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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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Abstract

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

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

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)-β-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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Materials and Methods

Fungal pathogens

A clinical isolate of *A. fumigatus* was used as described previously [14]. Resting conidia were obtained after *A. fumigatus* was subcultured on Sabouraud dextrose agar (Oxoid, Basingstoke, UK) at 37 °C for 7 days, and conidia were harvested using sterile physiological saline (Oxoid, Basingstoke, UK). Resting live conidia were used immediately or fixed in 4% PBS–formaldehyde for 10 min at room temperature (RT), washed, and resuspended in PBS. Fixed *A. fumigatus* conidia were stored at 4 °C for up to 1 month until further use. To fluorescently label *A. fumigatus* conidia, up to 10^9 particles were incubated for 24 h at 4°C with 0.5 mg mL^{-1} fluorescein isothiocyanate (FITC; Sigma) in FITC labelling buffer (500 mM NaHCO$_3$, 500 mM Na$_2$CO$_3$, 0.9% (w/v) NaCl), washed repeatedly with PBS and centrifuged at 775 g for 4 min until the supernatant was clear in colour.

Ethical approval

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. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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^{-1} penicillin and 50 µg ml^{-1}

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^{-1} penicillin and 50 µg ml^{-1} 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_2 atmosphere. FITC-

136 labelled fixed *A. fumigatus* conidia (FL1-A) were opsonized with 5 µg mL^{-1} ficolin-A for 1 h

137 at 37°C. Ficolin-A-opsonized *A. fumigatus* conidia (5 x 10^5) 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_2 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® 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^{-1} 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® 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-1β, IL-6, IL-10 and TNF-α 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 (Exλ 488 nm, Emλ 585/40nm) and (Exλ 633 nm, Emλ 780/30 nm) on a

178 BD Accuri C6 flow cytometer with BD CFlow® Software, collecting 1500 events as outlined

179 in the protocol.

180

181 *Statistical analysis*

182 Results were expressed as mean ± 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.

Results

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

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

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

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

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 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-1β, IL-6, IL-10 and TNF-α 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-α 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-1β, IL-6 and TNF-α (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-1β, and TNF-α from neutrophils (Fig. 4A, B, E).

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254

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Discussion

256 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-1β, IL-2, IL-5, IL-6,

311   IL-8, IL-10, IL-13, IL-17A, IL-22, IFN-γ, TNF-α, 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-1β, IL-6, IL-10 and TNF-α, whereas only IL-1β, IL-6 and TNF-α

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

Acknowledgements

Conflict of interest

The authors have no conflicts of interest.

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Figure legends

Fig. 1. Association and killing of ficolin-A opsonized *A. fumigatus* conidia with MDM. FITC-labelled *A. fumigatus* conidia (5 x 10^5) were opsonized with 5 µg mL^{-1} ficolin-A prior to incubation with MDM (conidia:MDM ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 2 h. To measure killing, live unlabelled conidia were incubated with MDM for 24 h prior to LIVE/DEAD staining. **A** Gating strategy used to distinguish macrophages. **B** MDM in the absence of FITC-labelled conidia. **C** MDM in the presence of FITC-labelled conidia. **D** The percentage of MDM associated with conidia in pH 5.7 and pH 7.4 conditions in the presence or absence of ficolin-A. **E** The relative number of associated FITC-labelled conidia (based upon the median fluorescence intensity; FL1-A) either un-opsonized (-ficolin-A) or following opsonization by ficolin-A (+ficolin-A). **F** Gating strategy used to measure fungal viability. **G** Fungal death after incubation with MDMs based upon the median fluorescence intensity of FL1-A. Heat killed conidia were used as a positive control for fungal killing. 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; $p < 0.05$.

Fig. 2. Association and killing of ficolin-A opsonized *A. fumigatus* conidia with neutrophils. FITC-labelled *A. fumigatus* conidia (5 x 10^5) were opsonized with 5 µg mL^{-1} ficolin-A prior to incubation with neutrophils (conidia:neutrophil ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 2 h. To measure killing, live unlabelled conidia were incubated with neutrophils for 24 h prior to LIVE/DEAD staining. **A** Gating strategy used to distinguish neutrophils. **B** Neutrophils in the absence of FITC-labelled conidia. **C** Neutrophils in the presence of FITC-labelled conidia. **D** The percentage of neutrophils associated with conidia 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 (5×10^5) either un-opsonized or

514 ficolin-A opsonized ($5 \ \mu g \ mL^{-1}$) 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-1β production.

516 **C** Quantity of IL-6 production. **D** Quantity of IL-10 production. **E** Quantity of TNF-α

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 x 10^5) either un-

528 opsonized or ficolin-A opsonized (5 µg 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-1β

530 production. **C** Quantity of IL-6 production. **D** Quantity of IL-10 production. **E** Quantity of

531 TNF-α 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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