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Differential interactions of serum and bronchoalveolar lavage complement proteins with conidia of airborne fungal pathogen *Aspergillus fumigatus*

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1 **Differential interactions of serum and bronchoalveolar lavage complement**
2 **proteins with conidia of airborne fungal pathogen *Aspergillus fumigatus***

3
4 **Running title:** Complement-system and *Aspergillus fumigatus* conidia

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6 Sarah Sze Wah Wong¹, Irene Daniel², Jean-Pierre Gangneux³, Jeya Maheshwari Jayapal²,
7 H el ene Guegan³, Sarah Delli ere^{1,4}, Prajna Lalitha⁵, Rajashri Shende⁶, Taruna Madan⁷, Jagadeesh
8 Bayry⁸, J. I naki Gujjarro⁹, Dharmalingam Kuppamuthu², Vishukumar Aimaganianda^{1*}

9
10 ¹Institut Pasteur, Molecular Mycology Unit, CNRS, UMR2000, Paris, France; ²Department of
11 Proteomics and Ocular Microbiology, Aravind Medical Research Foundation, Madurai, Tamil
12 Nadu, India; ³University of Rennes, CHU Rennes, Inserm, EHESP, Irset (Institut de Recherche
13 en sant e, environnement et travail) - UMR_S 1085, F-35000, Rennes, France; ⁴Parasitology-
14 Mycology Laboratory, Groupe Hospitalier Saint-Louis-Lariboisi ere-Fernand-Widal, Assistance
15 Publique-H opitaux de Paris, Universit e de Paris, Paris, France; ⁵Department of Ocular
16 Microbiology, Aravind Eye Hospital, Madurai, Tamil Nadu, India; ⁶National Centre for Cell
17 Sciences, University of Pune, Pune, Maharashtra, India; ⁷National Institute for Research in
18 Reproductive Health, Indian Council of Medical Research, Mumbai, Maharashtra, India; ⁸Institut
19 National de la Sant e et de la Recherche M edicale, Centre de Recherch e des Cordeliers, Sorbonne
20 Universit e, Universit e de Paris, Paris, France; ⁹Institut Pasteur, Biological NMR Technological
21 Platform, CNRS UMR 3528, Paris, France.

22
23 *Correspondance : V. Aimaganianda (E-mail : vkumar@pasteur.fr; Tel : +33-145688225)

24 **ABSTRACT**

25 Even though both cellular and humoral immunities contribute to host defense, the role played by
26 humoral immunity against the airborne opportunistic fungal pathogen *Aspergillus fumigatus* has
27 been underexplored. In this study, we aimed at deciphering the role of complement-system, the
28 major humoral immune component, against *A. fumigatus*. Mass-spectrometric analysis of the
29 proteins extracted from *A. fumigatus* conidial (asexual spores and infective propagules) surfaces
30 opsonized with human serum indicated that C3 is the major complement protein involved. Flow
31 cytometry and immunolabelling assays further confirmed C3b (activated C3) deposition on the
32 conidial surfaces. Assays using cell wall components of conidia indicated that the hydrophobin
33 RodAp, β -(1,3)-glucan (BG) and galactomannan (GM) could efficiently activate C3. Using
34 complement component-depleted sera, we show that while RodAp activated C3 by the
35 alternative pathway, BG and GM partially follow the classical and lectin pathways, respectively.
36 Opsonization facilitated conidial aggregation and phagocytosis, and complement receptors (CR3
37 and CR4) blockage on phagocytes significantly inhibited phagocytosis, indicating that the
38 complement-system exerts a protective role against conidia by opsonizing them and facilitating
39 their phagocytosis mainly through complement receptors. Conidial opsonization with human
40 bronchoalveolar lavage fluid (BALF) confirmed C3 to be the major complement protein
41 interacting. Nevertheless, complement C2 and mannose binding lectin (MBL), the classical and
42 lectin pathway components respectively, were not identified, indicating that BALF activates the
43 alternative pathway on the conidial surface. Moreover, the cytokine profiles were different upon
44 stimulation of phagocytes with serum or BALF opsonized conidia, highlighting the importance
45 of studying the conidial interaction in their biological niche.

46

47 **Keywords:** *Aspergillus fumigatus* conidia; cell wall; polysaccharides; humoral immunity;
48 complement-system; complement receptors.

49 **INTRODUCTION**

50 *Aspergillus fumigatus* is a saprophyte, but also an opportunistic human fungal pathogen. It
51 propagates through conidia that are airborne, and are constantly inhaled (1). To establish an
52 invasive infection, conidia have to cross respiratory barrier that includes epithelial/mucous layers
53 in the upper respiratory tract. While, conidia reaching the distal part (lung-alveoli) of the
54 respiratory system have to further confront with both cellular and humoral immune barriers. The
55 cellular immunity is provided by residing alveolar macrophages and recruited neutrophils. The
56 humoral immune system consists of the complement proteins, collectin, antimicrobial peptides,
57 acute phase proteins and immunoglobulins. Among these, the complement-system has been
58 speculated to play an important role against *A. fumigatus* conidia (2, 3).

59

60 The activation of the complement-system consists of a cascade of reactions through classical,
61 lectin or alternative pathways (4) that differ by their activation complexes formed, but converge
62 in C3b formation. With *A. fumigatus*, the main effect of complement-system is executed through
63 opsonization by C3b that has been shown to bind to the *A. fumigatus* conidial surface (5-7). It
64 was shown that *A. fumigatus* conidia activate the alternative pathway, whereas, swollen conidia
65 and mycelial morphotypes activate the classical and lectin pathways (7). *Aspergillus fumigatus*
66 conidia are covered by a cell wall (CW), consisting of a proteinaceous rodlet layer, a melanin
67 pigment layer, and an inner CW is composed of different polysaccharides including β -(1,3)-
68 glucan (BG), α -(1,3)-glucan, chitin and galactomannan (GM) (1, 8, 9). The identity of the
69 conidial cell wall ligands associated with the activation of different complement pathways
70 remain to be elucidated. Moreover, the complement activation should result in the formation of a
71 membrane attack complex (MAC), damaging the pathogen-membrane and causing lysis of the
72 pathogens. Nevertheless, the presence of a thick CW in fungi has been reasoned to prevent the
73 lysis of fungal cell (10), however experimental evidence is lacking.

74

75 Our study was aimed at identifying the complement components interacting with *A. fumigatus*
76 conidia, determining the role of conidial CW components in activating complement pathways,
77 and studying the role of the humoral immune system against *A. fumigatus*. We show that among
78 the proteins interacting with the conidial surface, complement protein C3 is the prominent
79 component. Assays using individual conidial CW components indicated that RodAp, BG and
80 GM were the main components involved in C3 activation. We observed that C3 opsonization
81 facilitates conidial aggregation and phagocytosis, and that complement receptors are mainly
82 involved in the conidial phagocytosis. Being airborne, conidia interact first with alveolar
83 environment; therefore, we compared conidial opsonization with human serum and BALF.
84 Although conidial opsonization with serum or bronchoalveolar lavage fluid (BALF) confirmed
85 C3 to be the major complement component binding to the conidial surface, there were significant
86 differences in the interaction of other complement proteins, and the cytokines secreted upon
87 phagocytosis of these opsonized conidia with human monocyte derived macrophages (hMDM),
88 indicating the importance of the source of humoral immune components in immune response.

89

90 **RESULTS**

91 *Complement proteins interact with A. fumigatus conidial surface*

92 **Table 1** lists the complement proteins extracted from the conidial surface opsonized with human
93 serum and identified using mass-spectrometric approach. Proteins extracted with NH₂OH
94 represent strongly bound ones, while those extracted by NaSCN are weakly bound proteins. The
95 peptide spectrum match (PSM; the total number of identified peptide spectra matched for a
96 protein) score was high for the NH₂OH extractable complement protein C3, suggesting that C3
97 strongly interacts with the conidial surface. Other complement components found in the NH₂OH
98 extract were (in decreasing order of abundance): Complement Factor-H (CFH), C4B, C1q, C1r,
99 C2, C5, C1s, C9, C6, C7, C8, Complement Factor-D (CFD), properdin, Complement Factor-I
100 (CFI), Mannose Binding Lectin (MBL), MBL-Associated Serine Proteases 1 and 2 (MASP1 and

101 MASP2). Although identified in the NH₂OH extract, C5, C9, C6, C7 and C8 were found more
102 abundantly in the NaSCN fraction, suggesting their weaker interaction with conidia.
103 Identification of the complement proteins C2, C4B, MBL, MASP1 and MASP2 was indicative
104 of the activation of the complement system by the classical and lectin pathways. However, the
105 absence of Complement Factor-B (CFB) was suggestive of the lack of alternative pathway
106 activation loop. The complement proteins C5, C6, C7, C8 and C9 are the components of the
107 MAC. However, the PSM scores for C6-C8 were lower, and that for C9 did not correspond to a
108 multimer, suggesting the absence of MAC. Indeed, opsonized conidial immunolabelling with
109 anti-MAC antibodies was negative (data not shown). Interestingly, ficolin, a component of the
110 lectin pathway and an alternative for MBL, was not found in both the NH₂OH and NaSCN
111 extracted fractions.

112

113 Flow cytometry analysis confirmed the deposition of C3b on the conidial surface upon
114 opsonization with serum (both in-house and commercial sera tested were positive; for clarity, the
115 data for the commercial sera is presented) and with purified C3 (**Figures 1A, 1B**). The direct
116 deposition of C3b upon interaction with C3 was suggestive of the activation of the alternative
117 pathway on the conidial surface. There was positive immunolabelling with anti-C3b antibodies
118 on conidia opsonized with C3 (**Figure 1C**), confirming C3 activation on the conidial surfaces.

119

120 ***Complement activation capacity of the *A. fumigatus* conidial cell wall components***

121 Since CW is the first component of *A. fumigatus* conidia interacting with the host immune
122 system, we then looked at the complement activation capacity of the individual CW components
123 of conidia; the readout was the conversion of C3 into C3b by the CW components. Of the
124 different CW-components, RodAp, BG and GM efficiently activated C3, but not melanin
125 pigment, chitin or α -(1,3)-glucan. GM showed the highest C3 activation, followed by BG and
126 RodAp (**Figure 2A**).

127

128 Conversion of C3 into C3b could take place via the classical, lectin and/or alternative pathways,
129 with the first two pathways being $\text{Ca}^{+2}\text{-Mg}^{+2}$ dependent (11). The C3 activation capacity of
130 RodAp was unaltered in the presence/absence of $\text{Ca}^{+2}\text{-Mg}^{+2}$, suggestive of C3 activation by
131 RodAp through the alternative pathway. In agreement, C3b deposition on RodAp was
132 significantly lower with CFB-depleted serum even in the presence of $\text{Ca}^{+2}\text{-Mg}^{+2}$. In contrast, in
133 the absence of $\text{Ca}^{+2}\text{-Mg}^{+2}$ there was a significant reduction in the C3 activation by both BG and
134 GM (**Figure 2B**), indicating that these two CW-polysaccharides activate C3 partially by
135 alternative and classical or lectin pathways, respectively.

136

137 Complement components C1q and C4 are associated with the classical pathway, MBL with the
138 lectin pathway; immunoglobulins are involved in both the classical and the alternative pathways,
139 while CFB participates only in the alternative pathway. When MBL-depleted serum was used,
140 there was a modest (~15%) reduction in the C3 activating capacity of BG, but C1q- or C4-
141 depleted sera resulted in about 50% reduction, confirming that BG activates C3 partially through
142 the classical pathway (**Figure 2C**). Immunoglobulin-depleted serum resulted in ~70% reduction
143 in the C3 activation by BG, indicating that the immunoglobulin mediated classical or alternative
144 pathways are the major contributor to C3 activation by BG. MBL-depleted serum resulted in
145 ~70% reduction in the C3 activation by GM, while with C1q, C4 and immunoglobulin-depleted
146 sera there was about 40-50% reduction, suggesting that GM activates C3 majorly through the
147 lectin pathway (**Figure 2D**). With CFB-depleted serum in gelatin-veronal buffer (GVB)
148 supplemented with $\text{Ca}^{+2}\text{-Mg}^{+2}$ (GVB++), there was only a partial decrease in the C3 activation
149 by BG and GM, confirming that BG and GM activate the complement system partially through
150 the classical and lectin pathways (**Figures 2C and 2D**).

151

152 ***Conidial surface rodlets interact with complement C3***

153 The deposition of C3b on the conidial surface upon opsonization with C3 was concentration
154 dependent (**Figure 3A**), suggesting a specific interaction between the conidial surface and C3.
155 The dormant conidial surface is covered by a rodlet layer, made up by the protein RodAp that
156 belongs to the family of hydrophobins; however, melanin is exposed at some areas on the
157 conidial surface (1, 12). Melanin did not activate any of the complement pathways (**Figure 1A**);
158 therefore, we looked at the possible interaction between RodAp and the complement C3. In an
159 ELISA assay, there was a concentration dependent interaction between RodAp and C3 (**Figure**
160 **3B**). To further test this interaction, opsonization, extraction of conidial surface RodAp from
161 conidia using hydrofluoric acid (HF) followed by SDS-PAGE (**Figure 3C**), Western blot of the
162 extract probed with either anti-RodAp (**Figure 3D**) or anti-C3/anti-C3b antibodies were
163 performed (**Figure 3E**). Western blots displayed multiple bands upon probing with anti-C3
164 antibody; importantly, a band at round a M_w of ~68 kDa, was observed when probed with either
165 anti-RodAp or anti-C3b antibodies, suggesting a covalent linkage between C3b and RodAp. At
166 the same time, when the blot was probed with anti-RodAp antibody, there were additional bands
167 to the one at 68 kDa, suggesting that conidial surface rodlets can interact with other components
168 of the complement-system/humoral immune components.

169

170 ***Complement proteins facilitate conidial phagocytosis via complement receptors***

171 Opsonization is described to lead to microbial aggregation, phagocytosis or MAC formation,
172 ultimately causing microbial lyses. Having ruled out MAC formation on *A. fumigatus* conidia
173 based on our proteomic/immunolabelling data, we tested other possible roles of opsonization.
174 Bright field microscopy showed conidial aggregation following opsonization by serum (**Figure**
175 **4A**). Upon interaction with hMDM, the opsonized conidia were phagocytosed at significantly
176 higher rate (**Figure 4B**), indicating that opsonization facilitates conidial phagocytosis.

177

178 Since C3 is the predominant complement protein interacting with conidia and opsonization
179 facilitated conidial phagocytosis, we hypothesized that complement receptors might play a role
180 in conidia phagocytosis. Accordingly, when complement receptors CR3 and CR4 on hMDM
181 were blocked using anti-CD11b, anti-CD11c and anti-CD18 antibodies, there was a significant
182 decrease in the phagocytosis of opsonized conidia (**Figures 4B, 4C**), suggesting that
183 complement receptors are indeed involved in conidia phagocytosis. We confirmed that the
184 CR3/CR4 blockage using antibodies did not affect the viability of hMDM. Nevertheless, even
185 after blocking the CR3 and CR4, there was still a significant uptake of unopsonized conidia
186 (**Figure 4B**), suggesting that conidial recognition and phagocytosis could occur independent of
187 complement opsonization and complement receptors.

188

189 *Human serum and bronchoalveolar lavage fluid (BALF) display differential complement*
190 *component interaction with A. fumigatus conidia*

191 **Table 2** shows the identification of complement proteins extracted from the conidial surface
192 opsonized with BALF. Similar to serum opsonized conidia, BALF opsonized conidia showed C3
193 as the major complement component interacting with the conidial surface. However, binding of
194 other complement components varied between serum and BALF opsonized conidia (**Table 3**).
195 Complement proteins C2, MBL, CFI, MASP1 and MASP2, found in the serum opsonized
196 conidial extract, were absent in the BALF opsonized conidial extract. Moreover, CFB was found
197 only in the conidial protein extract opsonized with BALF. These observations suggested
198 complement activation through the classical and lectin pathways upon opsonization with serum,
199 in contrast to the BALF opsonized conidia for which only the alternative pathway was
200 operational. Ficolin, a complement factor also involved in the activation of the lectin pathway,
201 was not found in either serum or BALF opsonized conidial surface protein extracts. In addition
202 to complement proteins, we also observed a differential binding of surfactant proteins A and D
203 (SP-A/SP-D, the C-type lectins belonging to the collectin family) upon conidial opsonization

204 with serum and BALF; SP-A and SP-D were extracted from the BALF opsonized conidia even
205 through their PSM scores were lower; however, they were not identified in the serum opsonized
206 conidial extract.

207

208 Flow cytometry analysis indicated that conidial opsonization with BALF results in C3b
209 deposition and conidial aggregation (**Figures 5A, 5B**), similarly to opsonization with serum. We
210 looked at the cytokine response upon stimulating hMDM with serum or BALF opsonized
211 conidia. Although significantly higher than the control (medium), the secretion of IL-10 (anti-
212 inflammatory cytokine) from hMDM stimulated with unopsonized and serum/BALF opsonized
213 conidia were not significantly different. Whereas, the secretion of pro-inflammatory cytokines
214 TNF- α and IL-6 was significantly higher upon stimulation of hMDM with serum-opsonized
215 conidia, IL-1 β secretion was higher with BALF-opsonized conidia, and IL-8 (chemoattractant)
216 secretion was higher with serum-opsonized conidia. Of note, the secretion of TNF- α , IL-6 and
217 IL-8 was not significantly different upon stimulating hMDM with unopsonized or BALF-
218 opsonized conidia (**Figure 5C**).

219

220 *Opsonization results in conidial killing through reactive intermediates*

221 We further studied the effect of opsonization on conidial killing efficiency of phagocytes. THP1
222 cells (human leukemic monocytes) were incubated with opsonized (serum/BALF) or
223 unopsonized conidia for 6 h; the viability of the conidia in the interaction mixture was then
224 examined. The percentage of live conidia was significantly lower in opsonized condition than in
225 the control and unopsonized conditions (**Figure 6A**). Moreover, THP1 cells co-incubated with
226 opsonized conidia produced significantly higher level of Reactive Oxygen Species (ROS)
227 compared to control THP1 cells (incubated with culture medium alone) or THP1 cells interacted
228 with unopsonized conidia (**Figure 6B**).

229

230 **DISCUSSION**

231 In the present study, we have identified (i) complement proteins interacting with *A. fumigatus*
232 conidia, (ii) conidial cell wall ligands interacting with C3, a central protein of the complement-
233 system, (iii) complement pathways activated by those cell wall components and (iv) the
234 biological importance of conidial opsonization (conidial aggregation, recognition, phagocytosis,
235 ROS production and killing). Being airborne, *A. fumigatus* conidia enter up to lung-alveoli
236 through breath; therefore, inhaled conidia first contact the alveolar environment. Nonetheless,
237 studies using human BALF were lacking. In our study, we used both human serum and BALF,
238 and compared complement proteins interacting with conidia. Although C3 is the major
239 complement component interacting with the conidial surface irrespective of the source (serum or
240 BALF), there were substantial differences in the abundance as well as the nature of other
241 complement proteins interacting with conidia. Furthermore, serum or BALF opsonized conidial
242 interactions with hMDM resulted in distinct cytokine profiles, indicating that the immune-
243 stimulation pathways elicited by conidia differ with its opsonization source.

244

245 It has been previously shown that *A. fumigatus* conidia predominantly activate the alternative
246 pathway (5, 7). Confirming that conidia activate C3 through the alternative pathway, we
247 observed that the depletion of Ca^{+2} - Mg^{+2} during interaction between C3 and RodAp, the protein
248 that coats the conidial surface, had no effect on C3 activation. C1q and MBL were also shown to
249 bind to dormant conidia of *A. fumigatus*, activating the classical and lectin pathways (13-15).
250 Ficolin, the other lectins responsible in activating the lectin pathway, was also shown to bind to
251 *A. fumigatus* (15-17). However, these observations were based on the interaction of conidia with
252 serum. In contrast to sera opsonized conidial studies, MBL and the mannose-binding serine
253 proteases (MASP1 and MASP2) were not pulled-out in our proteomic analysis of the BALF
254 opsonized conidia, suggesting the absence of the lectin pathway upon conidial opsonization with

255 BALF. Although C1q was identified in both serum and BALF opsonized conidial protein
256 extracts, C2, one of the complement components required for the activation of the classical
257 pathway, was absent in the BALF opsonized conidial extract. The importance of C1q binding to
258 the conidial surface is obscure with this data, as C1q is a component of the classical pathway.
259 The structure of C1q resembles that of collectins, the soluble pattern recognition receptors (PRR)
260 (18); the collectin receptors share binding sites with C1q (19). Therefore, C1q may function as a
261 conidia-recognizing PRR, although this hypothesis needs to be validated. Of note, it has been
262 reported that C1q knockout mice are susceptible to invasive aspergillosis (20). Pentraxin-3
263 (PTX3) is involved in the complement activation via classical pathway by recruiting C1q to the
264 microbial surfaces (21). We could identify PTX3 in the pooled BALF sample after subjecting it
265 to in-solution digestion followed by proteomic analysis, but not in the BALF-opsonized conidial
266 protein extract. This could be possibly due to the overwhelming C3 recruitment on the conidial
267 surface through the activation of alternative pathway or due to the technical issues (in-gel
268 digestion) in identifying PTX3 if its PSM score in the extracted sample is significantly low.

269

270 The studies of complement activation by *A. fumigatus* have always utilized intact fungus. As a
271 result, the conidial CW, the first fungal component to interact with the host immune system and
272 responsible for complement activation remained unclear up to date. The *A. fumigatus* CW is a
273 dynamic and immunomodulating component, of which the composition changes across the
274 fungal morphologies (22). Understanding the interaction between the complement-system and
275 the CW components is essential in elucidating how the fungal pathogen is eliminated in healthy
276 hosts, as inhaled conidial phagocytosis may not be an immediate process (23). We found that
277 RodAp and the two CW polysaccharides, BG and GM could activate the complement-system, in
278 contrast with the rest of the CW components. It has been shown that there is stage-specific
279 exposure of BG during conidial germination, and an atomic force microscopic analysis using a

280 ConA-ligated probe indicated at least 5-7% mannan positivity on the conidial surface (24),
281 suggesting BG and GM could play roles in the partial activation of the complement-system.

282

283 The melanin pigments present in the *A. niger* and *Cryptococcus neoformans* CWs are of
284 dihydroxyphenylalanine (DOPA) origin, and were shown to activate the alternative pathway and
285 to bind activated C3 (25). However, melanin pigment in the CW of *A. fumigatus* is dihydroxy-
286 naphthalene (DHN) derived (26), and in our assay it failed to activate the complement-system. In
287 *A. fumigatus* conidia, melanin pigments form a layer underneath the rodlet layer, and at places it
288 is exposed on the conidial surface (12). Upon disrupting a major enzyme in the *A. fumigatus*
289 DHN melanin biosynthesis, the deposition of C3 was enhanced, which suggested that the intact
290 DHN melanin indeed impairs C3 binding to the conidial surface (27). On the other hand, chitin,
291 a polymer of *N*-acetylglucosamine, should be a ligand for ficolin, a lectin containing a
292 collagenous domain and a fibrinogen domain that recognizes *N*-acetylated compounds (28).
293 Ficolin has been shown to bind to chitin and activate the complement-system (17) as well as to
294 activate the alternative pathway in human plasma (29). However, in the present work, although
295 significant compared to control, we did not observe a major C3b deposition on chitin upon
296 opsonization with human serum. This discrepancy could be explained by the fact that in the
297 earlier studies, crustacean chitin (extracted from crab or shrimp) was used, while we used chitin
298 isolated directly from *A. fumigatus* conidial cell wall.

299

300 In our view, the role of the complement-system in host defense against pathogenic fungi has not
301 been given enough attention. This could be due to the presumed resistance of the thick fungal
302 CW to the complement-based MAC complex. In accordance, we did not see MAC formation on
303 the *A. fumigatus* conidial surface. However, opsonization is known to render its effector function
304 causing microbial agglutination (19). We observed conidial aggregation upon opsonization with
305 serum as well as BALF. Moreover, the complement-system plays a critical role in host

306 elimination of the fungal pathogens through opsonin-mediated phagocytosis and facilitation of
307 the inflammatory response (10, 11, 30). In agreement, we observed that conidial opsonization
308 resulted in significant increase in the conidial phagocytosis as well as their killing.

309

310 The receptors involved in fungal recognition dictate immune responses. It has been shown that
311 Dectin-1 binds to BG to recognize fungi (31) and that Toll-Like Receptor-2 (TLR2) is implicated
312 in the crosstalk between fungal cell wall α -(1,3)-glucan and human dendritic cells (32).
313 Although, β -/ α -(1,3)-glucans are the major components of the conidial cell wall, they are
314 covered by the melanin and rodlet layers (1, 8). Recently, we showed that the C-type lectin
315 MelLec, expressed by the human myeloid immune cells, recognizes the *A. fumigatus* conidial
316 surface melanin pigment (12). Nevertheless, the study of *MELLE*C knockout mice and single
317 nucleotide polymorphism analysis in humans indicated that this receptor is implicated in the
318 dissemination of the fungal infection. Moreover, we also showed that Dectin-1 inhibition only
319 partially blocks BG-uptake by hMDM (33). Since immunoglobulins is rich in BALF (34), we
320 suspected the involvement of Fc γ R (receptors recognizing immunoglobulin G) in the uptake of
321 immunoglobulin-opsonized conidia. However, there was no difference in the opsonized conidial
322 uptake by hMDM with all the three Fc γ R blocked [CD16 (Fc γ R-III), CD32 (Fc γ R-II) and CD64
323 (Fc γ R-I) with respective monoclonal antibodies] compared to opsonized conidial uptake by
324 hMDM, in agreement with the *in vivo* data where the conidial uptake by alveolar neutrophils of
325 FcR- γ II knockout mice was comparable to that of wild-type mice (35). Thus, it was still unclear
326 which receptors were involved in the conidial recognition. Complement receptors CR3 and CR4
327 are the receptors known to recognize activated C3 fragments (36). In our study, upon blocking
328 these two CRs on hMDM, we observed a significant decrease in the opsonized conidial
329 phagocytosis, suggesting that CR3 and CR4 are the major receptors involved in the recognition
330 of *A. fumigatus* conidia, facilitating their phagocytosis. However, there was a significant (~35%)

331 amount of unopsonized as well as opsonized conidial phagocytosis even after complement
332 receptor blockage, suggesting that there are other receptors involved.

333

334 A common practice while performing *in vitro* cell culture assays is to supplement medium with
335 serum. However, *A. fumigatus* is an airborne pathogen and its conidia are first confronted with
336 the alveolar environment. With a panel of five cytokines, we showed here that the induction of
337 cytokines when hMDM encounters conidia opsonized with serum and BALF are different.
338 Higher TNF- α , IL-6 and IL-8 secretions and a lower secretion of IL-1 β with serum opsonized
339 conidia compared to BALF opsonized conidia were observed, with no significant difference in
340 the IL-10 secretion. TNF- α , IL-1 β and IL-6 are involved in the proinflammatory response. IL-1 β
341 contributes to the augmentation of antimicrobial properties of phagocytes as well as to the
342 differentiation of T cells in to Th1/Th17 cells (37, 38) and expansion of Th17 cells (39), TNF- α
343 maintains a normal innate immune response when an infection is encountered (40, 41), whereas
344 IL-6 has been shown to induce IL-17 production upon *Aspergillus* infection (42). Thus, a
345 significantly higher secretion of IL-1 β by hMDM, compared to other cytokines, upon interaction
346 with BALF-opsonized conidia may indicate a beneficial role in the clearance of *A. fumigatus*
347 conidia through multiple axes. Classically, inflammasomes are thought to be critical for the
348 release of IL-1 β ; however, IL-1 β release could also be TLR-mediated (38). Also, it has been
349 demonstrated that TLR2 aggregates at the site of conidial phagocytosis (43). Therefore, even
350 after the blockage of complement receptors, a significant conidial phagocytosis by hMDM leads
351 us to speculate that there might be cross-talks between complement proteins/humoral immunity
352 and TLRs.

353

354 We demonstrated that *A. fumigatus* conidial surface rodlet layer masks conidial recognition by
355 immune cells (1). In agreement, there was no cytokine production when *para*-formaldehyde

356 (PFA)-fixed (inactivated) conidia were made to interact with hMDM in a medium supplemented
357 with human serum, suggesting that metabolically active conidia are essential for phagocytes to
358 mount an antifungal defense mechanism. CR3 mediated phagocytosis has been considered to be
359 a silent mode of entry for pathogens, resulting in limited induction of pro-inflammatory
360 cytokines (44, 45). In line, even at hMDM and conidial multiplicity-of-infection ratio of 2:1, the
361 proinflammatory cytokines secreted by hMDM in our study system was not very high [the
362 positive control lipopolysaccharide (10 ng/well) used in our study resulted in the secretion of
363 6792 ± 69 pg/mL of TNF- α , 1503 ± 58 pg/mL of IL6 and 6789 ± 332 pg/mL of IL8], suggesting that
364 *A. fumigatus* conidia may also utilize complement receptor-mediated route of phagocytosis for a
365 silent-entry. On the other hand, it could be the metabolic activeness of conidia and recognition of
366 pathogen-associated molecular patterns in the phagolysosome following conidial swelling that
367 stimulates phagocytes to produce reactive intermediates, a host-defense mechanism that results
368 in conidial killing. It should be noted that hMDM could uptake significant number of
369 unopsonized conidia; nevertheless, conidial killing and ROS production were lower in this
370 conditions. Thus, our study suggest that the *A. fumigatus* conidial phagocytosis and killing are
371 two independent processes, in agreement with the earlier observation (23).

372

373 Altogether, our data indicate that the complement-system is activated on the *A. fumigatus*
374 conidial surface through the alternative pathway, facilitating conidial opsonization, aggregation
375 and phagocytosis. *Aspergillus fumigatus* conidial interaction with the complement-system in the
376 alveolar environment results in the activation of phagocytosis and enhancing antimicrobial
377 properties, facilitating the conidial clearance in healthy host. However, we could not associate
378 the functional role played by some of the complement proteins interacting with conidia in our
379 study, for example MBL. It could be either owing to a competition of MBL with the two
380 structurally similar lung collectins, SP-A and SP-D, which, as shown here, interact with conidia
381 or, to the absence of MBL in the BALF from uninfected lungs (46). However, MBL has been

382 reported to be the activator of the complement under low immunoglobulin levels in aspergillosis
383 (15). Furthermore, genetic polymorphism leading to MBL deficiency have been reported to be
384 associated with chronic pulmonary and severe invasive aspergilloses (47, 48). Significantly
385 lower level of serum MBL was found in invasive aspergillosis patients compared to those of the
386 control, suggesting an association between MBL deficiency and invasive aspergillosis (48).
387 Interestingly, mRNA transcripts related to complement components C3 and CFB in primary
388 human bronchial epithelial cells were down-regulated upon *A. fumigatus* infection (49). These
389 observations demand further investigation of the role played by the complement/humoral
390 immune system against *A. fumigatus* during infection.

391

392 **MATERIALS AND METHODS**

393 *Aspergillus fumigatus* strain, and preparation of cell wall components

394 The *A. fumigatus* CBS144-89 clinical isolate (50) was maintained on 2% malt extract agar-slants
395 at ambient temperature; conidia were harvested from the agar-slants after 12-15 days of growth.
396 RodAp and melanin pigment were obtained as described earlier (8, 51). β -(1,3)- α -(1,3)-Glucans
397 were extracted from the alkali-insoluble and alkali-soluble conidial CW fractions, respectively,
398 following the protocol we described (32, 52, 53). Chitin was obtained from the conidial
399 morphotype following the protocol that we described for the mycelia (54), whereas
400 galactomannan (GM) was isolated from the *A. fumigatus* plasma-membrane fraction (55).

401

402 *Chemicals, buffers, serum and bronchoalveolar lavage fluid (BALF) samples*

403 Human complement C3 (purified from serum), lipopolysaccharide (LPS) from *Escherichia coli*
404 and Polymyxin B-agarose were purchased from Sigma-Aldrich/Merck Millipore. Buffers for
405 different complement activation pathways were prepared as described earlier (56). Whole blood
406 samples were collected from five healthy donors, incubated at 37°C for 30 min, centrifuged at
407 3,000 rpm for 5 min, the blood cell-pellet was discarded and the collected serum samples were

408 pooled. Pooled serum sample was also obtained from Zen-Bio Inc (France). Complement C4,
409 Factor B and immunoglobulin depleted sera were purchased from CompTech (Texas, USA), and
410 C1q-depleted serum was obtained from Merck Chemicals. Mannose binding lectin (MBL)-
411 depleted serum was prepared using pooled serum sample as described earlier (57). BALF from
412 four donors negative for fungal culture and nucleic acids were obtained from the Centre
413 Hospitalier Universitaire de Rennes, Hôpital Pontchaillou. Briefly, these donors, as they were
414 suspected for infection, underwent bronchoscopy following local anesthesia, and the BALF were
415 collected by installing 40-50 mL saline, aspirating at least 50% of the installed saline, repeating
416 this procedure for three times, and collecting each fraction separately. Collected fractions were
417 centrifuged (300 g, 5 min) to separate BAL-cells, and if necessary, the supernatant was passed
418 through a nylon mesh of 70 μm to remove any fibrillar material. All these fractions were stored
419 at -80°C until further use. The galactomannan index in these BALF samples (0.173-0.233) were
420 below the EORTC/MSG cut-off value (≥ 1.0) (58) and they were associated to other negative
421 biomarkers, allowing their exclusion from probable *Aspergillus* infection. Of the four individuals
422 from whom BALF was collected, two had suspected pneumonia, one with infectious lung lesions
423 in the CT scan and the other with acute respiratory failure. However, these four BALF samples
424 had a bacterial count of $<10^3$ CFU/mL [uninfected (59)]; three of them were negative for viral
425 loads, and the fourth individual had an asymptomatic viral load for cytomegalovirus [2.37 \log_{10}
426 IU/mL; interquartile range, 0-2.5 \log_{10} IU/mL (60)], and we pooled these four BALF samples in
427 our study. Each BALF sample used in our study was an aliquot of the fractions 1 of the three
428 fractions aspirated from an individual.

429

430 ***Conidial opsonization and extraction of conidial surface bound proteins***

431 Conidia harvested from 12-15 days old agar slants were washed twice with aqueous Tween-20
432 (0.05%) and once with MilliQ water. Conidia (2.5×10^8) were then incubated with 100 μL of

433 pooled serum [in-house/commercial, diluted to 20% in phosphate buffered saline (PBS); 223 μg
434 protein/100 μL] or BALF (102.0 μg protein/100 μL) at 37°C for 30 min, with vortexing for
435 every 5 min. After centrifugation (5000 rpm, 5 min), the supernatants were discarded, conidia
436 were washed five times with PBS, and conidial surface bound proteins were extracted with 200
437 μL of either NH_2OH (1 M in 0.2 M NaHCO_3 , pH 10.0) or 3.5 M NaSCN (pH 7.0) in a rotator to
438 ensure efficient extraction. Extracted proteins were collected by centrifugation (10000 rpm, 10
439 min), desalted and concentrated using 3 kDa Amicon membrane filters (Sigma-Aldrich).
440 Proteomic analysis was performed (three technical replicates) subjecting the samples to shotgun
441 proteomic identification using a nanoLC-Orbitrap mass spectrometer (61).

442

443 The concentrations of proteins [estimated with Bradford reagent (BioRad) using bovine serum
444 albumin standard] were (in μg): in the NH_2OH extract, serum–19.35 \pm 1.80, BALF–18.01 \pm 1.81,
445 unopsonized–1.79 \pm 0.45 and in the NaSCN extract, serum–18.01 \pm 1.12 and BALF–22.47 \pm 2.00,
446 unopsonized–2.50 \pm 0.26.

447

448 *Conidial Immunolabelling and flow-cytometric analysis*

449 Conidia (1×10^6) were incubated with complement C3 (5 $\mu\text{g}/\text{mL}$ in PBS with 1% bovine serum
450 albumin) for 1 h at 37°C. Conidia were then washed thrice with PBS supplemented with 0.005%
451 Tween-20, and incubated with FITC-conjugated anti-C3 antibody (mouse monoclonal, clone 2
452 D8; Invitrogen) for 1 h at 37°C, washed with PBS-0.005% Tween-20 (twice), PBS (once),
453 mounted on glass slides and observed under confocal microscope. Labelled conidia were also
454 subjected to flow cytometry analysis using LSR II equipment (BD Biosciences); the data were
455 analyzed using BD FACS DIVA (BD Biosciences) and FlowJo™. Anti-MAC labelling was
456 performed using anti-human C5b-9 antibody (mouse monoclonal, clone aE11; Abcam) and
457 secondary FITC-conjugated anti-mouse IgG (Sigma-Aldrich).

458

459 ***Conidial opsonization, RodAp extraction using hydrofluoric acid and Western-blot analyses***

460 As described above, conidia were opsonized with pooled serum (diluted to 20% in PBS) for 30
461 min, washed five-times with PBS and subjected to RodAp extraction using hydrofluoric acid
462 (HF) (1), but only for 6 h. Extracted samples were subjected to SDS-PAGE on 12% gel and
463 revealed Coomassie-brilliant blue staining or Western-blot by separating proteins on 15% gel
464 and using mouse polyclonal anti-RodAp antibodies (62), anti-C3 (LF-MA0132, mouse
465 monoclonal, clone 28A1, targets C3b; ThermoFisher Scientific) or anti-C3b antibodies [mouse
466 monoclonal, MA1-40155; Invitrogen; targets iC3b-part, checked using human serum as the
467 positive control and C3-deficient serum as the negative control and we cross-checked it with
468 human serum and C3-depleted serum (CompTech, Complement Technology Inc.,
469 Catalog#A314)] as the primary antibodies, and peroxidase conjugated anti-mouse IgG as the
470 secondary antibody (1:1000 dilution; Sigma-Aldrich). The blot-detection was performed using
471 ECL-chemiluminescence kit (Amersham, GE Healthcare Life Sciences).

472

473 ***Conidial cell wall components and complement activation***

474 *Aspergillus fumigatus* CW components (for polysaccharides or melanin pigments – 50 µg/mL,
475 and 10 µg/mL for RodAp, in 50 mM bicarbonate buffer, pH 9.6) were coated on 96-well plates
476 (100 µL/well) overnight at ambient temperature. The positive control wells were coated with
477 LPS (4 µg/well). After discarding the supernatants, wells were blocked with 300 µL block-buffer
478 [5% non-fat milk in gelatin-veronal buffer (GVB)] at ambient temperature for 1 h, washed once
479 with 200 µL wash buffer (PBS with 0.05% Tween-20). Plates with 8 µL sera and 92 µL GVB
480 per well were incubated at ambient temperature for 1h and washed with 200 µL wash buffer
481 (3X). Deposited C3b/iC3b in the wells were determined with monoclonal anti-human C3b/iC3b
482 antibody (MA1-82814, 1:1000 diluted in block-buffer, 100 µL/well, ThermoFisher Scientific)
483 and incubating at ambient temperature for 1 h. Wells were washed (3X) with PBS-Tween

484 followed by the addition of secondary antibodies (peroxidase-conjugated anti-mouse IgG,
485 1:1000 diluted in block-buffer) and incubation at ambient temperature for 1 h. After washing
486 with PBS-Tween (3X), 100 μ L substrate solution (*O*-phenylenediamine) was added per well,
487 reactions were arrested with 50 μ L 4% H₂SO₄ and the absorbance was read at 492 nm.

488

489 ***Phagocytosis and blockade of the complement receptors***

490 Human monocyte derived macrophages (hMDM) were obtained and the conidial opsonization
491 was performed as described earlier (53). In brief, 500 μ L of 2×10^6 per mL peripheral blood
492 mononuclear cell (PBMC) suspension in incomplete RPMI medium was seeded in to a 24-well
493 cell-culture plate. After 3 h incubation in a CO₂ incubator maintained at 37°C, the supernatant
494 was discarded, wells were washed twice with PBS and added with 500 μ L of RPMI
495 supplemented with 10% human serum and granulocyte macrophage–colony-stimulating factor
496 (GM-CSF; 10 ng/mL; R&D Systems) and incubated in a CO₂ incubator at 37°C for 6-days with
497 replacement of the medium after 3-days. Differentiated macrophages were washed twice with
498 PBS, incubated with conidial suspension (5×10^5 /well, opsonized or unopsonized) in a CO₂
499 incubator at 37°C for 1 h, supernatants were discarded and the wells were washed with PBS
500 twice to remove non-phagocytosed conidia. Following, hMDM were disrupted by adding 1%
501 Triton-X 100 and incubation at 4°C for 30 min. Phagocytosed conidia were collected upon gentle
502 scraping and washing the wells twice with 1% Triton-X 100 and subjected to gentle sonication in
503 a water-bath. Upon appropriate dilutions, conidial suspensions were spread on malt-agar plates,
504 incubated at 37°C until the growth of colonies, followed by colony count. Conidia interacting
505 with hMDM but not phagocytosed were identified upon Calcofluor-white staining (56); their
506 percentage was deducted from the colony count to determine the percent of phagocytosed
507 conidia. Conidia-hMDM interaction was also performed in RPMI without serum; unopsonized
508 and serum or BALF opsonized conidia were used for the interaction, the culture supernatants
509 were collected after 24 h incubation in a CO₂ incubator and analyzed for cytokines.

510

511 To study the complement receptor blockage followed by phagocytosis, hMDM were blocked
512 with anti-CD11b (mouse monoclonal isotype IgG1, clone CBRM1/5, BioLegend), anti-CD11c
513 (mouse monoclonal, clone 3.9, CliniSciences) and anti-CD18 (mouse monoclonal, clone IB4,
514 Merck Chemicals) antibodies in PBS for 30 min in a CO₂ incubator at 37°C followed by washing
515 twice with PBS, addition of conidia and determination of colony count as above. The viability of
516 hMDM after receptor blockage was assessed by lactate dehydrogenase assay (63).

517

518 *Conidial killing and reactive oxygen intermediate (ROS) production assays*

519 Experiments were performed using THP1 cell-line (human leukemic monocytes; Merck/Sigma-
520 Aldrich); cells from the frozen stock were propagated as per the manufacturer's instruction.
521 Following, collected THP1 cells were seeded into 24 well culture plates (2x10⁶/well) in
522 incomplete RPMI medium and added with unopsonized or opsonized (serum/BALF) conidia
523 (1x10⁶/well) and incubated in a in a CO₂ incubator maintained at 37°C. For killing assay, conidia
524 added to the wells containing only incomplete medium served as the control; whereas for ROS
525 estimation, wells with only THP1 cells in the medium served as the control. Estimation of ROS
526 produced was performed as described earlier (53), and for killing assay, after 6 h of conidia-
527 THP1 interaction, contents from the wells were collected and centrifuged to collect the pellet;
528 wells were washed twice with 1% Triton-X100 (each time with 0.5 mL) with gentle scraping and
529 the contents were transferred to the tubes containing respective cell pellets. After vortexing,
530 collected cells contents were kept at 4°C for 30 min and subjected to brief sonication in a water-
531 bath to disrupt THP1 cells releasing phagocytosed conidia and also to disaggregate conidia if
532 any. Upon appropriate dilutions, conidial suspensions were spread on malt-agar plates, incubated
533 at 37°C until the growth of colonies, followed by colony count; the colony forming unit (CFU)
534 counts were expressed as the percentage of live conidia, considering conidial numbers added per
535 well as 100%.

536

537 *Statistical analysis*

538 One-way ANOVA analysis was performed using the Prism-8 software (GraphPad Software, Inc.,
539 La Jolla, CA, USA).

540

541 **ETHICAL STATEMENT**

542 Blood samples from healthy individuals were obtained from Etablissement Français du Sang
543 Saint-Louis (Paris, France) with written and informed consent as per the guidelines provided by
544 the Institutional Ethics Committee, Institut Pasteur (convention 12/EFS/023). For the BALF
545 samples used in this study, according to the French Public Health Law (Code de la Santé
546 Publique ; Décret n° 2017-884 du 9 mai 2017 modifiant certaines dispositions réglementaires
547 relatives aux recherches impliquant la personne humaine | Légifrance. Article R.1121-1-1, 2017;
548 <https://www.legifrance.gouv.fr/eli/decret/2017/5/9/AFSP1706303D/jo/texte>), the protocol of
549 using the BALF did not require approval from an ethical committee and was exempted from the
550 requirement for formal informed consent.

551

552 **DISCLOSURE STATEMENT**

553 No conflict of interest to disclose.

554

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564

565 **AUTHOR-CONTRIBUTION STATEMENT**

566 VA – Conceptualization, Project-administration, Supervision, Visualization and Writing (the
567 original-draft); SSWW, ID, JMJ, SD, RS, JB and VA – Investigation and Methodologies; JPG,
568 HG, TM and JIG – Resources; VA, JMJ, PL and KD – Funding acquisition; SSWW, ID, JMJ,
569 SD, JB and VA – Data curation and Formal analysis; All the authors – Validation, Writing
570 (editing and reviewing the draft manuscript). The final version of this manuscript was reviewed,
571 edited as well as approved by all the authors.

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765 **Table 1:** Complement proteins extracted from the conidial surface opsonized with serum (pooled and diluted to 20% with PBS) from healthy donors
 766 and identified using mass spectrometry (PSM – Peptide Spectrum Match)
 767

Protein description	UNIPROT ID	M _w	NH ₂ OH	NaSCN
			extract	extract
PSM				
Complement C3B	CO3B_HUMAN	187 kDa	3451	3571
Complement factor H	CFAH_HUMAN	139 kDa	1590	929
Complement C4B	CO4B_HUMAN	193 kDa	1413	641
Complement C1q subcomponent subunit A	C1QA_HUMAN	26 kDa	542	-
Complement C1r subcomponent	B4DPQ0_HUMAN	82 kDa	382	359
Complement C2	CO2_HUMAN	83 kDa	344	216
Complement C5	CO5_HUMAN	188 kDa	334	424
Complement C1q subcomponent subunit B	C1QB_HUMAN	27 kDa	330	17
Complement C1s subcomponent	A0A087X232_HUMAN	76 kDa	289	224
Complement factor H-related protein 2	FHR2_HUMAN	28 kDa	265	-
Complement component C9	CO9_HUMAN	63 kDa	262	434
Complement component C6	CO6_HUMAN	105 kDa	177	316
Complement component C7	CO7_HUMAN	94 kDa	173	346
Complement component C8 alpha chain	CO8A_HUMAN	65 kDa	140	210
Complement component C8 beta chain	CO8B_HUMAN	67 kDa	124	183
Complement C1q subcomponent subunit C	C1QC_HUMAN	26 kDa	117	64
Complement factor D	CFAD_HUMAN	27 kDa	100	2
Complement component C8 gamma chain	CO8G_HUMAN	22 kDa	93	73
Complement factor H-related protein 5	FHR5_HUMAN	64 kDa	90	190
Properdin	PROP_HUMAN	51 kDa	62	44
Complement factor I, isoform CRA_b	G3XAM2_HUMAN	65 kDa	25	12
Mannose-binding protein C	MBL2_HUMAN	26 kDa	25	-
Mannose-binding lectin serine protease 1	MASP1_HUMAN	79 kDa	11	23
Mannose-binding lectin serine protease 2	MASP2_HUMAN	76 kDa	4	-

768 **Table 2:** Complement proteins extracted from the conidial surface opsonized with bronchoalveolar lavage (pooled) from healthy donors and
 769 identified using mass spectrometry (PSM – Peptide Spectrum Match)
 770

Protein Description	UNIPROT ID	M _w	NH ₂ OH extract	NaSCN extract
			PSM	
Complement C3B	CO3B_HUMAN	187 kDa	784	178
Complement factor H	CFAH_HUMAN	139 kDa	237	1003
Complement C4B	CO4B_HUMAN	193 kDa	196	30
Complement C5	CO5_HUMAN	188 kDa	110	5
Complement C1q subcomponent subunit B	C1QB_HUMAN	27 kDa	32	15
Complement C1q subcomponent subunit A	C1QA_HUMAN	26 kDa	27	8
Complement factor B	B4E1Z4_HUMAN	141 kDa	26	54
Complement component C7	CO7_HUMAN	94 kDa	24	29
Complement C1q subcomponent subunit C	C1QC_HUMAN	26 kDa	14	4
Complement factor H-related protein 2	FHR2_HUMAN	28 kDa	13	36
Properdin	PROP_HUMAN	51 kDa	12	14
Complement C1r subcomponent	B4DPQ0_HUMAN	82 kDa	11	26
Complement factor D	CFAD_HUMAN	27 kDa	10	14
Complement C1s subcomponent	C1S_HUMAN	77 kDa	8	13
Complement component C8 gamma chain	CO8G_HUMAN	22 kDa	6	1
Complement component C9	CO9_HUMAN	63 kDa	4	6
Complement component C6	CO6_HUMAN	105 kDa	3	20
Complement factor H-related protein 5	FHR5_HUMAN	64 kDa	2	34
Complement component C8 beta chain	CO8B_HUMAN	67 kDa	-	5

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777 **Table 3:** The complement proteins from human bronchoalveolar lavage and serum differentially interacting with *A. fumigatus* conidia and identified
 778 using mass spectrometry (PSM – Peptide Spectrum Match)
 779

Protein description	UNIPROT ID	M _w	Serum		BALF	
			NH ₂ OH extract	NaSCN extract	NH ₂ OH extract	NaSCN extract
			PSM		PSM	
Complement C2	CO2_HUMAN	83 kDa	344	216	Not identified	Not identified
Complement factor B	B4E1Z4_HUMAN	141 kDa	Not identified	Not identified	26	54
Complement factor I, isoform CRA_b	G3XAM2_HUMAN	65 kDa	25	12	Not identified	Not identified
Mannose-binding protein C	MBL2_HUMAN	26 kDa	25	-	Not identified	Not identified
Mannose-binding lectin serine protease 1	MASP1_HUMAN	79 kDa	11	23	Not identified	Not identified
Mannose-binding lectin serine protease 2	MASP2_HUMAN	76 kDa	4	-	Not identified	Not identified

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791 **FIGURE LEGENDS**

792 **Figure 1: Complement proteins C3 interacts with *A. fumigatus* conidial surface. (A)**

793 **Flow cytometry showing the deposition C3b on the conidial surfaces.** Conidia were

794 opsonized with pooled human serum and probed with monoclonal anti-C3b antibodies

795 followed by FITC-conjugated secondary antibodies; opsonized conidia probed with FITC-

796 conjugated secondary antibodies (Sec. Ab-FITC) served as the control. **(B) Flow cytometry**

797 **showing direct interaction of conidia with C3.** Conidia opsonized with C3 were probed

798 with anti-C3b antibodies and then with FITC-conjugated secondary antibodies; C3 opsonized

799 conidia probed with FITC-conjugated secondary antibodies served as the control. **(C)**

800 **Confocal microscopy of the immunolabelled conidia showing C3b deposition on the**

801 **conidial surface.** C3 opsonized conidia were probed with monoclonal anti-C3b antibodies

802 followed by FITC-conjugated secondary antibody, and subjected to fluorescent microscopy;

803 control was the opsonized conidia probed only with FITC-conjugated secondary antibodies.

804

805 **Figure 2: Complement activation by the *A. fumigatus* conidial cell wall components.** Cell

806 wall components were coated onto microtiter plates and serum (8 μ L) diluted in gelatin

807 veronal buffer (GVB; 92 μ L) without (GVB--) or supplemented with Ca^{2+} - Mg^{2+} (GVB++)

808 that permits activation of only alternative or all three pathways, respectively. After

809 incubation, the contents were discarded and the wells were washed in wash buffer for three

810 times. The level of complement activation by each cell wall component was determined by

811 the amount of deposited C3b, by anti-human C3b antibodies and peroxidase-conjugated anti-

812 mouse IgG antibodies; *O*-phenylenediamine was used as the peroxidase substrate, the reaction

813 was arrested using 4% H_2SO_4 and the optical density (OD) was measured at 492 nm. **(A)**

814 **Complement activation capacity of the individual cell wall components found in *A.***

815 ***fumigatus* conidia.** A pooled serum sample from nine healthy donors was used and the

816 experiment was performed with three technical-two biological replications. **(B) Complement**

817 pathway activation by the cell wall components RodAp, β -(1,3)-glucan and
818 galactomannan. RodAp converted C3 into C3b both in GVB++ and GVB--, but showed a
819 significant decrease in the C3 activation with Complement Factor-B depleted serum in
820 GVB++; whereas, β -(1,3)-glucan and galactomannan showed a significant decrease in the
821 conversion of C3 into C3b in GVB--. (C & D) Complement factors required by β -(1,3)-
822 glucan and galactomannan. Complement depleted sera showing the requirement of different
823 complement factors for the complement activation by β -(1,3)-glucan/galactomannan (MBL,
824 mannose binding lectin; CFB, Complement Factor-B).

825

826 **Figure 3: C3 interacts with *A. fumigatus* conidial surface in a concentration dependent**
827 **manner. (A) Flow-cytometry, (B) ELISA showing concentration dependent C3 binding to**
828 **conidial surface (C3 concentration – 1.95-250 ng/mL); Unopsonized and serum opsonized**
829 **conidia were subjected to RodAp extraction using hydrofluoric acid (HF). Extracted protein**
830 **was monitored by (C) SDS-PAGE (12% gel; PM – protein markers, L1 – unopsonized**
831 **conidial HF-extract, L2 – serum opsonized conidial HF-extract; Coomassie-brilliant blue**
832 **staining) and Western blot (15% gel for protein separation) using (D) polyclonal anti-RodAp**
833 **(L3 – recombinant RodAp, L4 – serum opsonized conidial HF-extract, L5 – unopsonized**
834 **conidial HF-extract) (E) monoclonal anti-C3 (L6 – serum opsonized conidial HF-**
835 **extract)/anti-C3b antibodies (L7 – purified complement C3, L8 – serum opsonized conidial**
836 **HF-extract, L9 – unopsonized conidial HF-extract). Arrow marks in the Western blots (D)**
837 **and (E) indicate a common band recognized by both anti-C3b and anti-RodAp antibodies,**
838 **suggesting a covalent interaction between RodAp and the complement protein C3.**

839

840 **Figure 4: Opsonization facilitates *A. fumigatus* conidial aggregation and complement**
841 **receptors are involved in conidial phagocytosis. (A) Bright-field microscopy showing**

842 conidial aggregation upon opsonization with pooled human serum (the arrows indicate
843 conidial aggregation), **(B)** Serum opsonized conidia suspended in incomplete RPMI were
844 added to human monocyte derived macrophages (hMDM; opsonized) or complement
845 receptor-blocked hMDM (CR3-block, CR4-block and both CR3&4-block); unopsonized
846 conidia added to hMDM served as the control (unopsonized). After 1 h incubation at 37°C in
847 a CO₂ incubator, supernatants were discarded, wells were washed twice with PBS, hMDM
848 were disrupted using 1% Triton-X 100, contents were collected and sonicated gently in a
849 water-bath to separate any conidia aggregated. After appropriate dilutions, collected conidial
850 suspensions were spread over malt-agar, and incubated at 37°C until the growth of colonies,
851 followed by colony forming unit (CFU) counts; hMDM from three donors and two technical
852 replicates were used for CFU counts and the mean ± SD values are presented (**p<0.0001,
853 **p<0.0001 and *p<0.01). **(C)** Bright-field microscopy showing significantly increased
854 conidial phagocytosis upon conidial opsonization with serum, and decrease in the
855 phagocytosis upon blockage of the complement receptors CR3 and CR4 on hMDM (the
856 arrows indicate phagocytosed conidia; inset in the opsonized conidial phagocytosis image
857 indicates the capacity of hMDM to uptake aggregated conidia, and that in the CR3-CR4
858 blocked image indicates incapability of hMDM to phagocytose aggregated conidia as well).

859

860 **Figure 5: Cytokine secretion upon stimulation of human monocyte derived macrophages**
861 **(hMDM) with conidia. (A) Flow cytometry showing the deposition of C3b on the**
862 **conidial surfaces;** conidia were opsonized with pooled human BALF and probed with
863 monoclonal anti-C3b antibodies followed by FITC-conjugated secondary antibody.
864 Opsonized conidia probed with FITC-conjugated secondary antibody served as the control.
865 **(B)** Bright-field microscopy showing conidial aggregation upon opsonization with pooled
866 human BALF. **(C)** Human monocyte derived macrophages (hMDM; obtained upon seeding
867 2x10⁶ PBMCs per well) were stimulated with unopsonized, serum-opsonized or BALF-

868 opsonized conidia suspended in RPMI without serum (500 μ L/well with 2.5×10^4 conidia) and
869 incubated at 37°C for 20 h in a CO₂ incubator. The supernatants were recovered from the
870 microtiter plate wells and subjected to cytokine analyses (TNF- α , IL-1 β , IL-6, IL-8 and IL-
871 10). The values are from four donors with two technical replicates; hMDM from each donor
872 were tested for all four groups of samples, i.e., medium control, unopsonized conidia, serum-
873 opsonized conidia and BALF-opsonized conidia (**p<0.01, ***p<0.001, ****p<0.0001).

874

875 **Figure 6: Opsonization facilitates conidial killing by phagocytes, possibly through**

876 **reactive oxygen intermediates. (A)** In cell-culture plate wells, human leukemic monocytes

877 (THP1 cells) were made to interact with serum/BALF opsonized or unopsonized conidia for 6

878 h at 37°C in a CO₂ incubator. From wells, the entire reaction mixtures were collected and

879 centrifuged, discarding the supernatant to collect cell pellet (both conidia + THP1 cells).

880 Wells were washed twice with 1% Triton-X100, each time with 0.5 mL; the contents were

881 transferred to respective tubes containing cell-pellets and incubated at 4°C for 30 min.

882 Following, the tubes were sonicated for 60 sec in a water-bath to disrupt THP1 cells releasing

883 phagocytosed conidia. Conidial suspensions in the tubes were diluted appropriately, spread

884 over malt-agar plates and incubated at 37°C until fungal colony grows. The fungal colony

885 counts were expressed as percentage of live conidia. **(B)** THP1 cells were incubated with

886 unopsonized or serum/BALF opsonized conidia for 2 h in a CO₂ incubator, after which added

887 100 μ M of 2,7-dichlorofluorescein diacetate, incubated further for 1 h and the fluorescent

888 were read using a using Tecan Infinite[®] 200 PRO plate reader, with excitation-emissions at

889 485 and 530 nm, respectively and the results were expressed as fluorescence intensities. For

890 both killing and ROS production assays, incomplete RPMI was used; conidia added to the

891 well containing only the culture medium served as control for killing assay and well with

892 THP1 cells suspended in incomplete RPMI was the control for ROS-assay; experiments were

893 performed in triplicates (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure 1

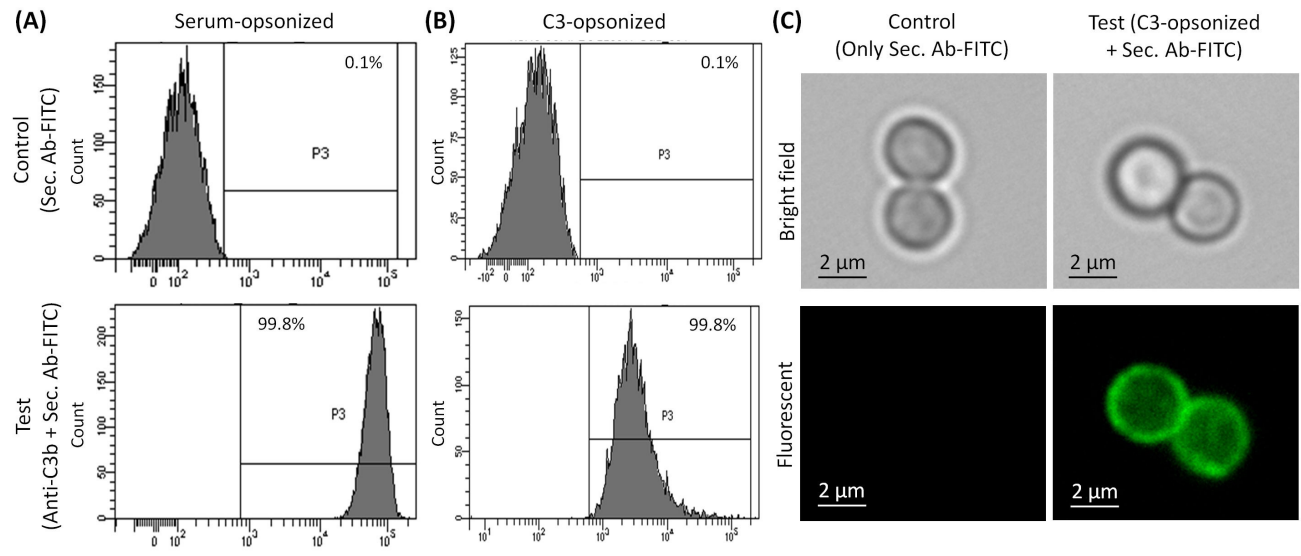


Figure 2

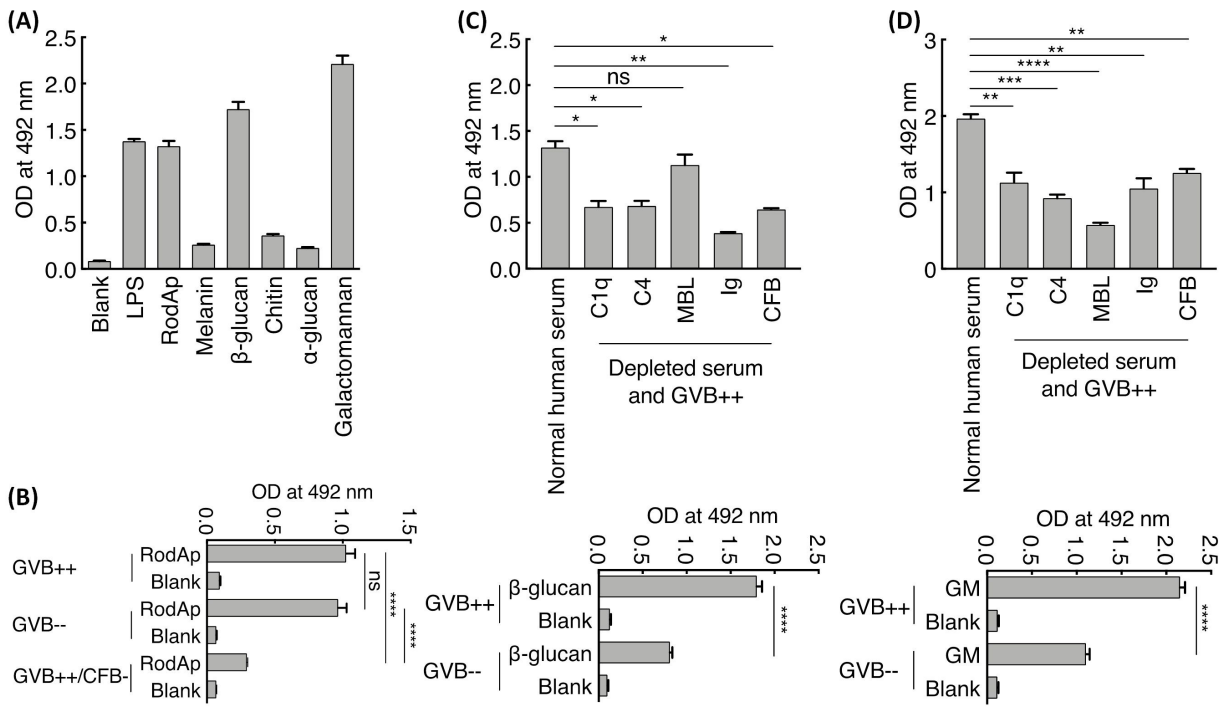


Figure 3

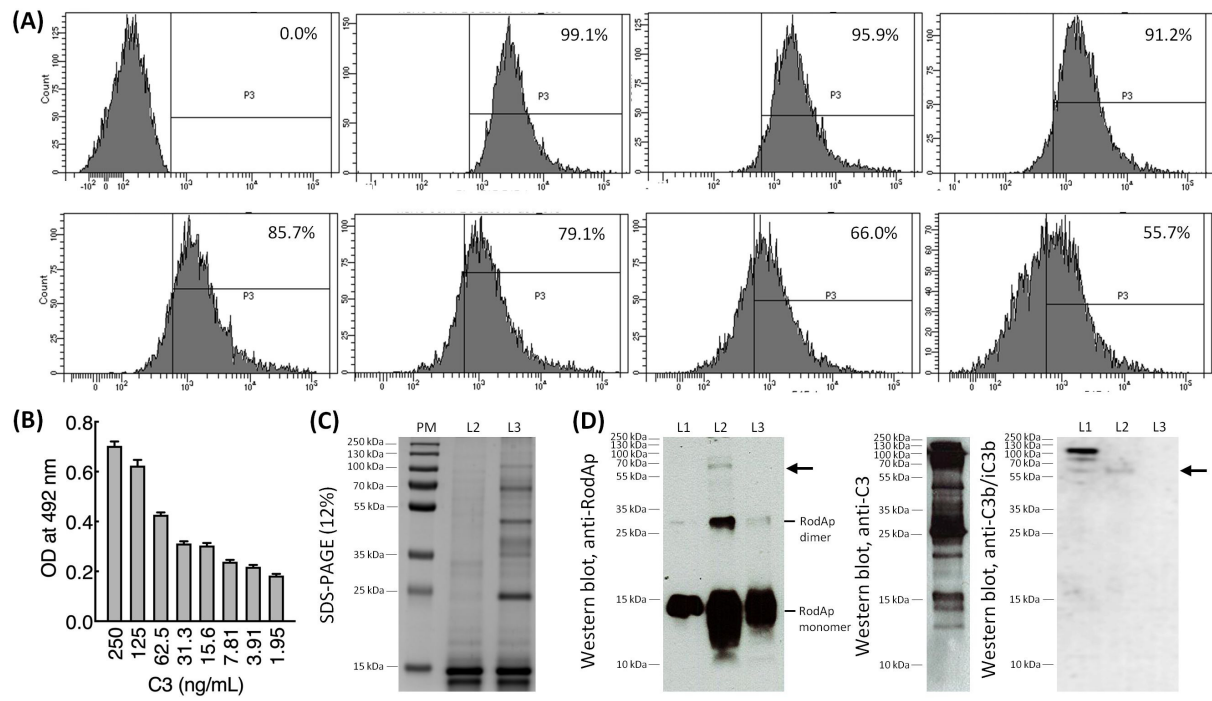


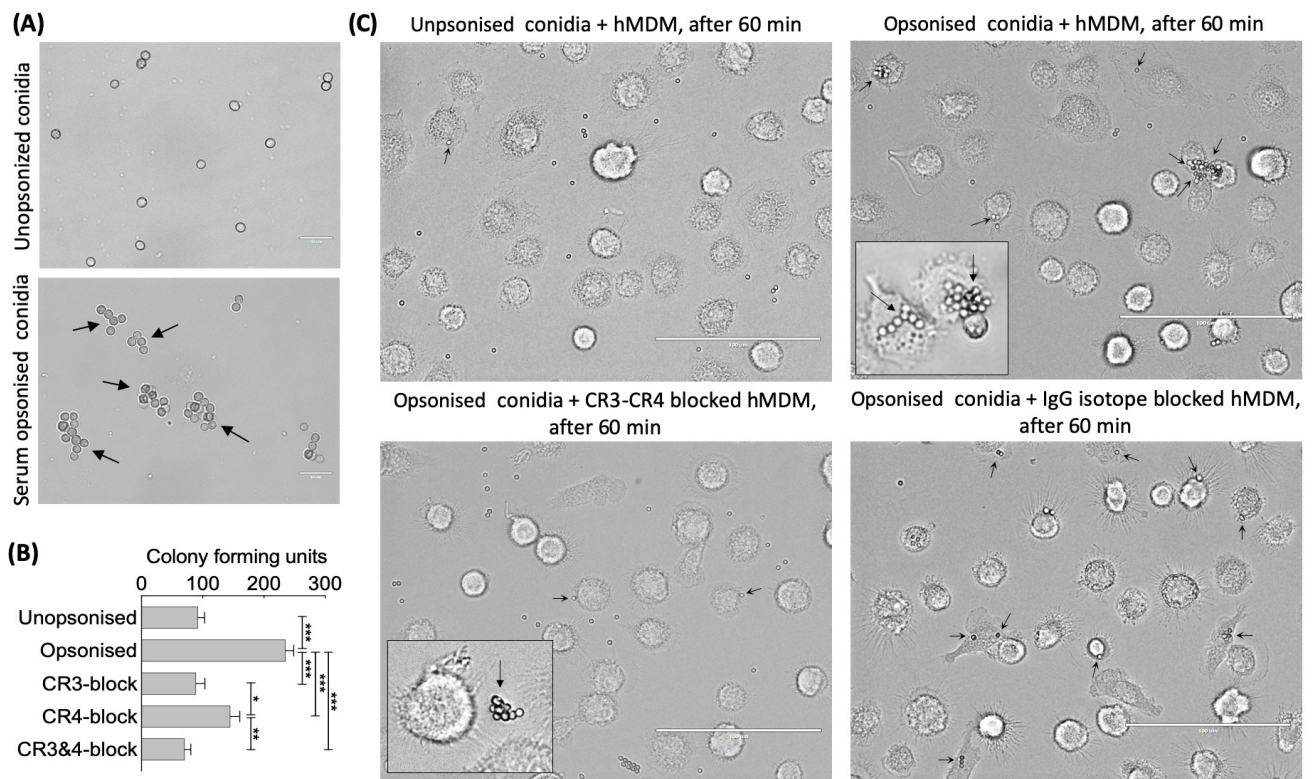
Figure 4

Figure 5

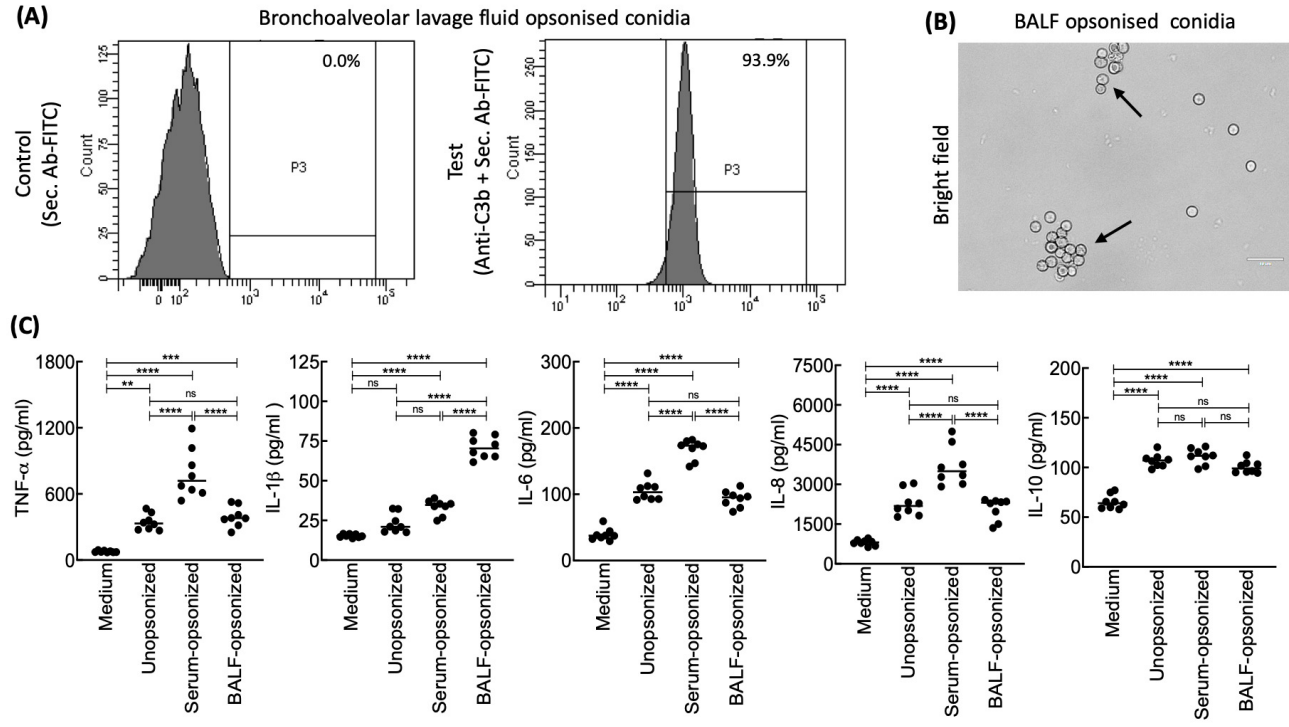


Figure 6

