensity; FL1-A) either un-opsonized (-ficolin-A) or following  
485 opsonization by ficolin-A (+ficolin-A). **F** Gating strategy used to measure fungal viability. **G**  
486 Fungal death after incubation with MDMs based upon the median fluorescence intensity of  
487 FL1-A. Heat killed conidia were used as a positive control for fungal killing. Results are  
488 representative of the average of all data points gained from three independent experiments.  
489 Error bars represent SD and significance was determined via two-tailed Students *t*-test. An  
490 asterisk indicates a significant difference;  $p < 0.05$ .

491

492 **Fig. 2.** Association and killing of ficolin-A opsonized *A. fumigatus* conidia with neutrophils.  
493 FITC-labelled *A. fumigatus* conidia ( $5 \times 10^5$ ) were opsonized with  $5 \mu\text{g mL}^{-1}$  ficolin-A prior  
494 to incubation with neutrophils (conidia:neutrophil ratio of 5:1) in pH 5.7 and pH 7.4  
495 conditions for 2 h. To measure killing, live unlabelled conidia were incubated with  
496 neutrophils for 24 h prior to LIVE/DEAD staining. **A** Gating strategy used to distinguish  
497 neutrophils. **B** Neutrophils in the absence of FITC-labelled conidia. **C** Neutrophils in the  
498 presence of FITC-labelled conidia. **D** The percentage of neutrophils associated with conidia  
499 in pH 5.7 and pH 7.4 conditions in the presence or absence of ficolin-A. **E** The relative

500 number of associated FITC-labelled conidia (based upon the median fluorescence intensity;  
501 FL1-A) either un-opsonized (-ficolin-A) or following opsonization by ficolin-A (+ficolin-A).  
502 **F** Hyphal germination following incubation of un-opsonized conidia in pH 5.7 or. **G** in pH  
503 7.4. **H** Hyphal germination following incubation of ficolin-A opsonized conidia in pH 5.7 or.  
504 **I** in pH 7.4. **J** Gating strategy used to measure fungal viability. **K** Fungal death after  
505 incubation with neutrophils based upon the median fluorescence intensity of FL1-A. Heta  
506 killed conidia were used as a positive control for fungal killing. Results are representative of  
507 the average of all data points gained from three independent experiments. Error bars represent  
508 SD and significance was determined via two-tailed Students *t*-test. An asterisk indicates a  
509 significant difference;  $p < 0.05$ .

510

511 **Fig. 3.** Inflammatory cytokine release from MDM following challenge with un-opsonized or  
512 ficolin-A opsonized conidia. Supernatants were collected after 8 h and 24 h time points  
513 during challenge of MDM with live *A. fumigatus* conidia ( $5 \times 10^5$ ) either un-opsonized or  
514 ficolin-A opsonized ( $5 \mu\text{g mL}^{-1}$ ) prior to the conduction of cytometric bead arrays  
515 (conidia:MDM ratio of 5:1). **A** Quantity of IL-8 production. **B** Quantity of IL-1 $\beta$  production.  
516 **C** Quantity of IL-6 production. **D** Quantity of IL-10 production. **E** Quantity of TNF- $\alpha$   
517 production. Following *A. fumigatus* challenge. **MDM** is representative of MDM alone. +  
518 **ficolin-A** represents MDM in the presence of ficolin-A alone. + **AF** and +**AF +ficolin-A** are  
519 representative of un-opsonized *A. fumigatus* or ficolin-A opsonized conidia, respectively.  
520 Results are representative of the average of all the data points gained from three independent  
521 experiments. Error bars represent the SD. Significance was determined via one-way ANOVA  
522 and pair-wise comparisons were conducted using the Student-Newman-Keuls method. An  
523 asterisk indicates a significant difference:  $p < 0.05$ .

524

525 **Fig. 4.** Inflammatory cytokine release from neutrophils following challenge with un-  
526 opsonized or ficolin-A opsonized conidia. Supernatants were collected after 8 h and 24 h time  
527 points during challenge of neutrophils with live *A. fumigatus* conidia ( $5 \times 10^5$ ) either un-  
528 opsonized or ficolin-A opsonized ( $5 \mu\text{g mL}^{-1}$ ) prior to the conduction of cytometric bead  
529 arrays (conidia:neutrophil ratio of 5:1). **A** Quantity of IL-8 production. **B** Quantity of IL-1 $\beta$   
530 production. **C** Quantity of IL-6 production. **D** Quantity of IL-10 production. **E** Quantity of  
531 TNF- $\alpha$  production. Following *A. fumigatus* challenge. **Neutrophils** is representative of  
532 neutrophils alone. + **ficolin-A** represents neutrophils in the presence of ficolin-A alone. + **AF**  
533 and +**AF +ficolin-A** are representative of un-opsonized *A. fumigatus* or ficolin-A opsonized  
534 conidia, respectively. Results are representative of the average of all the data points gained  
535 from three independent experiments. Error bars represent the SD. Significance was  
536 determined via one-way ANOVA and pair-wise comparisons were conducted using the  
537 Student-Newman-Keuls method. An asterisk indicates a significant difference:  $p < 0.05$ .  
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