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Differential Interactions of Serum and Bronchoalveolar Lavage Fluid Complement Proteins with Conidia of Airborne Fungal Pathogen *Aspergillus fumigatus*

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

KEYWORDS *Aspergillus fumigatus* conidia, cell wall, polysaccharides, humoral immunity, complement system, complement receptors, *Aspergillus fumigatus*, cytokines, host-pathogen interactions, macrophages, proteomics

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

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

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

RESULTS

Complement proteins interact with the *A. fumigatus* conidial surface. Table 1 lists the complement proteins extracted from the conidial surface opsonized with human serum and identified using a mass-spectrometric approach. Proteins extracted with NH_2OH represent strongly bound ones, while those extracted by NaSCN are weakly bound proteins. The peptide-spectrum match (PSM; the total number of identified peptide spectra matched for a protein) score was high for the NH_2OH-extractable complement protein C3, suggesting that C3 strongly interacts with the conidial surface. Other complement components found in the NH_2OH extract were (in decreasing order of abundance) complement factor H (CFH), C4B, C1q, C1r, C2, C5, C1s, C9, C6, C7, C8, complement factor D (CFD), properdin, complement factor I (CFI), mannose-binding lectin (MBL), and MBL-associated serine proteases 1 and 2 (MASP1 and MASP2). Although identified in the NH_2OH extract, C5, C9, C6, C7, and C8 were found more

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TABLE 1 Complement proteins extracted from the conidial surface opsonized with serum from healthy donors and identified using mass spectrometry[a]

Protein description	UNIPROT ID	Mol mass (kDa)	PSM score	
			NH_2OH extract	NaSCN extract
Complement C3B	CO3B_HUMAN	187	3,451	3,571
Complement factor H	CFAH_HUMAN	139	1,590	929
Complement C4B	CO4B_HUMAN	193	1,413	641
Complement C1q subcomponent subunit A	C1QA_HUMAN	26	542	
Complement C1r subcomponent	B4DPQ0_HUMAN	82	382	359
Complement C2	CO2_HUMAN	83	344	216
Complement C5	CO5_HUMAN	188	334	424
Complement C1q subcomponent subunit B	C1QB_HUMAN	27	330	17
Complement C1s subcomponent	A0A087X232_HUMAN	76	289	224
Complement factor H-related protein 2	FHR2_HUMAN	28	265	
Complement component C9	CO9_HUMAN	63	262	434
Complement component C6	CO6_HUMAN	105	177	316
Complement component C7	CO7_HUMAN	94	173	346
Complement component C8 alpha chain	CO8A_HUMAN	65	140	210
Complement component C8 beta chain	CO8B_HUMAN	67	124	183
Complement C1q subcomponent subunit C	C1QC_HUMAN	26	117	64
Complement factor D	CFAD_HUMAN	27	100	2
Complement component C8 gamma chain	CO8G_HUMAN	22	93	73
Complement factor H-related protein 5	FHR5_HUMAN	64	90	190
Properdin	PROP_HUMAN	51	62	44
Complement factor I, isoform CRA_b	G3XAM2_HUMAN	65	25	12
Mannose-binding protein C	MBL2_HUMAN	26	25	
Mannose-binding lectin serine protease 1	MASP1_HUMAN	79	11	23
Mannose-binding lectin serine protease 2	MASP2_HUMAN	76	4	

[a]Serum was pooled and diluted to 20% with PBS. PSM, peptide-spectrum match.

abundantly in the NaSCN fraction, suggesting their weaker interaction with conidia. Identification of the complement proteins C2, C4B, MBL, MASP1, and MASP2 was indicative of the activation of the complement system by the classical and lectin pathways. However, the absence of complement factor B (CFB) was suggestive of the lack of an alternative pathway activation loop. The complement proteins C5, C6, C7, C8, and C9 are the components of the MAC. However, the PSM scores for C6 to C8 were lower, and that for C9 did not correspond to a multimer, suggesting the absence of the MAC. Indeed, immunolabeling of opsonized conidia with anti-MAC antibodies was negative (data not shown). Interestingly, ficolin, a component of the lectin pathway and an alternative for MBL, was not found in the NH_2OH- and NaSCN-extracted fractions.

Flow cytometry analysis confirmed the deposition of C3b on the conidial surface upon opsonization with serum (both in-house and commercial sera tested were positive; for clarity, the data for the commercial sera are presented) and with purified C3 (Fig. 1A and B). The direct deposition of C3b upon interaction with C3 was suggestive of the activation of the alternative pathway on the conidial surface. There was positive immunolabeling with anti-C3b antibodies on conidia opsonized with C3 (Fig. 1C), confirming C3 activation on the conidial surfaces.

Complement activation capacity of the *A. fumigatus* conidial cell wall components. Since CW is the first component of *A. fumigatus* conidia interacting with the host immune system, we next looked at the complement activation capacity of the individual CW components of conidia; the readout was the conversion of C3 to C3b by the CW components. Of the different CW components, RodAp, BG, and GM efficiently activated C3, but melanin pigment, chitin, and α-(1,3)-glucan did not. GM showed the highest C3 activation, followed by BG and RodAp (Fig. 2A).

Conversion of C3 to C3b could take place via the classical, lectin, and/or alternative pathways, with the first two pathways being Ca^{2+}-Mg^{2+} dependent (11). The C3 activation capacity of RodAp was unaltered in the presence versus the absence of Ca^{2+}-Mg^{2+}, suggestive of C3 activation by RodAp through the alternative pathway. In agreement, C3b deposition on RodAp was significantly lower with CFB-depleted serum even in the presence of Ca^{2+}-Mg^{2+}. In contrast, in the absence of Ca^{2+}-Mg^{2+}, there

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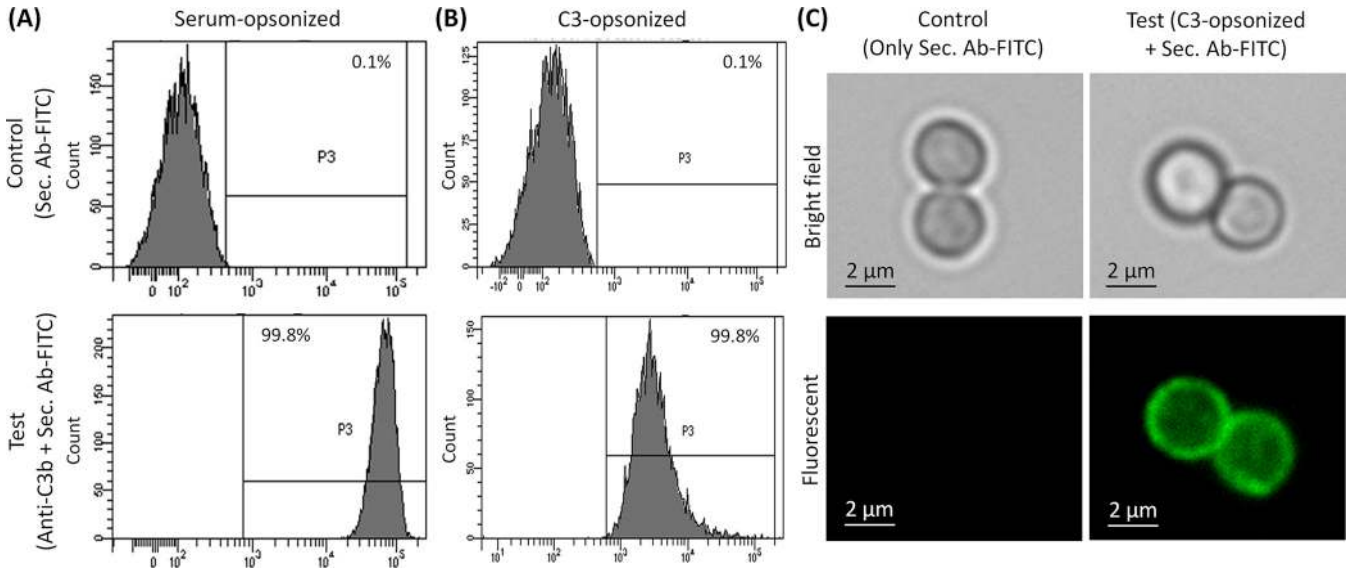

FIG 1 Complement C3 interacts with the *A. fumigatus* conidial surface. (A) Flow cytometry showing the deposition of C3b on the conidial surfaces. Conidia were opsonized with pooled human serum and probed with monoclonal anti-C3b antibodies followed by FITC-conjugated secondary antibodies; opsonized conidia probed with FITC-conjugated secondary antibodies (Sec. Ab-FITC) served as the control. (B) Flow cytometry showing direct interaction of conidia with C3. Conidia opsonized with C3 were probed with anti-C3b antibodies and then with FITC-conjugated secondary antibodies; C3-opsonized conidia probed with FITC-conjugated secondary antibodies served as the control. (C) Confocal microscopy of the immunolabeled conidia showing C3b deposition on the conidial surface. C3-opsonized conidia were probed with monoclonal anti-C3b antibodies followed by FITC-conjugated secondary antibody and subjected to fluorescence microscopy; the control was the opsonized conidia probed only with FITC-conjugated secondary antibodies.

was a significant reduction in C3 activation by both BG and GM (Fig. 2B), indicating that these two CW polysaccharides activate C3 partially by alternative and classical or lectin pathways, respectively.

The complement components C1q and C4 are associated with the classical pathway, MBL with the lectin pathway; immunoglobulins are involved in both the classical and the alternative pathways, while CFB participates only in the alternative pathway. When MBL-depleted serum was used, there was a modest (~15%) reduction in the C3-activating capacity of BG, but C1q- or C4-depleted sera resulted in about 50% reduction, confirming that BG activates C3 partially through the classical pathway (Fig. 2C). Immunoglobulin-depleted serum resulted in an ~70% reduction in C3 activation by BG, indicating that the immunoglobulin-mediated classical and alternative pathways are the major contributors to C3 activation by BG. MBL-depleted serum resulted in an ~70% reduction in C3 activation by GM, while with C1q-, C4-, and immunoglobulin-depleted sera, there was about a 40 to 50% reduction, suggesting that GM activates C3 primarily through the lectin pathway (Fig. 2D). With CFB-depleted serum in gelatin-Veronal buffer (GVB) supplemented with Ca^{2+}-Mg^{2+} (GVB++), there was only a partial decrease in C3 activation by BG and GM, confirming that BG and GM activate the complement system partially through the classical and lectin pathways (Fig. 2C and D).

Conidial surface rodlets interact with complement C3. The deposition of C3b on the conidial surface upon opsonization with C3 was concentration dependent (Fig. 3A), suggesting a specific interaction between the conidial surface and C3. The dormant conidial surface is covered by a rodlet layer, made up of the protein RodAp, which belongs to the family of hydrophobins; however, melanin is exposed at some areas on the conidial surface (1, 12). Melanin did not activate any of the complement pathways (Fig. 1A); therefore, we looked at the possible interaction between RodAp and the complement C3. In an enzyme-linked immunosorbent assay (ELISA), there was a concentration-dependent interaction between RodAp and C3 (Fig. 3B). To further test this interaction, conidial surface RodAp was opsonized and extracted from conidia using hydrofluoric acid (HF) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3C), and the extract was subjected to Western blotting

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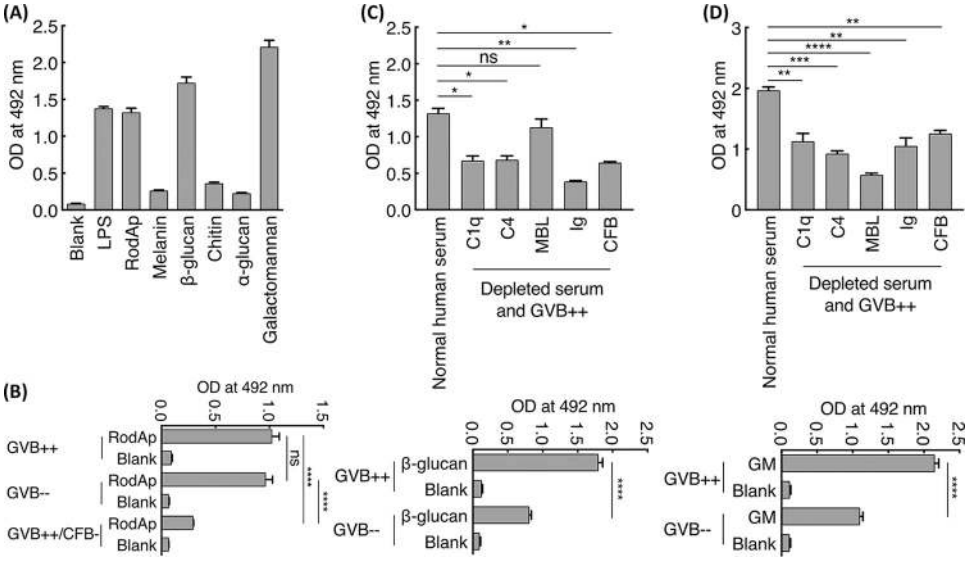

FIG 2 Complement activation by the *A. fumigatus* conidial cell wall components. Cell wall components were used to coat microtiter plates, and serum (8 μl) was diluted in gelatin-Veronal buffer (GVB; 92 μl) without supplement (GVB−) or supplemented with Ca^{2+}-Mg^{2+} (GVB++), which permitted activation of only the alternative pathway or all three pathways, respectively. After incubation, the contents were discarded and the wells were washed in wash buffer three times. The level of complement activation by each cell wall component was determined by measuring the amount of deposited C3b, by using anti-human C3b antibodies and peroxidase-conjugated anti-mouse IgG antibodies. *O*-Phenylenediamine was used as the peroxidase substrate, the reaction was arrested using 4% H_2SO_4, and the optical density (OD) was measured at 492 nm. (A) Complement activation capacity of the individual cell wall components found in *A. fumigatus* conidia. A pooled serum sample from nine healthy donors was used, and the experiment was performed with three technical and two biological replications. (B) Complement pathway activation by the cell wall components RodAp, β-(1,3)-glucan, and galactomannan. RodAp converted C3 to C3b in both GVB++ and GVB– but showed a significant decrease in C3 activation with complement factor B-depleted serum in GVB++, whereas β-(1,3)-glucan and galactomannan showed a significant decrease in the conversion of C3 to C3b in GVB–. (C and D) Complement factors required by β-(1,3)-glucan and galactomannan. Complement-depleted sera showed the requirement of different complement factors for the complement activation by β-(1,3)-glucan and galactomannan. MBL, mannose-binding lectin; CFB, complement factor B.

and probed with anti-RodAp, anti-C3, or anti-C3b antibodies (Fig. 3D). Western blots displayed multiple bands upon probing with anti-C3 antibody; importantly, a band with a molecular mass of ~68 kDa was observed when blots were probed with either anti-RodAp or anti-C3b antibodies, suggesting a covalent linkage between C3b and RodAp. At the same time, when the blot was probed with anti-RodAp antibody, there were bands in addition to the one at 68 kDa, suggesting that conidial surface rodlets can interact with other components of the complement system and humoral immune components.

Complement proteins facilitate conidial phagocytosis via complement receptors. Opsonization is described as leading to microbial aggregation, phagocytosis, or MAC formation, ultimately causing microbial lysis. Having ruled out MAC formation on *A. fumigatus* conidia based on our proteomic and immunolabeling data, we tested other possible roles of opsonization. Bright-field microscopy showed conidial aggregation following opsonization by serum (Fig. 4A). Upon interaction with hMDM, the opsonized conidia were phagocytosed at a significantly higher rate (Fig. 4B), indicating that opsonization facilitates conidial phagocytosis.

Since C3 is the predominant complement protein interacting with conidia and opsonization facilitated conidial phagocytosis, we hypothesized that complement receptors might play a role in conidial phagocytosis. Accordingly, when the complement receptors CR3 and CR4 on hMDM were blocked using anti-CD11b, anti-CD11c, and anti-CD18 antibodies, there was a significant decrease in the phagocytosis of opsonized conidia (Fig. 4B and C), suggesting that complement receptors are indeed involved in conidial phagocytosis. We confirmed that the CR3 and CR4 blockage using antibodies did not affect the viability of hMDM. Nevertheless, even after blocking the CR3 and CR4,

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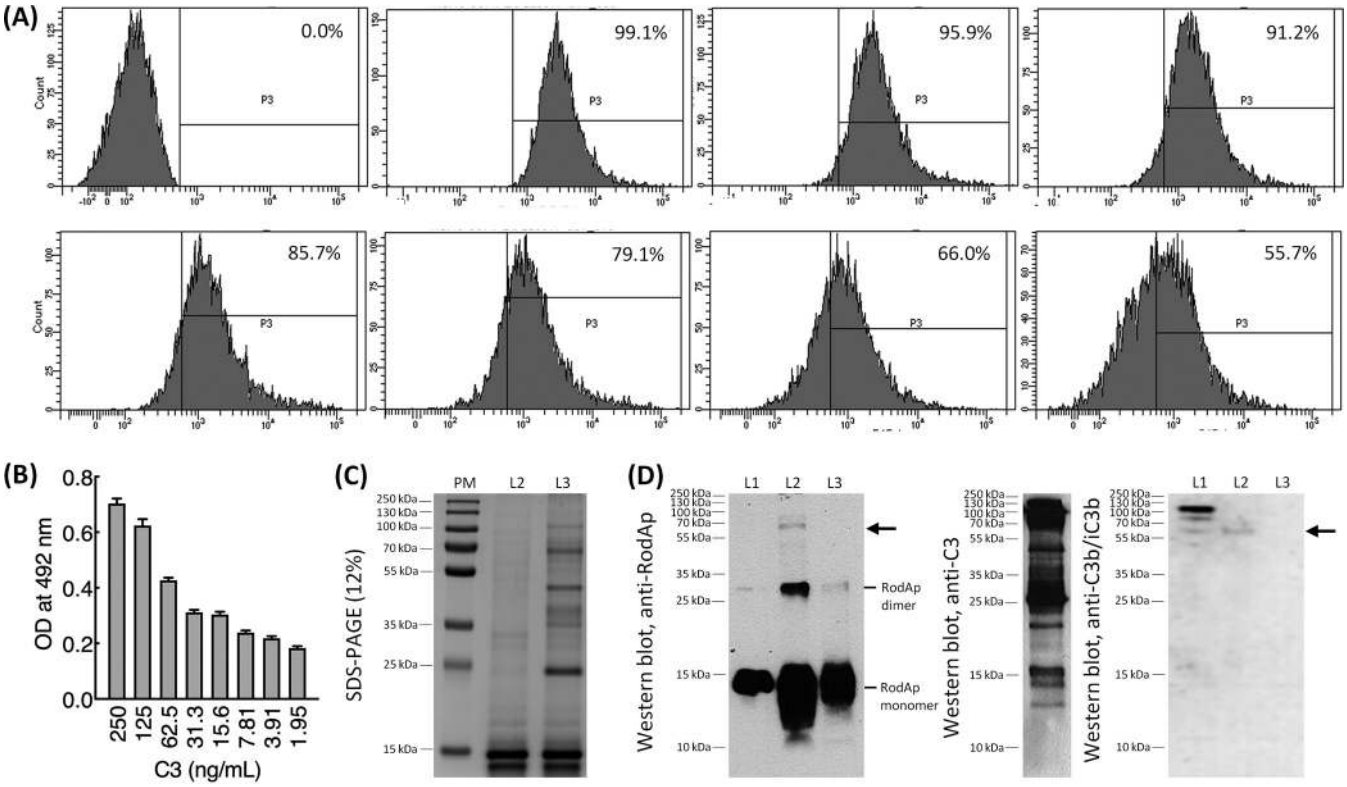

FIG 3 C3 interacts with *A. fumigatus* conidial surface in a concentration-dependent manner. (A) Flow cytometry. (B) ELISA showing concentration-dependent C3 binding to the conidial surface (C3 concentration, 1.95 to 250 ng/ml). (C) Unopsonized and serum-opsonized conidia were subjected to RodAp extraction using hydrofluoric acid (HF), and extracted protein was monitored by SDS-PAGE (12% gel with Coomassie brilliant blue staining). PM, protein markers; L2, unopsonized conidial HF extract; L3, serum-opsonized conidial HF extract. (D) Conidia were subjected to Western blotting (15% gel for protein separation) using polyclonal anti-RodAp antibodies (left: L1, recombinant RodAp; L2, serum-opsonized conidial HF extract; L3, unopsonized conidial HF extract), monoclonal anti-C3 antibodies (middle: serum-opsonized conidial HF extract), and monoclonal anti-C3b antibodies (right: L1, purified complement C3; L2, serum-opsonized conidial HF extract; L3, unopsonized conidial HF extract). Arrows indicate a common band recognized by both anti-C3b and anti-RodAp antibodies, suggesting a covalent interaction between RodAp and the complement protein C3.

there was still a significant uptake of unopsonized conidia (Fig. 4B), suggesting that conidial recognition and phagocytosis could occur independently of complement opsonization and complement receptors.

Human serum and BALF display differential complement component interactions with *A. fumigatus* conidia. Table 2 shows the identification of complement proteins extracted from the conidial surface opsonized with bronchoalveolar lavage fluid (BALF). Similar to serum-opsonized conidia, BALF-opsonized conidia showed C3 as the major complement component interacting with the conidial surface. However, binding of other complement components varied between serum- and BALF-opsonized conidia (Table 3). Complement proteins C2, MBL, CFI, MASP1, and MASP2, which were found in the serum-opsonized conidial extract, were absent in the BALF-opsonized conidial extract. Moreover, CFB was found only in the protein extract of conidia opsonized with BALF. These observations suggested complement activation through the classical and lectin pathways upon opsonization with serum, in contrast to the BALF-opsonized conidia, for which only the alternative pathway was operational. Ficolin, a complement factor also involved in the activation of the lectin pathway, was not found in either serum- or BALF-opsonized conidial surface protein extracts. In addition to complement proteins, we also observed a differential binding of surfactant proteins A and D (SP-A and SP-D, the C-type lectins belonging to the collectin family) upon conidial opsonization with serum and BALF; SP-A and SP-D were extracted from the BALF-opsonized conidia even though their PSM scores were low; however, they were not identified in the serum-opsonized conidial extract.

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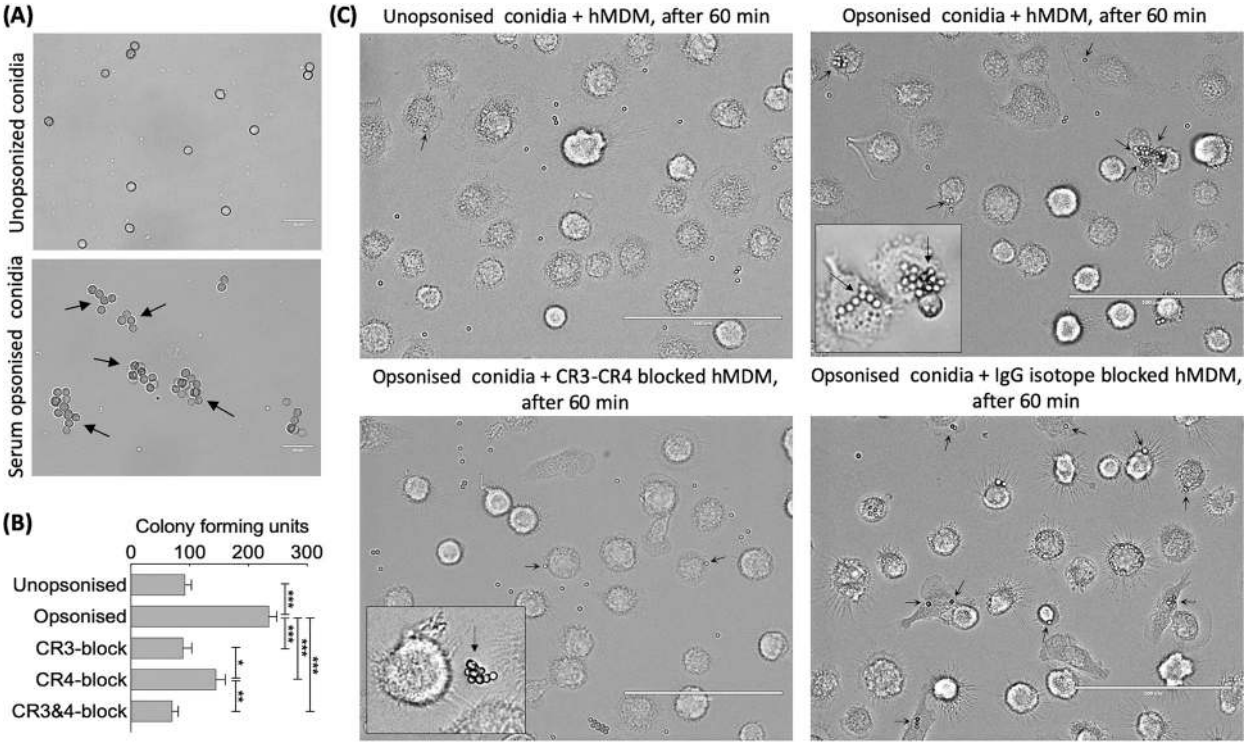

FIG 4 Opsonization facilitates *A. fumigatus* conidial aggregation, and complement receptors are involved in conidial phagocytosis. (A) Bright-field microscopy showing conidial aggregation upon opsonization with pooled human serum (arrows indicate conidial aggregation). (B) Serum-opsonized conidia suspended in incomplete RPMI were added to human monocyte-derived macrophages (hMDM; opsonized) or complement receptor-blocked hMDM (CR3-block, CR4-block, and CR3&4-block); unopsonized conidia added to hMDM served as the control (unopsonized). After 1 h of incubation at 37°C in a CO_2 incubator, supernatants were discarded, wells were washed twice with PBS, hMDM were disrupted using 1% Triton X-100, and the contents were collected and sonicated gently in a water bath to separate any aggregated conidia. After appropriate dilutions, collected conidial suspensions were spread over malt agar and incubated at 37°C until the growth of colonies, followed by CFU counting; hMDM from three donors and two technical replicates were used for CFU counts, and the means and standard deviations are presented (***, $P < 0.0001$; **, $P < 0.0001$; *, $P < 0.01$). (C) Bright-field microscopy showing significantly increased conidial phagocytosis upon conidial opsonization with serum and a decrease in the phagocytosis upon blockage of the complement receptors CR3 and CR4 on hMDM (arrows indicate phagocytosed conidia). The inset in the image of opsonized conidial phagocytosis indicates the capacity of hMDM to take up aggregated conidia, and that in the image of CR3- and CR4-blocked hMDM indicates the inability of hMDM to phagocytose aggregated conidia as well.

Flow cytometry analysis indicated that conidial opsonization with BALF results in C3b deposition and conidial aggregation (Fig. 5A and B), similarly to opsonization with serum. We looked at the cytokine response upon stimulating hMDM with serum- or BALF-opsonized conidia. Although significantly higher than the control (medium), the levels of secretion of interleukin 10 (IL-10; an anti-inflammatory cytokine) from hMDM stimulated with unopsonized and serum- and BALF-opsonized conidia were not significantly different. In contrast, the levels of secretion of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and IL-6 were significantly higher upon stimulation of hMDM with serum-opsonized conidia, IL-1β secretion was higher with BALF-opsonized conidia, and IL-8 (chemoattractant) secretion was higher with serum-opsonized conidia. Of note, the secretion of TNF-α, IL-6, and IL-8 was not significantly different upon stimulation of hMDM with unopsonized or BALF-opsonized conidia (Fig. 5C).

Opsonization results in conidial killing through reactive intermediates. We further studied the effect of opsonization on conidial killing efficiency of phagocytes. THP1 cells (human leukemic monocytes) were incubated with opsonized (serum or BALF) or unopsonized conidia for 6 h; the viability of the conidia in the interaction mixture was then examined. The percentage of live conidia was significantly lower with opsonization than in the controls and unopsonized cells (Fig. 6A). Moreover, THP1 cells coincubated with opsonized conidia produced significantly higher levels of reactive

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TABLE 2 Complement proteins extracted from the conidial surface opsonized with bronchoalveolar lavage fluid (pooled) from healthy donors and identified using mass spectrometry[a]

Protein description	UNIPROT ID	Mol mass (kDa)	PSM score	
			NH_2OH extract	NaSCN extract
Complement C3B	CO3B_HUMAN	187	784	178
Complement factor H	CFAH_HUMAN	139	237	1,003
Complement C4B	CO4B_HUMAN	193	196	30
Complement C5	CO5_HUMAN	188	110	5
Complement C1q subcomponent subunit B	C1QB_HUMAN	27	32	15
Complement C1q subcomponent subunit A	C1QA_HUMAN	26	27	8
Complement factor B	B4E1Z4_HUMAN	141	26	54
Complement component C7	CO7_HUMAN	94	24	29
Complement C1q subcomponent subunit C	C1QC_HUMAN	26	14	4
Complement factor H-related protein 2	FHR2_HUMAN	28	13	36
Properdin	PROP_HUMAN	51	12	14
Complement C1r subcomponent	B4DPQ0_HUMAN	82	11	26
Complement factor D	CFAD_HUMAN	27	10	14
Complement C1s subcomponent	C1S_HUMAN	77	8	13
Complement component C8 gamma chain	CO8G_HUMAN	22	6	1
Complement component C9	CO9_HUMAN	63	4	6
Complement component C6	CO6_HUMAN	105	3	20
Complement factor H-related protein 5	FHR5_HUMAN	64	2	34
Complement component C8 beta chain	CO8B_HUMAN	67		5

[a]PSM, peptide-spectrum match.

oxygen species (ROS) than control THP1 cells (incubated with culture medium alone) or THP1 cells incubated with unopsonized conidia (Fig. 6B).

DISCUSSION

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

It was shown previously that *A. fumigatus* conidia predominantly activate the alternative pathway (5, 7). Confirming that conidia activate C3 through the alternative

TABLE 3 Complement proteins from human bronchoalveolar lavage fluid and serum differentially interacting with *A. fumigatus* conidia and identified using mass spectrometry[a]

Protein description	UNIPROT ID	Mol mass (kDa)	PSM score			
			Serum		BALF	
			NH_2OH extract	NaSCN extract	NH_2OH extract	NaSCN extract
Complement C2	CO2_HUMAN	83	344	216	Not identified	Not identified
Complement factor B	B4E1Z4_HUMAN	141	Not identified	Not identified	26	54
Complement factor I, isoform CRA_b	G3XAM2_HUMAN	65	25	12	Not identified	Not identified
Mannose-binding protein C	MBL2_HUMAN	26	25		Not identified	Not identified
Mannose-binding lectin serine protease 1	MASP1_HUMAN	79	11	23	Not identified	Not identified
Mannose-binding lectin serine protease 2	MASP2_HUMAN	76	4		Not identified	Not identified

[a]PSM, peptide-spectrum match.

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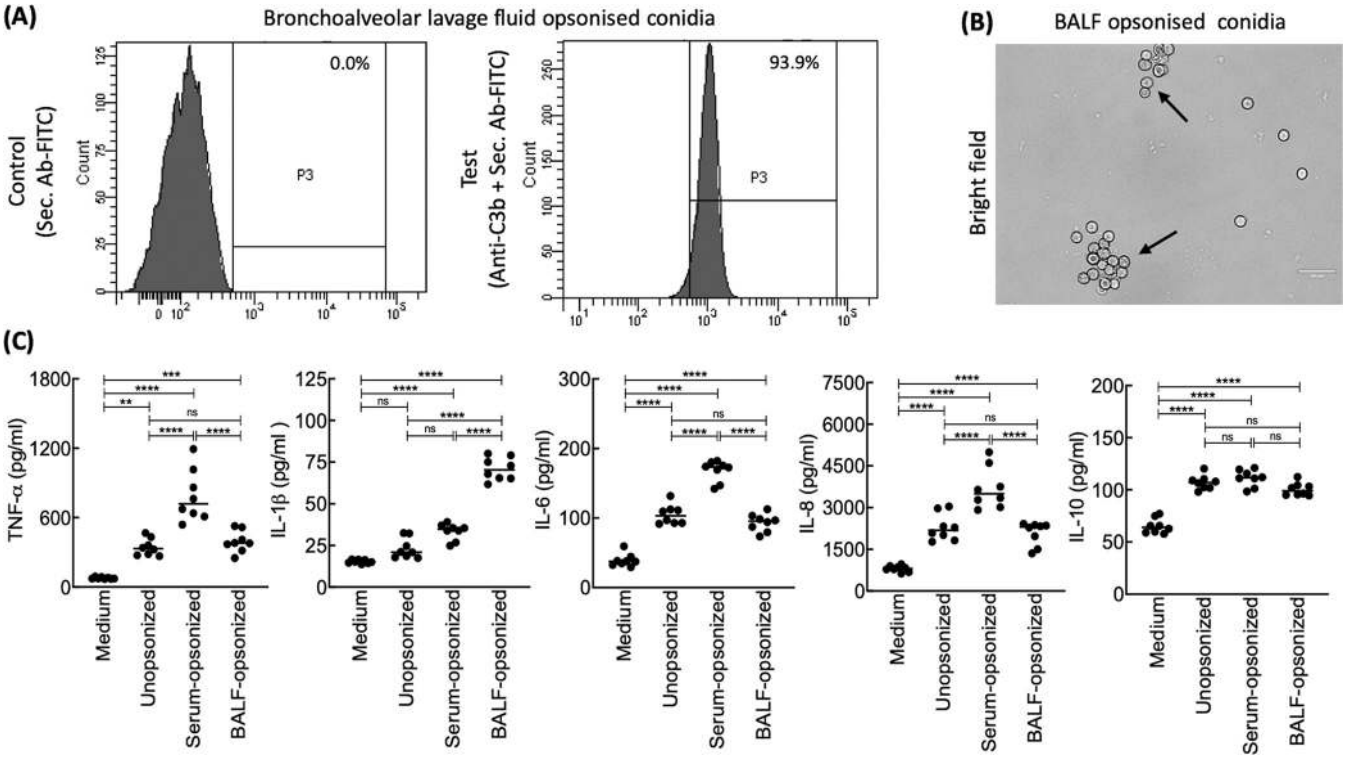

FIG 5 Cytokine secretion upon stimulation of human monocyte-derived macrophages (hMDM) with conidia. (A) Flow cytometry showing the deposition of C3b on the conidial surfaces; conidia were opsonized with pooled human BALF and probed with monoclonal anti-C3b antibodies followed by FITC-conjugated secondary antibody. Opsonized conidia probed with FITC-conjugated secondary antibody served as the control. (B) Bright-field microscopy showing conidial aggregation upon opsonization with pooled human BALF. (C) hMDM (obtained upon seeding 2×10^6 PBMCs per well) were stimulated with unopsonized, serum-opsonized, or BALF-opsonized conidia suspended in RPMI without serum (500 μl/well with 2.5×10^4 conidia) and incubated at 37°C for 20 h in a CO_2 incubator. The supernatants were recovered from the microtiter plate wells and subjected to cytokine analyses (TNF-α, IL-1β, IL-6, IL-8, and IL-10). The values are from four donors with two technical replicates; hMDM from each donor were tested for all four groups of samples, i.e., medium control, unopsonized conidia, serum-opsonized conidia, and BALF-opsonized conidia. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

pathway, we observed that the depletion of Ca^{2+}-Mg^{2+} during interaction between C3 and RodAp, the protein that coats the conidial surface, had no effect on C3 activation. C1q and MBL were also shown to bind to dormant conidia of *A. fumigatus*, activating the classical and lectin pathways (13–15). Ficolin, the other lectin responsible for activating the lectin pathway, was also shown to bind to *A. fumigatus* (15–17). However, these observations were based on the interaction of conidia with serum. In contrast to studies with serum-opsonized conidia, MBL and the mannose-binding serine proteases (MASP1 and MASP2) were not identified in our proteomic analysis of the BALF-opsonized conidia, suggesting the absence of the lectin pathway upon conidial opsonization with BALF. Although C1q was identified in both serum- and BALF-opsonized conidial protein extracts, C2, one of the complement components required for the activation of the classical pathway, was absent in the BALF-opsonized conidial extract. The importance of C1q binding to the conidial surface is obscure with these data, as C1q is a component of the classical pathway. The structure of C1q resembles that of collectins, the soluble pattern recognition receptors (PRR) (18); the collectin receptors share binding sites with C1q (19). Therefore, C1q may function as a conidium-recognizing PRR, although this hypothesis needs to be validated. Of note, it has been reported that C1q knockout mice are susceptible to invasive aspergillosis (20). Pentraxin 3 (PTX3) is involved in the complement activation via classical pathway by recruiting C1q to the microbial surfaces (21). We could identify PTX3 in the pooled BALF sample after subjecting it to in-solution digestion followed by proteomic analysis but not in the protein extract from BALF-opsonized conidia. This could be due to the overwhelming C3 recruitment on the conidial surface through the activation of the alternative

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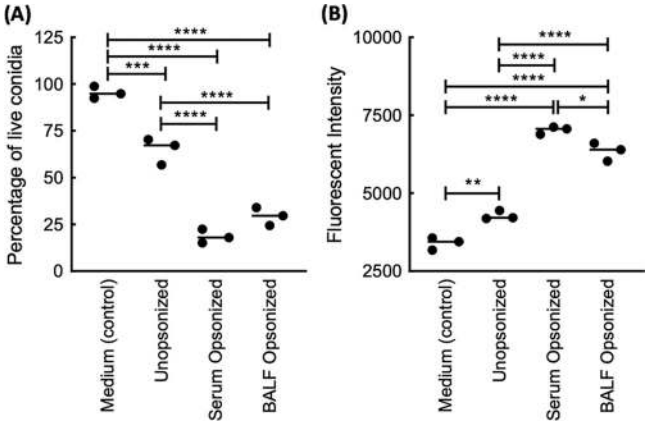

FIG 6 Opsonization facilitates conidial killing by phagocytes, possibly through reactive oxygen intermediates. (A) In cell culture plate wells, human leukemic monocytes (THP1 cells) were made to interact with serum- or BALF-opsonized or unopsonized conidia for 6 h at 37°C in a CO_2 incubator. From wells, the entire reaction mixtures were collected and centrifuged; the supernatant was discarded, and the cell pellet was collected (both conidia and THP1 cells). Wells were washed twice with 1% Triton X-100 (0.5 ml each time); the contents were transferred to tubes containing cell pellets and incubated at 4°C for 30 min. Next, the tubes were sonicated for 60 s in a water bath to disrupt THP1 cells, thus releasing phagocytosed conidia. Conidial suspensions in the tubes were diluted appropriately, spread over malt agar plates, and incubated at 37°C until fungal colony growth. The fungal colony counts were expressed as percent live conidia. (B) THP1 cells were incubated with unopsonized or serum- or BALF-opsonized conidia for 2 h in a CO_2 incubator. Next, 100 μM 2,7-dichlorofluorescein diacetate was added, cells were incubated for 1 h, and fluorescence was read using a Tecan Infinite 200 PRO plate reader, with excitation and emission at 485 and 530 nm, respectively. The results were expressed as fluorescence intensities. For both killing and ROS production assays, incomplete RPMI was used; conidia added to the well containing only the culture medium served as controls for the killing assay, and wells with THP1 cells suspended in incomplete RPMI were the controls for the ROS assay. Experiments were performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

pathway or due to the technical issues (in-gel digestion) in identifying PTX3 if its PSM score in the extracted sample is significantly low.

To date, studies of complement activation by *A. fumigatus* have always utilized intact fungus. As a result, the role of conidial CW, the first fungal component to interact with the host immune system and the one responsible for complement activation, remained unclear. The *A. fumigatus* CW is a dynamic and immunomodulating component whose composition changes across fungal morphologies (22). Understanding the interaction between the complement system and the CW components is essential for elucidating how the fungal pathogen is eliminated in healthy hosts, as phagocytosis of inhaled conidia may not be an immediate process (23). We found that RodAp and the CW polysaccharides BG and GM could activate the complement system, in contrast to the rest of the CW components. It has been shown that there is stage-specific exposure of BG during conidial germination, and an atomic force microscopy analysis using a ConA-ligated probe indicated at least 5 to 7% mannan positivity on the conidial surface (24), suggesting that BG and GM could play roles in the partial activation of the complement system.

The melanin pigments present in the *Aspergillus niger* and *Cryptococcus neoformans* CWs are of dihydroxyphenylalanine (DOPA) origin, and were shown to activate the alternative pathway and to bind activated C3 (25). However, melanin pigment in the CW of *A. fumigatus* is dihydroxynaphthalene (DHN) derived (26), and in our assay it failed to activate the complement system. In *A. fumigatus* conidia, melanin pigments form a layer underneath the rodlet layer, and at places it is exposed on the conidial surface (12). Upon disruption of a major enzyme in the *A. fumigatus* DHN melanin biosynthesis, the deposition of C3 was enhanced, which suggested that the intact DHN melanin indeed impairs C3 binding to the conidial surface (27). On the other hand, chitin, a polymer of *N*-acetylglucosamine, should be a ligand for ficolin, a lectin containing a collagenous domain and a fibrinogen domain that recognizes

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N-acetylated compounds (28). Ficolin has been shown to bind to chitin and activate the complement system (17) as well as to activate the alternative pathway in human plasma (29). However, in the present work, although it was significant compared to the control, we did not observe a major C3b deposition on chitin upon opsonization with human serum. This discrepancy could be explained by the fact that in the earlier studies, crustacean chitin (extracted from crab or shrimp) was used, while we used chitin isolated directly from *A. fumigatus* conidial cell wall.

In our view, the role of the complement system in host defense against pathogenic fungi has not been given enough attention. This could be due to the presumed resistance of the thick fungal CW to the complement-based MAC. In accordance, we did not see MAC formation on the *A. fumigatus* conidial surface. However, opsonization is known to render its effector function, causing microbial agglutination (19). We observed conidial aggregation upon opsonization with serum as well as BALF. Moreover, the complement system plays a critical role in host elimination of the fungal pathogens through opsonin-mediated phagocytosis and facilitation of the inflammatory response (10, 11, 30). In agreement with this, we observed that conidial opsonization resulted in a significant increase in phagocytosis of conidia as well as their killing.

The receptors involved in fungal recognition dictate immune responses. It has been shown that Dectin-1 binds to BG to recognize fungi (31) and that Toll-like receptor-2 (TLR2) is implicated in the cross talk between fungal cell wall α-(1,3)-glucan and human dendritic cells (32). Although β- and α-(1,3)-glucans are the major components of the conidial cell wall, they are covered by the melanin and rodlet layers (1, 8). Recently, we showed that the C-type lectin MelLec, expressed by human myeloid immune cells, recognizes the *A. fumigatus* conidial surface melanin pigment (12). Nevertheless, the study of *MELLEC* knockout mice and single nucleotide polymorphism analysis in humans indicated that this receptor is implicated in the dissemination of the fungal infection. Moreover, we also showed that Dectin-1 inhibition only partially blocks BG uptake by hMDM (33). Since immunoglobulins are rich in BALF (34), we suspected the involvement of FcR-γ (receptors recognizing immunoglobulin G) in the uptake of immunoglobulin-opsonized conidia. However, there was no difference in the uptake of opsonized conidia by hMDM with all three FcR-γ blocked (CD16 [FcR-γIII], CD32 [FcR-γII], and CD64 [FcR-γI] with respective monoclonal antibodies) and opsonized conidial uptake by hMDM, in agreement with the *in vivo* data showing that conidial uptake by alveolar neutrophils of FcR-γII knockout mice was comparable to that of wild-type mice (35). Thus, it was still unclear which receptors were involved in conidial recognition. Complement receptors CR3 and CR4 are the receptors known to recognize activated C3 fragments (36). In our study, upon blocking these two CRs on hMDM, we observed a significant decrease in phagocytosis of opsonized conidia, suggesting that CR3 and CR4 are the major receptors involved in the recognition of *A. fumigatus* conidia, facilitating their phagocytosis. However, there was a significant ($\sim$35%) amount of phagocytosis of unopsonized as well as opsonized conidia even after complement receptor blockage, suggesting that other receptors are involved.

A common practice in performing *in vitro* cell culture assays is to supplement medium with serum. However, *A. fumigatus* is an airborne pathogen, and its conidia first encounter the alveolar environment. With a panel of five cytokines, we showed here that the levels of induction of cytokines when hMDM encounters conidia opsonized with serum and BALF are different. Levels of TNF-α, IL-6, and IL-8 secretion were higher and the level of secretion of IL-1β was lower with serum-opsonized conidia than BALF-opsonized conidia, with no significant difference in IL-10 secretion. TNF-α, IL-1β, and IL-6 are involved in the proinflammatory response. IL-1β contributes to the augmentation of antimicrobial properties of phagocytes as well as to the differentiation of T cells in to Th1/Th17 cells (37, 38) and expansion of Th17 cells (39), and TNF-α maintains a normal innate immune response when an infection is encountered (40, 41), whereas IL-6 has been shown to induce IL-17 production upon *Aspergillus* infection (42). Thus, a significantly higher secretion of IL-1β than other cytokines by hMDM upon interaction with BALF-opsonized conidia may indicate a beneficial role in the clearance

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of *A. fumigatus* conidia through multiple axes. Classically, inflammasomes are thought to be critical for the release of IL-1β; however, IL-1β release could also be TLR mediated (38). Also, it has been demonstrated that TLR2 aggregates at the site of conidial phagocytosis (43). Therefore, even after the blockage of complement receptors, a significant conidial phagocytosis by hMDM leads us to speculate that there might be cross talk between complement proteins and/or other humoral immune components and TLRs.

We demonstrated that the *A. fumigatus* conidial surface rodlet layer masks conidial recognition by immune cells (1). In agreement, there was no cytokine production when paraformaldehyde (PFA)-fixed (inactivated) conidia were made to interact with hMDM in a medium supplemented with human serum, suggesting that metabolically active conidia are essential for phagocytes to mount an antifungal defense mechanism. CR3-mediated phagocytosis has been considered a silent mode of entry for pathogens, resulting in limited induction of proinflammatory cytokines (44, 45). In line with this, even at an hMDM-conidium ratio of 2:1, the rate of secretion of proinflammatory cytokines by hMDM in our study system was not very high (the positive-control lipopolysaccharide [10 ng/well] used in our study resulted in the secretion of 6,792 $\pm$ 69 pg/ml of TNF-α, 1,503 $\pm$ 58 pg/ml of IL-6, and 6,789 $\pm$ 332 pg/ml of IL-8), suggesting that *A. fumigatus* conidia may also utilize a complement receptor-mediated route of phagocytosis for a silent entry. On the other hand, it could be the metabolic activeness of conidia and recognition of pathogen-associated molecular patterns in the phagolysosome following conidial swelling that stimulate phagocytes to produce reactive intermediates, a host defense mechanism that results in conidial killing. It should be noted that hMDM could take up a significant number of unopsonized conidia; nevertheless, conidial killing and ROS production were lower under these conditions. Thus, our study suggests that *A. fumigatus* conidial phagocytosis and killing are two independent processes, in agreement with the earlier observation (23).

Altogether, our data indicate that the complement system is activated on the *A. fumigatus* conidial surface through the alternative pathway, facilitating conidial opsonization, aggregation, and phagocytosis. *Aspergillus fumigatus* conidial interaction with the complement system in the alveolar environment results in the activation of phagocytosis and enhanced antimicrobial properties, facilitating conidial clearance in healthy hosts. However, we could not identify the functional role played by some of the complement proteins interacting with conidia in our study, for example, MBL. This could be due either to competition of MBL with the structurally similar lung collectins SP-A and SP-D, which, as shown here, interact with conidia, or to the absence of MBL in the BALF from uninfected lungs (46). However, MBL has been reported to be the activator of complement in the presence of low immunoglobulin levels in aspergillosis (15). Furthermore, genetic polymorphism leading to MBL deficiency has been reported to be associated with chronic pulmonary and severe invasive aspergilloses (47, 48). A significantly lower level of serum MBL was found in invasive-aspergillosis patients than in controls, suggesting an association between MBL deficiency and invasive aspergillosis (48). Interestingly, mRNA transcripts related to complement components C3 and CFB in primary human bronchial epithelial cells were downregulated upon *A. fumigatus* infection (49). These observations demand further investigation of the role played by the complement and humoral immune system against *A. fumigatus* during infection.

MATERIALS AND METHODS

Ethics statement. Blood samples from healthy individuals were obtained from Etablissement Français du Sang Saint-Louis (Paris, France) with written and informed consent per the guidelines provided by the Institutional Ethics Committee, Institut Pasteur (convention 12/EFS/023). For the BALF samples used in this study, according to French public health law (https://www.legifrance.gouv.fr/eli/decret/2017/5/9/AFSP1706303D/jo/texte), the protocol of using the BALF did not require approval from an ethical committee and was exempted from the requirement for formal informed consent.

***Aspergillus fumigatus* strain and preparation of cell wall components.** The *A. fumigatus* CBS144-89 clinical isolate (50) was maintained on 2% malt extract agar slants at ambient temperature; conidia were harvested from the agar slants after 12 to 15 days of growth. RodAp and melanin pigment were obtained as described earlier (8, 51). β-(1,3)- and α-(1,3)-glucans were extracted from the alkali-

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insoluble and alkali-soluble conidial CW fractions, respectively, following the protocol we and others described previously (32, 52, 53). Chitin was obtained from the conidial morphotype following the protocol that we described for the mycelia (54), whereas galactomannan (GM) was isolated from the *A. fumigatus* plasma membrane fraction (55).

Chemicals, buffers, and serum and BALF samples. Human complement C3 (purified from serum), lipopolysaccharide (LPS) from *Escherichia coli*, and polymyxin B-agarose were purchased from Sigma-Aldrich/Merck Millipore. Buffers for different complement activation pathways were prepared as described earlier (56). Whole-blood samples were collected from five healthy donors, incubated at 37°C for 30 min, and centrifuged at 3,000 rpm for 5 min; the blood cell pellet was discarded, and the collected serum samples were pooled. Pooled serum samples were also obtained from Zen-Bio, Inc. (France). Complement C4-, factor B-, and immunoglobulin-depleted sera were purchased from Complement Technology, Inc. (Texas, USA), and C1q-depleted serum was obtained from Merck Chemicals. Mannose-binding lectin (MBL)-depleted serum was prepared using pooled serum samples as described earlier (57). Bronchoalveolar lavage fluid (BALF) from four donors negative for fungal culture and nucleic acids was obtained from the Centre Hospitalier Universitaire de Rennes, Hôpital Pontchaillou. Briefly, these donors, who were suspected of having infection, underwent bronchoscopy following local anesthesia, and the BALF samples were collected by instilling 40 to 50 ml saline, aspirating at least 50% of the instilled saline, repeating this procedure three times, and collecting each fraction separately. Collected fractions were centrifuged (300 $\times$ *g*, 5 min) to separate BAL cells, and if necessary, the supernatant was passed through a nylon mesh of 70 μm to remove any fibrillar material. All these fractions were stored at -80°C until further use. The galactomannan indices in these BALF samples (0.173 to 0.233) were below the EORTC/MSG (European Organization for Research and Treatment of Cancer and the National Institute of Allergy and Infectious Diseases Mycoses Study Group) cutoff value ($\geq$1.0) (58), and they were associated with other negative biomarkers, allowing us to exclude probable *Aspergillus* infection. Of the four individuals from whom BALF was collected, two had suspected pneumonia, one with infectious lung lesions in a computed tomography (CT) scan and the other with acute respiratory failure. However, these four BALF samples had a bacterial count of $<10^3$ CFU/ml (uninfected [59]); three of them were negative for viral loads, and the fourth individual had an asymptomatic cytomegalovirus load (2.37 $\log_{10}$ IU/ml; interquartile range, 0 to 2.5 $\log_{10}$ IU/ml [60]). We pooled these four BALF samples in our study. Each BALF sample used in our study was an aliquot of fraction 1 of the three fractions aspirated from an individual.

Conidial opsonization and extraction of conidial surface-bound proteins. Conidia harvested from 12- to 15- day-old agar slants were washed twice with aqueous Tween 20 (0.05%) and once with MilliQ water. Conidia (2.5×10^8) were then incubated with 100 μl of pooled serum (in-house/commercial, diluted to 20% in phosphate-buffered saline [PBS]; 223 μg protein/100 μl) or BALF (102.0 μg protein/100 μl) at 37°C for 30 min, with vortexing for every 5 min. After centrifugation (5,000 rpm, 5 min), the supernatants were discarded, conidia were washed five times with PBS, and conidial surface-bound proteins were extracted with 200 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 ensure efficient extraction. Extracted proteins were collected by centrifugation (10,000 rpm, 10 min), desalted, and concentrated using 3-kDa Amicon membrane filters (Sigma-Aldrich). Proteomic analysis was performed (three technical replicates) subjecting the samples to shotgun proteomic identification using a nanoLC-Orbitrap mass spectrometer (61).

The concentrations of proteins (estimated with Bradford reagent [Bio-Rad] using bovine serum albumin standard) in the NH_2OH extract (in micrograms) were as follows: serum-opsonized conidia, 19.35 $\pm$ 1.80; BALF-opsonized conidia, 18.01 $\pm$ 1.81; unopsonized conidia, 1.79 $\pm$ 0.45. Those in the NaSCN extract were as follows: serum-opsonized conidia, 18.01 $\pm$ 1.12; BALF-opsonized conidia, 22.47 $\pm$ 2.00; and unopsonized conidia, 2.50 $\pm$ 0.26.

Conidial immunolabeling and flow-cytometric analysis. Conidia (1×10^6) were incubated with complement C3 (5 μg/ml in PBS with 1% bovine serum albumin) for 1 h at 37°C. Conidia were then washed three times with PBS supplemented with 0.005% Tween 20 and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-C3 antibody (mouse monoclonal, clone 2 D8; Invitrogen) for 1 h at 37°C, washed with PBS–0.005% Tween 20 (twice) and PBS (once), mounted on glass slides, and observed under a confocal microscope. Labeled conidia were also subjected to flow cytometry analysis using LSR II equipment (BD Biosciences); the data were analyzed using BD FACSDiva (BD Biosciences) and FlowJo. Anti-MAC labeling was performed using anti-human C5b-9 antibody (mouse monoclonal, clone aE11; Abcam) and secondary FITC-conjugated anti-mouse IgG (Sigma-Aldrich).

Conidial opsonization, RodAp extraction using hydrofluoric acid, and Western blot analyses. As described above, conidia were opsonized with pooled serum (diluted to 20% in PBS) for 30 min, washed five times with PBS, and subjected to RodAp extraction using hydrofluoric acid (HF) (1), but only for 6 h. Extracted samples were subjected to SDS-PAGE on 12% gels and revealed by Coomassie-brilliant blue staining or Western blotting by separating proteins on a 15% gel and using mouse polyclonal anti-RodAp antibodies (62), anti-C3 antibodies (LF-MA0132, mouse monoclonal, clone 28A1 [Thermo Fisher Scientific]; targets C3b), or anti-C3b antibodies (mouse monoclonal, MA1-40155 [Invitrogen]; targets iC3b; checked by using human serum as the positive control and C3-deficient serum as the negative control and cross-checked with human serum and C3-depleted serum [catalog no. A314; Complement Technology, Inc.]) as the primary antibodies and peroxidase conjugated anti-mouse IgG as the secondary antibody (1:1,000 dilution; Sigma-Aldrich). The blot detection was performed with an enhanced chemiluminescence kit (ECL; Amersham, GE Healthcare Life Sciences).

Conidial cell wall components and complement activation. *Aspergillus fumigatus* CW components (50 μg/ml for polysaccharides or melanin pigments and 10 μg/ml for RodAp, in 50 mM bicarbonate buffer, pH 9.6) were used to coat 96-well plates (100 μl/well) overnight at ambient temperature. The

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positive-control wells were coated with LPS (4 μg/well). After the supernatants were discarded, wells were blocked with 300 μl blocking buffer (5% nonfat milk in gelatin-Veronal buffer [GVB]) at ambient temperature for 1 h and washed once with 200 μl wash buffer (PBS with 0.05% Tween 20). Plates with 8 μl sera and 92 μl GVB per well were incubated at ambient temperature for 1 h and washed with 200 μl wash buffer (three times). Deposited C3b/iC3b in the wells was assessed with monoclonal anti-human C3b/iC3b antibody (MA1-82814 [Thermo Fisher Scientific], diluted 1:1,000 in blocking buffer, 100 μl/well) and incubating at ambient temperature for 1 h. Wells were washed (three times) with PBS-Tween followed by the addition of secondary antibodies (peroxidase-conjugated anti-mouse IgG, diluted 1:1,000 in blocking buffer) and incubation at ambient temperature for 1 h. After three washes with PBS-Tween, 100 μl substrate solution (*O*-phenylenediamine) was added per well, reactions were arrested with 50 μl 4% H_2SO_4, and the absorbance was read at 492 nm.

Phagocytosis and blockade of the complement receptors. Human monocyte-derived macrophages (hMDM) were obtained and conidial opsonization was performed as described earlier (53). In brief, 500 μl (2×10^6 per ml) of a peripheral blood mononuclear cell (PBMC) suspension in incomplete RPMI medium was seeded into a 24-well cell culture plate. After 3 h of incubation in a CO_2 incubator maintained at 37°C, the supernatant was discarded, wells were washed twice with PBS, 500 μl of RPMI supplemented with 10% human serum and granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml; R&D Systems) was added, and wells were incubated in a CO_2 incubator at 37°C for 6 days with replacement of the medium after 3 days. Differentiated macrophages were washed twice with PBS and incubated with a conidial suspension (5×10^5/well, opsonized or unopsonized) in a CO_2 incubator at 37°C for 1 h; supernatants were discarded, and the wells were washed with PBS twice to remove nonphagocytosed conidia. Next, hMDM were disrupted by adding 1% Triton X-100 and incubating at 4°C for 30 min. Phagocytosed conidia were collected upon gentle scraping and washing of the wells twice with 1% Triton X-100 and subjected to gentle sonication in a water bath. Upon appropriate dilutions, conidial suspensions were spread on malt agar plates and incubated at 37°C until the growth of colonies, followed by colony counting. Conidia interacting with hMDM but not phagocytosed were identified upon calcofluor white staining (56); their percentage was deducted from the colony count to determine the percent phagocytosed conidia. Conidium-hMDM interaction was also assessed in RPMI without serum; unopsonized and serum- or BALF-opsonized conidia were used, and the culture supernatants were collected after 24 h in a CO_2 incubator and analyzed for cytokines.

To study the complement receptor blockage followed by phagocytosis, hMDM were blocked with anti-CD11b (mouse monoclonal isotype IgG1, clone CBRM1/5; BioLegend), anti-CD11c (mouse monoclonal, clone 3.9; CliniSciences), and anti-CD18 (mouse monoclonal, clone IB4; Merck Chemicals) antibodies in PBS for 30 min in a CO_2 incubator at 37°C followed by two washes with PBS, addition of conidia, and determination of colony counts, as described above. The viability of hMDM after receptor blockage was assessed by lactate dehydrogenase assay (63).

Conidial killing and ROS production assays. Experiments were performed using THP1 cells (human leukemic monocytes; Merck/Sigma-Aldrich); cells from the frozen stock were propagated as per the manufacturer's instruction. Next, collected THP1 cells were seeded into 24-well culture plates (2×10^6/well) in incomplete RPMI medium and added with unopsonized or opsonized (serum/BALF) conidia (1×10^6/well) and incubated in a CO_2 incubator maintained at 37°C. For the killing assay, conidia added to the wells containing only incomplete medium served as the control, whereas for reactive oxygen intermediate (ROS) estimation, wells with only THP1 cells in the medium served as the control. ROS production was estimated as described earlier (53), and for the killing assay, after 6 h of conidium-THP1 interaction, contents from the wells were collected and centrifuged to collect the pellet; wells were washed twice with 1% Triton X-100 (each time with 0.5 ml) with gentle scraping and the contents were transferred to the tubes containing respective cell pellets. After vortexing, collected cells contents were kept at 4°C for 30 min and subjected to brief sonication in a water bath to disrupt THP1 cells releasing phagocytosed conidia and also to disaggregate conidia if any. Upon appropriate dilutions, conidial suspensions were spread on malt agar plates and incubated at 37°C until the growth of colonies, followed by colony counting; the CFU counts were expressed as the percent live conidia, considering the number of conidia added per well as 100%.

Statistical analysis. One-way analysis of variance (ANOVA) was performed using Prism-8 software (GraphPad Software, Inc., La Jolla, CA, USA).

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