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Effective Neutrophil Phagocytosis of *Aspergillus fumigatus* Is Mediated by Classical Pathway Complement Activation

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Key Words
Complement · Host defense · Neutrophils · Phagocytosis

Abstract
Aspergillus fumigatus is an important airborne fungal pathogen and a major cause of invasive fungal infections. Susceptible individuals become infected via the inhalation of dormant conidia. If the immune system fails to clear these conidia, they will swell, germinate and grow into large hyphal structures. Neutrophils are essential effector cells for controlling *A. fumigatus* infection. In general, opsonization of microbial particles is crucial for efficient phagocytosis and killing by neutrophils. Although the antibodies present in human serum do bind to all fungal morphotypes, we observed no direct antibody-mediated phagocytosis of *A. fumigatus*. We show that opsonization, phagocytosis and killing by neutrophils of *A. fumigatus* is complement-dependent. Using human sera depleted of key complement components, we investigated the contribution of the different complement initiation pathways in complement activation on the fungal surface. We describe the classical complement pathway as the main initiator of complement activation on *A. fumigatus* swollen conidia and germ tubes. Antibodies play an important role in complement activation and efficient innate recognition, phagocytosis and killing of *A. fumigatus* by neutrophils.

Introduction

Aspergillus species are saprophytic fungi that are globally distributed and amongst the most common molds encountered. The genus *Aspergillus* includes over 200 different species, of which only a small number is associated with human or animal diseases. More than 90% of such diseases are caused by *Aspergillus fumigatus*, thus making it one of the most important airborne fungal pathogens. *A. fumigatus* induces a wide range of clinical syndromes. The disease spectrum varies from hypersensitivity and allergic pulmonary aspergillosis to severe invasive fungal disease. Although invasive aspergillosis is classically associated with specific risk factors (prolonged neutropenia, neutrophil dysfunction, cytotoxic chemotherapy, immune suppression and transplantation), there is a growing incidence of invasive aspergillosis in nonapparent immune-compromised hosts [1].

A. fumigatus mainly reproduces asexually by the production of mitotic spores called conidia. These conidia have a diameter of 2–3 μm and are ubiquitously present in the atmosphere. Dormant conidia are very well suited for air dispersal because of their small size and the presence of a hydrophobic outer cell wall layer. Due to their high concentrations in air (10–400 CFU/m^3) all humans inhale several hundred conidia per day [2]. Due to their small size, conidia can bypass the normal defenses of the

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nasal and bronchial cavities and can reach the lung alveoli without being cleared by ciliated mucus membranes. Dormant conidia are metabolically quiescent and become active under favorable conditions. This process starts with swelling and, within 4–5 h, the swollen conidia lose the outermost hydrophobic rodlet layer and become metabolically active. In the next phase, they germinate, followed by elongation of the germ tubes to produce hyphae.

As conidia are not cleared and remain in the alveoli, they have to be cleared by humoral and cellular immune responses. The innate immune system plays a crucial role in the clearance of conidia and the defense against *Aspergillus* invasion. Together with antimicrobial peptides, resident leukocytes present in the alveolar lung tissue (alveolar macrophages and dendritic cells) form the first line of defense against invasive aspergillosis. However, additional recruitment of neutrophils is essential for the efficient clearing of *A. fumigatus* [3]. A qualitative or quantitative defect in the neutrophils is one of the most important predisposing factors for invasive aspergillosis [4–6]. Killing of *A. fumigatus* by neutrophils is mediated by the production of reactive oxygen species (ROS) and the release of the neutrophil granular content [7–10]. Patients with chronic granulomatous disease, a genetic lesion causing defective production of ROS, have an increased risk for the development of invasive fungal infections, particularly invasive aspergillosis [4].

The recognition of *A. fumigatus* by phagocytes is either direct via complement receptor 3 (CR3, i.e. CD11b/CD18) or dectin-1 or indirect via opsonization [8, 11, 12]. In general, for efficient clearance by neutrophils, microbes need to be opsonized by humoral factors to establish effective phagocytosis and intracellular killing. The important opsonins in blood and tissue are proteins of the complement system and antibodies. The complement system can be activated via 3 different pathways: the classical pathway, the lectin pathway and the alternative pathway. The classical pathway is initiated by the binding of antibodies followed by the binding of C1 [13]. The C1 complex ($C1qr_2s_2$) consists of C1q, which binds to antibody-antigen complexes, and the serine proteases C1r and C1s, that initially processes C4 and subsequently C2 to generate the C3 convertase (C4b2a) [13, 14]. C1q can also recognize and bind microbial surfaces directly or indirectly via the interaction of pentraxins to activate the classical pathway independent of antibodies [15]. Mannose-binding lectin (MBL) and ficolins bind sugar moieties on the microbial surface and initiate activation of the lectin pathway that also result in the formation of C4b2a [16]. The alternative pathway mainly acts as an amplification loop but also shows a low level of spontaneous activation through hydrolysis of C3. Just like the other 2 pathways, the alternative pathway also results in the formation of a C3 convertase (C3bBb) that cleaves C3 to massively label the microbial surface with C3b molecules. C3b acts as an important opsonin for efficient phagocytosis and is the preferential ligand of CR1 (CD35) that is present on the neutrophil surface. Complement activation also results in the attraction of leukocytes to the infected tissue via the formation of C5a, an anaphylatoxin and a potent chemoattractant for the recruitment of neutrophils.

Although complement is known to stimulate the phagocytosis of many invading pathogens, its role in the clearance of *Aspergillus* infections is not well understood. Here, we show the essential role of the different complement activation pathways in the opsonization and phagocytosis by neutrophils of the different morphologic forms of *A. fumigatus* and describe the classical pathway as the main initiator of complement activation on swollen conidia and germ tubes.

Material and Methods

Fungal Strains and Culture Conditions

Af293 was used to perform deposition studies and a red fluorescent strain, Af-mRFP1, a kind gift of Michelle Momany (University of Athens, Ga., USA), was used to study phagocytosis. A green fluorescent protein (GFP)-expressing strain A1258 [17] obtained from the Fungal Genetics Stock Center (Kansas City, Mo., USA) was used to perform growth studies. All strains were cultured on minimal medium agar plates supplemented with 1% of glucose. After 2 days, dormant conidia were isolated with PBS-Tween 0.05% and the concentration was determined by optical density [18]. To obtain swollen conidia or germ tubes, minimal medium, supplemented with yeast extract, casamino acids and 2% glucose, was inoculated with fresh isolated conidia and grown at 37°C for 4 and 8 h, respectively. Swollen conidia and germ tubes were collected and washed twice with PBS and concentrations were determined by microscopic counting. All 3 morphotypes were gently sonicated for 2 min to break up clumps and stored at –20°C until further use.

Sera

Normal human serum (NHS) was isolated from at least 20 healthy donors. Heat-inactivated (HI) NHS was obtained by heating at 56°C for 30 min. To examine the different complement pathways, we used deficient sera lacking different complement components. C2-deficient serum (C0913, Sigma-Aldrich) and C1q-deficient serum (C8567, Calbiochem) were purchased. The C1q-deficient serum was subjected to a standard CH50 hemolytic assay to investigate the residual C1q activity (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000369493). Factor D (FD)-deficient serum was prepared as described earlier [19]. The sera of healthy donors were screened for naturally occurring polymorphisms in MBL that re-

sult in the activation of the abrogated lectin complement pathway [20]. Serum from 1 donor with impaired lectin pathway activity was used as MBL-deficient serum. To reconstitute deficient sera, purified proteins were added at concentrations that were 2-fold higher than normal serum levels, i.e. 28 μg/ml of FD, 140 μg/ml of C1q, 400 μg/ml of C2 and 10 μg/ml of MBL (in undiluted serum). FD (A136, CompTech), C1q (A099, CompTech) and C2 (204882, Calbiochem) were purchased commercially and MBL was kindly provided by Prof. A. Ezekowitz.

Deposition Assay

To study C3b deposition, all 3 morphotypes, i.e. dormant conidia, swollen conidia and germ tubes were incubated with different concentrations of NHS, HI NHS and deficient sera in RPMI supplemented with 0.05% human serum albumin (RPMI-HSA) for 25 min at 37°C. Fungal particles were washed twice with 1% BSA-PBS and stained with mouse-anti-C3 (1 μg/ml, WM-1, ATCC) in 1% BSA-PBS for 30 min at 4°C. The WM-1 anti-C3 antibody detects the C3c domain that is present in the C3 activation products C3b and iC3b (important ligands for CR1 and CR3 on phagocytic cells), but not C3a or C3d/C3dg (ligand for CR2 on B cells). The particles were then washed twice and stained with goat-anti-mouse-FITC (1 μg/ml, F0479, DAKO) in 1% BSA-PBS for 30 min at 4°C. They were washed once and fixed with 1% paraformaldehyde in 1% BSA-PBS and fluorescence was then measured by flow cytometry (FACSCanto, BD).

To study IgG deposition, all 3 morphotypes were incubated with HI NHS in 1% BSA-PBS-Tween 0,005% for 25 min at 4°C, washed twice and stained with goat-anti-human-IgG-FITC (5 μg/ml, 2040-02, SouthernBiotech) in 1% BSA-PBS-Tween 0.005% for 30 min at 4°C. After washing and fixation, fluorescence was examined by flow cytometry (FACSCalibur, BD).

Confocal Microscopy

To study the deposition of C3b and IgG by confocal microscopy, the different morphological forms were opsonized as described in the deposition assay. After opsonization, the morphotypes were washed and stained with mouse-anti-C3 (1 μg/ml, WM-1, ATCC) in 1% BSA-PBS for 30 min at 4°C and then with goat-anti-mouse-Alexa Fluor 488 (1 μg/ml, A-11001, Invitrogen) in 1% BSA-PBS for 30 min at 4°C. IgG staining was performed with protein A Alexa Fluor 488 (1 μg/ml, P-11047, Invitrogen) or with goat-anti-human IgG-FITC (5 μg/ml, 2040–02, SouthernBiotech) in 1% BSA-PBS for 30 min at 4°C. Both staining protocols gave similar results. All morphotypes were simultaneously incubated with wheat germ agglutinin Alexa Fluor 647 (2.5 μg/ml, W32466, Invitrogen) to stain the cell wall of the fungus. After the staining procedures, all morphotypes were washed and fixed with 1% paraformaldehyde in cold HBSS. Microscopic images were acquired using the 488- and 633-nm lasers of a Leica TCS SP5 microscope (HCX PL APO CS ×63/1.40–0.60 OIL objective, Leica Microsystems).

Phagocytosis Assay

Written informed consent was obtained from all subjects and was provided according to the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands). Fresh neutrophils were isolated from healthy volunteers as earlier described [21] and the membrane was stained with DiO (1 μl to 4×10^6 neutrophils/ml, V-22886, Invitrogen) for 10 min at 4°C and washed 3 times with cold RPMI-HSA. The Af-mRFP1 dormant conidia, swollen conidia and germ tubes were preopsonized with different sera (in the presence or absence of complement proteins) for 10 min at 37°C on a shaking platform (1,000 rpm). Subsequently, DiO-labeled neutrophils were added in an effector-to-target ratio of 1:5 and incubated for 15 min at 37°C on a shaking platform (700 rpm). After incubation, cells were fixed with ice-cold RPMI-HSA with 1% paraformaldehyde and phagocytosis was measured by flow cytometry (FACSCanto, BD). The DiO fluorescent population was gated as neutrophils. Phagocytosis was expressed as the mean fluorescence intensity (MFI) of mRFP1 within the neutrophil population or the percentage of mRFP1-positive neutrophils.

C5a Measurement

To study the generation of C5a, the different morphological forms were opsonized as described in the deposition assay. After opsonization, samples were centrifuged and supernatant was collected and stored at –20°C until further use. To measure the release of C5a, we labeled the C5aR-expressing U937 cells [22] with a calcium indicator Fluo-3 AM (0.5 μM to 5×10^6 cells/ml, F1241, Invitrogen) by agitation for 20 min at room temperature. U937 cells were washed once and resuspended in RPMI-HSA to a concentration of 1×10^6 cells/ml. The transient intracellular calcium mobilization was measured in time by flow cytometry (FACSVerse, BD). Labeled U937 cells were measured for 10 s to determine the basal level of fluorescence. Subsequently, 10 μl of supernatant was added and gently resuspended, and the fluorescence was measured over 80 s in total. Viable U937 cells were gated on forward- and side-scatter. The absolute calcium mobilization was calculated as the Fluo-3 fluorescence after the addition of supernatant minus the basal level of fluorescence. C5a activation was expressed as a percentage of calcium mobilization in complement-deficient serum compared to reconstituted complement-deficient serum.

Growth Rebound

A GFP-expressing strain A1258 was used to perform growth studies. A1258 conidia were incubated with 10% NHS or HI NHS in RPMI-HSA. Human neutrophils were added in a cell-to-conidia ratio of 3:1 and incubated for 33 h at 37°C. GFP fluorescence (Ex 485 nm, Em 520 nm) was measured every 10 min with a FLUOstar Omega plate reader (BMG Labtech). Per measurement, each condition was performed in triplicate and controlled for blank wells containing medium only. To quantify differences, the area under the curve (AUC) was calculated over the total time with Graphpad Prism. The experiment was performed independently 3 times.

Statistics

Statistical analysis was performed using an unpaired Student's t test by Graphpad Prism.

Results

C3b and Immunoglobulin G Deposition on Different Aspergillus Morphotypes

Since all different morphological forms of *A. fumigatus* will encounter serum components upon entering the

host, the deposition of the serum components C3b and immunoglobulin G (IgG) was studied. C3 deposition was investigated with an antibody recognizing both C3b and iC3b. When dormant conidia, swollen conidia and germ tubes were incubated in normal human serum, C3b was deposited in a dose-dependent manner on all the different morphotypes (fig. 1a). Significantly more C3b molecules were deposited on the germ tubes than on the swollen conidia, while C3b deposition on the dormant conidia was much lower. C3b deposition was not observed in the presence of HI NHS, lacking complement activity (fig. 1b). To study IgG deposition, without the interference of activated complement, the 3 morphotypes were incubated with HI NHS. In contrast to C3b, IgG was deposited in equal amounts on the swollen conidia and the germ tubes when incubated with 1% HI NHS (fig. 1c), while the binding to the dormant conidia was very low. Confocal microscopy confirmed these results, showing that C3b was only deposited in serum-incubated conditions and not when the morphotypes were incubated with buffer or HI NHS (fig. 1d). IgG was detected in all serum conditions, in both NHS and HI NHS, and not in the buffer control. Of note, C3b and IgG were deposited in a patchy manner on the dormant conidia, but were evenly distributed on the other morphotypes.

Complement Induces Phagocytosis of A. fumigatus

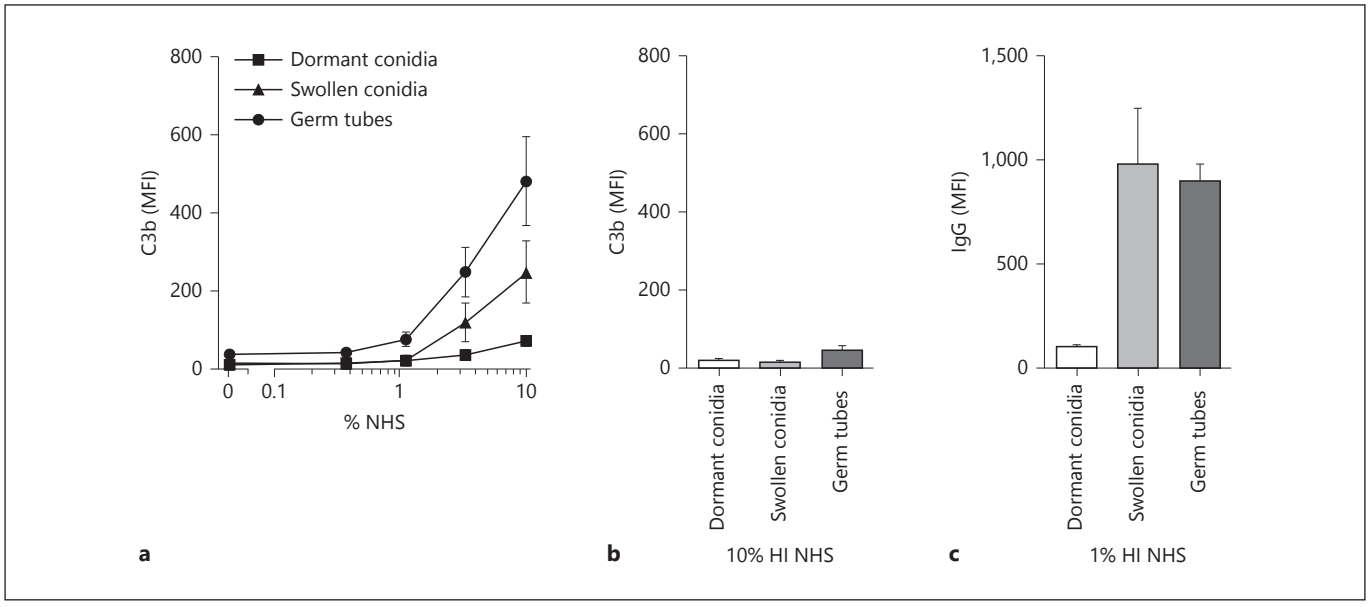
To investigate the importance of the serum components for the recognition and subsequent phagocytosis of the different morphotypes, neutrophils were incubated with preopsonized fungal particles. After 15 min of incubation, phagocytosis was measured, by quantifying either the percentages of neutrophils harboring a fungal particle (% of mRFP1-positive neutrophils) or the total fluorescence within the neutrophil population (MFI). Preopsonization with NHS resulted in 65–70% phagocytosis of all morphotypes whereas barely any phagocytosis was observed when the particles were preincubated in buffer or HI NHS (fig. 2a). Similar observations were made when the MFI of mRFP1 was plotted (fig. 2b). Almost no differences were observed between the different morphotypes. To ensure that the particles were ingested by the neutrophils, not only attached to them, confocal microscopy was performed. All the morphological forms were ingested by neutrophils when preopsonized with NHS (fig. 2c). Buffer and HI NHS preincubation did not result in the uptake of any of the morphotypes by the neutrophils, again confirming a critical role for complement in phagocytosis.

Complement Induces Killing of A. fumigatus

Next, the importance of complement on the killing of *A. fumigatus* by neutrophils was investigated. Therefore, constitutively fluorescent conidia were incubated with serum and neutrophils. GFP expression was monitored in time to observe the growth rebound of the fungus. Conidia incubated with NHS or HI NHS only showed a similar growth curve (fig. 3a). The addition of neutrophils to the HI NHS-incubated conidia did not result in differences in the outgrowth of the fungus. However, the conidia incubated with NHS and neutrophils showed a clear delay in growth. This difference was quantified by the calculation of the AUC during the complete incubation time, showing a significant delay in growth rebound (fig. 3b).

The Importance of the Classical Complement Pathway in the Opsonization of A. fumigatus

Previous results have shown the essence of complement activity in the processes of opsonization and phagocytosis of the 3 morphotypes. Since complement can be initiated via 3 different pathways, the importance of each pathway in the initiation of the complement cascade was investigated for all of the morphotypes. Dormant conidia, swollen conidia and germ tubes were incubated with different deficient sera and the amount of C3b deposition and phagocytosis was examined. FD- and C2-deficient sera distinguish between complement initiation via the alternative pathway, abolished when FD is absent, and via the lectin and classical pathways, abrogated when C2 is lacking. C1q- and MBL-deficient serum can elucidate the importance of the classical and the lectin pathway, respectively. Dormant conidia showed a decreased amount of C3b when incubated with FD- and C2-deficient sera (fig. 4a). The deposition of C3b on the dormant conidia was restored when FD- and C2-deficient sera were reconstituted with FD or C2, respectively. Incubation of the dormant conidia with C1q- and MBL-deficient serum did not result in a significant reduction of C3b deposition. Some residual classical complement activity was observed in C1q-deficient serum (online suppl. fig. 1), leading to an underestimation of the importance of the classical complement pathway. The phagocytosis of the dormant conidia preopsonized with any deficient serum did not show a significant reduction. However, a trend of reduced phagocytosis was clearly visible when dormant conidia were preopsonized with FD- and C2-deficient serum (fig. 4d), suggesting that both the alternative and the classical complement pathways are important for the opsonization and phagocytosis of the dor-



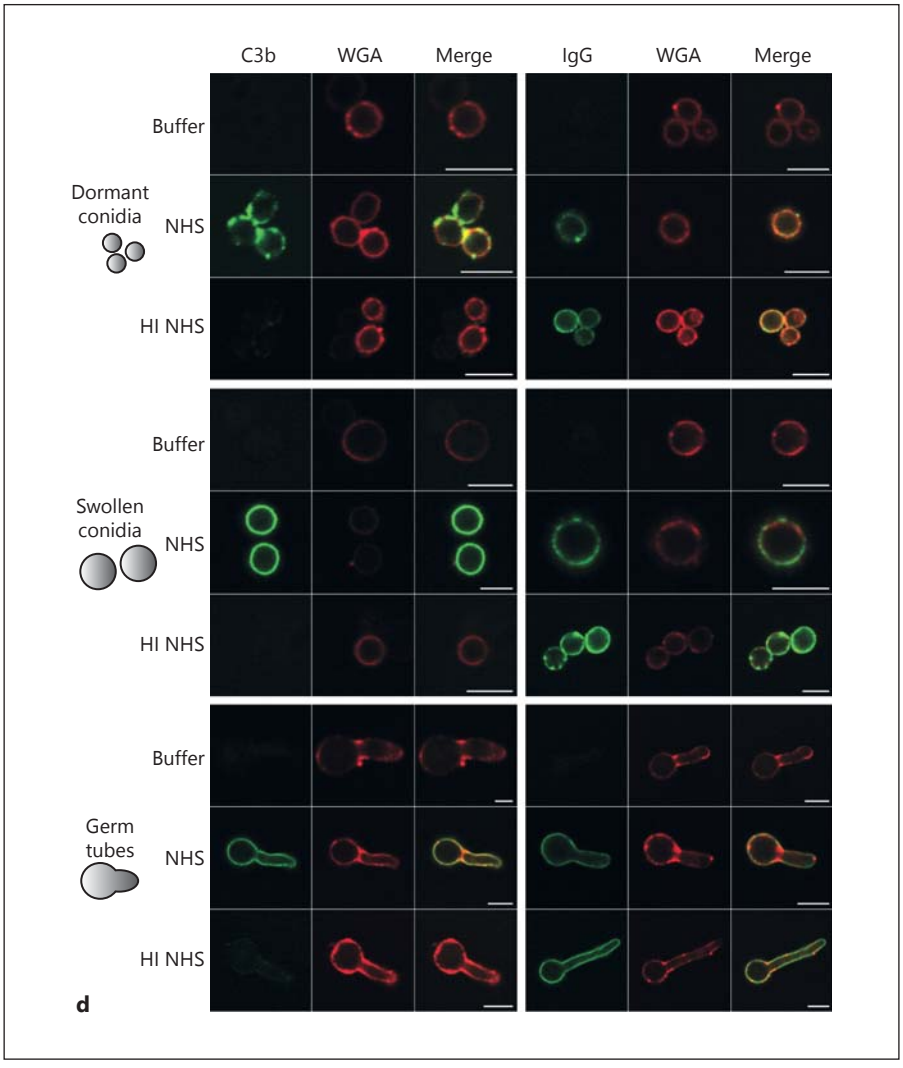


Fig. 1. C3b and IgG deposition on different *Aspergillus* morphotypes. **a** Different morphotypes (dormant conidia, swollen conidia and germ tubes) were incubated with NHS, and C3b deposition was detected by flow cytometry. **b** The different morphotypes were incubated with 10% HI NHS and C3b deposition was measured by flow cytometry. **c** The morphotypes were incubated with 1% HI NHS and IgG deposition was measured by flow cytometry. **a–c** The assays were performed at least 3 times and the mean ± SD is shown. **d** The morphotypes were incubated with buffer, 10% NHS and 10% HI NHS. C3b and IgG deposition was visualized using confocal microscopy after staining with mouse-anti-C3 and protein A Alexa Fluor 488 or anti-IgG-FITC antibodies, respectively. Wheat germ agglutinin (WGA) Alexa Fluor 647 was used to visualize the fungal cell wall. The confocal microscopy assay was performed 3 times and representative images are shown. Scale bar: 5 μm.

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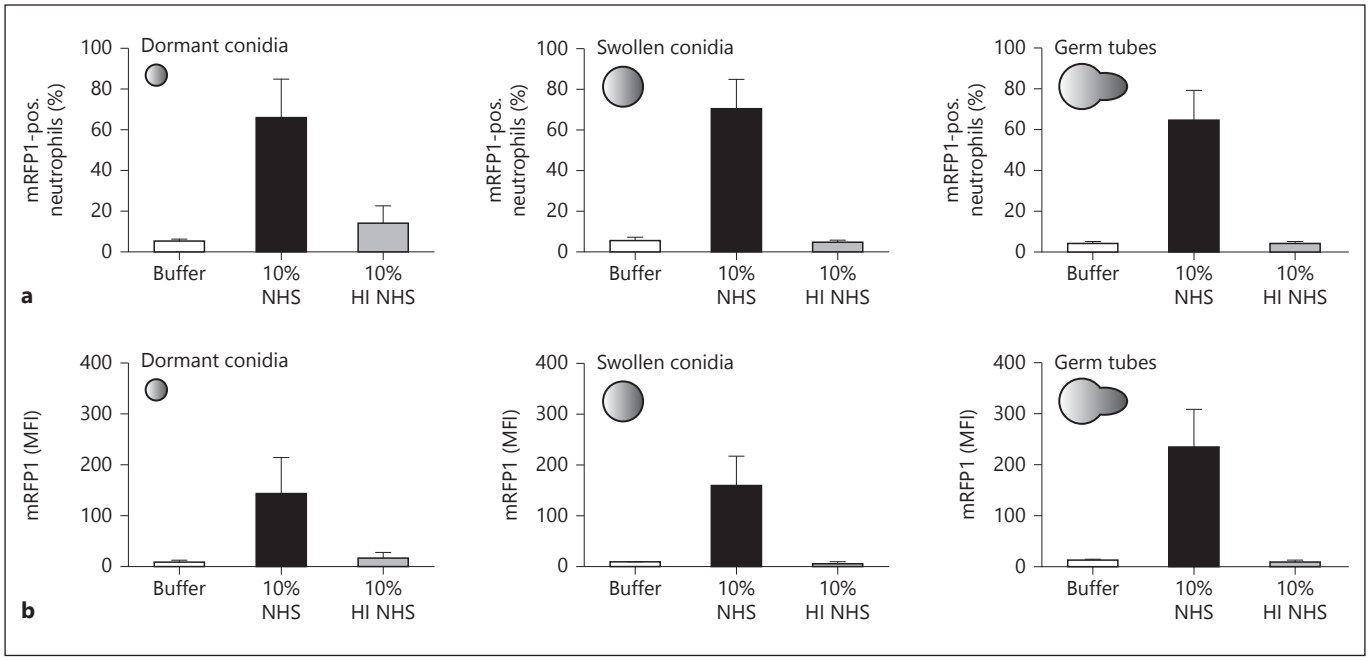

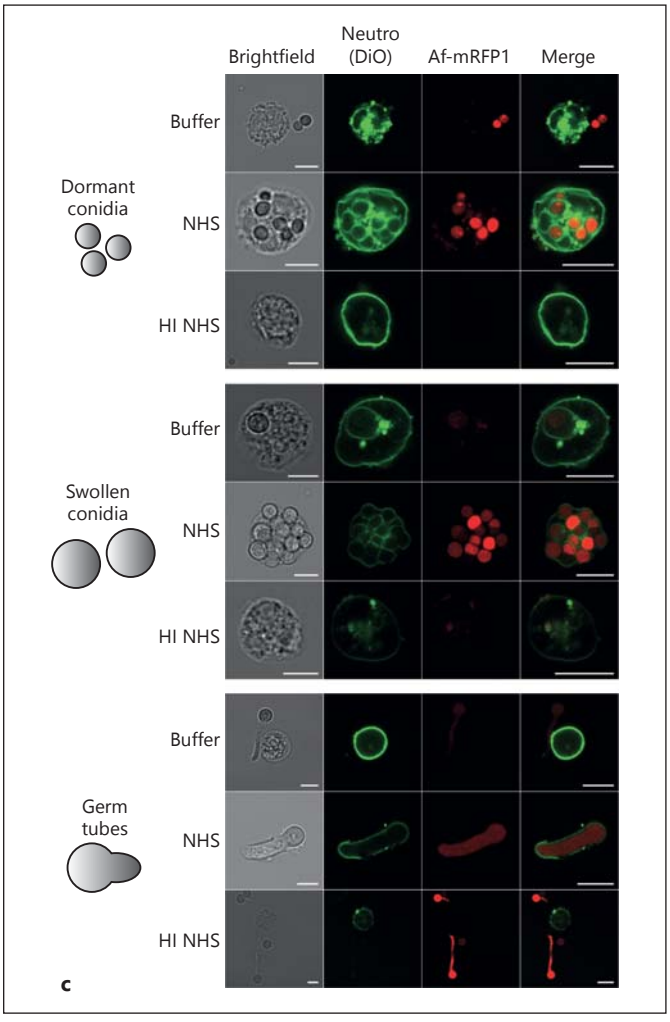

Fig. 2. Complement induces phagocytosis of *A. fumigatus*. **a–c** Different morphotypes (dormant conidia, swollen conidia and germ tubes) of a red fluorescent strain Af-mRFP1 were preopsonized with buffer, 10% NHS and 10% HI NHS. DiO-labeled neutrophils (neutro) were added in an effector-to-target ratio of 1:5. After an additional incubation of 15 min, the cells were fixed and analyzed by flow cytometry (**a**, **b**) or confocal microscopy (**c**). Phagocytosis is expressed as the percentage of mRFP1-positive (mRFP1-pos.) neutrophils (**a**) or the MFI of mRFP1 within the DiO-positive population (**b**). Both assays show the mean ± SD of 3 independent experiments. The ingestion of the mRFP1 morphotypes was visualized by confocal microscopy (**c**), showing representative images of 3 independent experiments. Scale bar: 10 μm.

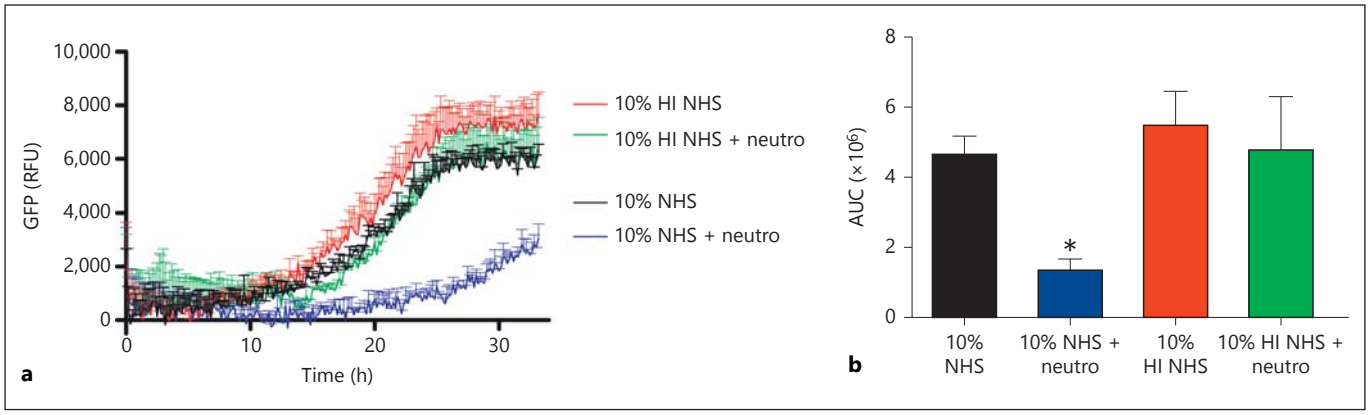

Fig. 3. Complement induces the neutrophil-dependent killing of *A. fumigatus*. **a**, **b** GFP-expressing dormant conidia were incubated with 10% NHS or 10% HI NHS with or without the addition of neutrophils (neutro) in an effector-to-target ratio of 3:1. GFP fluorescence was measured every 10 min for 33 h and plotted as relative fluorescent units (RFU). The assay was performed 3 times in duplicate and a representative plot with mean ± SEM is shown (**a**). The AUC was calculated over the total incubation time and the mean ± SD of 3 independent experiments is shown (**b**). * $p < 0.05$.

mant conidia. The swollen conidia and germ tubes were also examined for C3b binding and phagocytosis. C3b deposition was not significantly reduced on the swollen conidia or germ tubes incubated with FD-deficient serum (fig. 4b, c). In contrast, incubation with C2-deficient serum caused an enormous reduction of the amount of C3b deposition. This suggests that either the classical or the lectin pathway could be important in the complement pathway initiation on swollen conidia and germ tubes. Indeed, C1q-deficient serum showed a similar reduction in C3b deposition, while incubation with the MBL-deficient serum did not affect it. Similar observations were made when phagocytosis of the swollen conidia and germ tubes was studied. C2- and C1q-deficient serum almost completely abolished phagocytosis whereas FD- and MBL-deficient serum did not impede phagocytosis (fig. 4e, f).

Generation of Chemoattractant C5a

Complement activation on the surface results in the recognition, opsonization and phagocytosis of the fungal particle. Another function of complement activation is the release of chemotactic molecules, like C5a, to enhance the migration of leukocytes to the site of infection. Since neutrophil recruitment is essential in the host defense against *A. fumigatus* [3, 23], we studied the role of the different complement pathways in the generation of C5a. Dormant conidia, swollen conidia and germ tubes were incubated in complement-deficient serum with or without the reconstitution of the complement protein. The supernatant was examined for the presence and amount of C5a. The incubation of all morphotypes with the FD-deficient serum resulted in a diminished generation of C5a molecules compared to its reconstituted serum (fig. 5). This effect is more pronounced in the supernatant of the incubated dormant conidia than in the swollen conidia and germ tubes. In addition, C2- and C1q-deficient serum showed a decrease in the generation of C5a. In contrast, the incubation of the different morphotypes with MBL-deficient serum did not alter the generation of fluid-phase C5a.

Discussion

For efficient phagocytosis by neutrophils, opsonization of the foreign particle is crucial. Antibodies bind antigenic structures at the surface of invading pathogens. These antibody-antigen complexes are recognized by the Fcγ receptors (FcγR) on the neutrophil surface. Activation of the complement system by any of the 3 initiation pathways leads to C3b deposition. C3b and its inactivated form iC3b are the most active opsonins and are recognized by neutrophils via CR1 (CD35) and CR3 (CD11b/CD18), respectively. We observed that the incubation of conidia with human serum leads to instant complement activation and a rapid deposition of C3b on the conidial surface. This effect was concentration-dependent since the lowering of the serum concentration correlated with a decrease in C3b deposition and less efficient phagocytosis (data not shown). The dormant conidia showed a relatively low level of complement activation, also shown

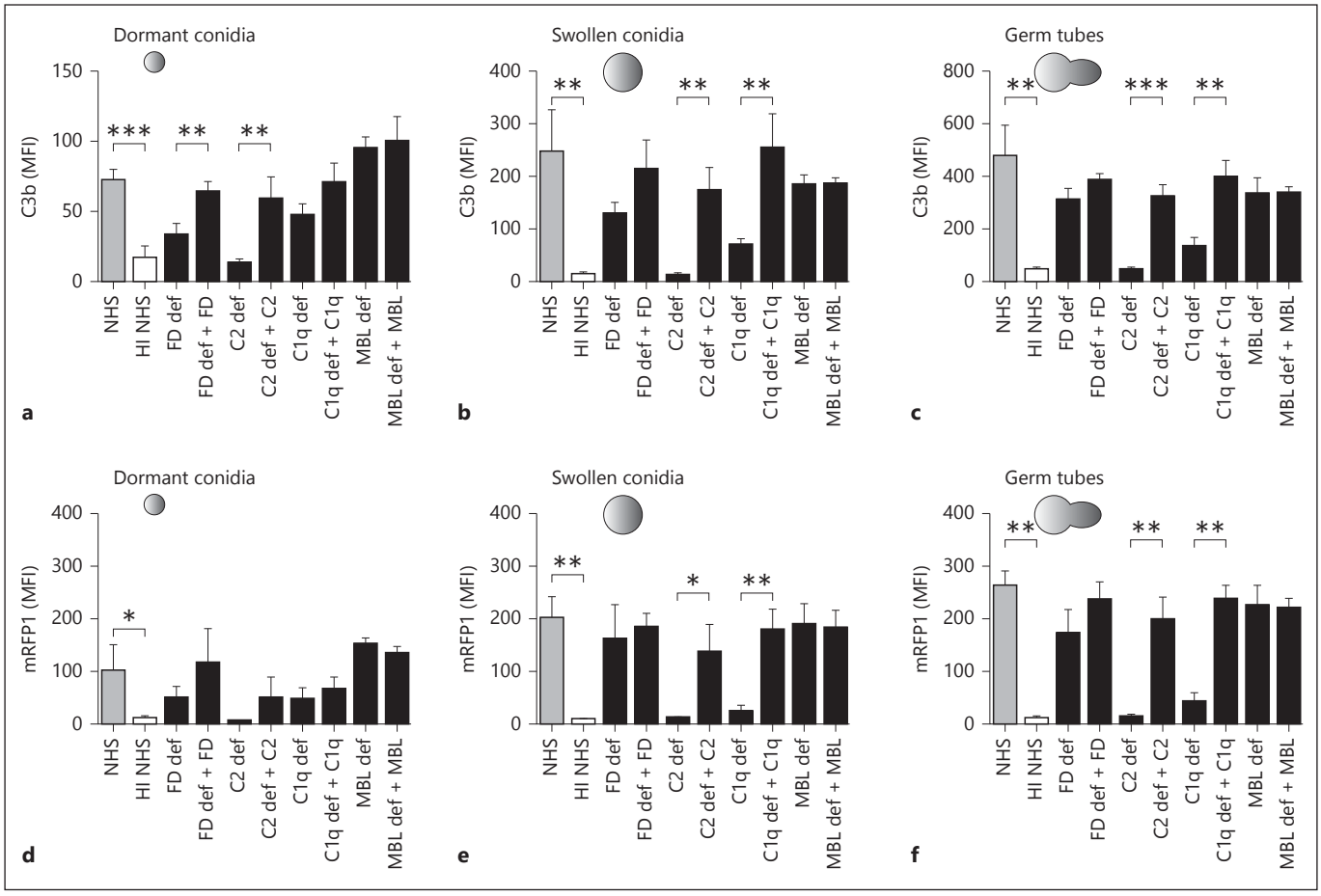

Fig. 4. Opsonization and phagocytosis of *Aspergillus* morphotypes in complement-deficient (def) sera. **a–c** Different morphotypes, i.e. dormant conidia (**a**), swollen conidia (**b**) and germ tubes (**c**) were incubated with NHS, HI NHS or complement-deficient serum with or without purified complement protein. C3b deposition was detected by flow cytometry. **d–f** Different morphotypes of a red fluorescent strain Af-mRFP1, i.e. dormant conidia (**d**), swollen conidia (**e**) and germ tubes (**f**) were preopsonized with NHS, HI NHS or complement-deficient serum with or without reconstitution of complement protein. DiO-labeled neutrophils were added in an effector-to-target ratio of 1:5. After an additional incubation of 15 min, cells were fixed and analyzed by flow cytometry. Phagocytosis is expressed as the MFI of mRFP1 within the DiO-positive population. Both assays show the mean ± SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

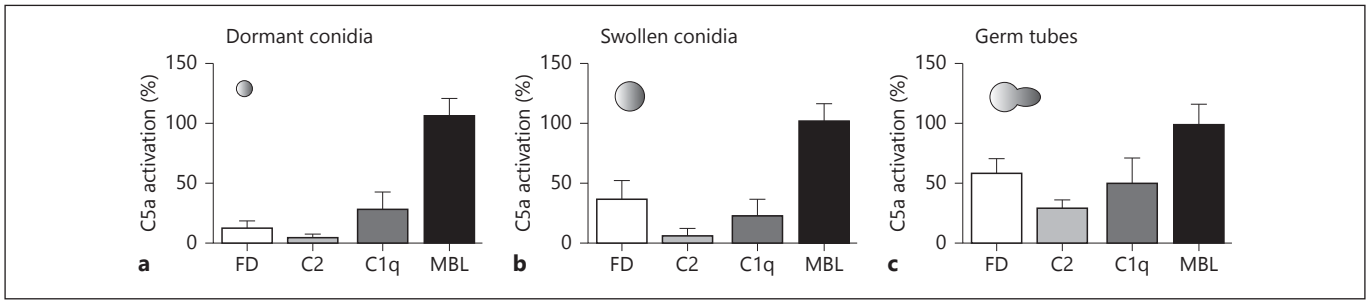

Fig. 5. Generation of chemoattractant C5a. **a–c** Different morphotypes, i.e. dormant conidia (**a**), swollen conidia (**b**) and germ tubes (**c**) were incubated with complement-deficient serum with or without reconstitution of complement protein. Supernatant was examined on the presence of C5a as explained in the Material and Methods. C5a activation is expressed as the amount of C5a generation in deficient serum compared to C5a generation in deficient serum reconstituted with purified complement protein. The assay was performed 3 times and the mean ± SD is shown.

in other studies [24, 25]. The same studies demonstrated that the alternative complement pathway, via the low spontaneous hydrolysis of C3 or the MBL-dependent C2 bypass mechanism, is the main initiator of complement activation on the dormant conidia. However, serum concentrations of at least 20% or serum without anti-*Aspergillus* antibodies were required to observe these effects. Dormant conidia are inhaled and deposited deep inside the respiratory tract. Dormant conidia are metabolically inactive and therefore one can argue whether the complement activation on dormant conidia is physiologically relevant. Activation to swollen conidia and subsequently hyphae is essential for invasion and progression to invasive disease. The total amount of C3b deposited on the dormant conidia is small compared to that on the swollen conidia and germ tubes, but, strikingly, this does not lead to decreased phagocytosis by neutrophils. This could mean that dormant conidia activate neutrophils in addition to and independently of the complement receptors. Another possibility could be that swollen conidia and germ tubes exhibit factors that can influence and inhibit the phagocytosis process. Interestingly, we found that the C3b deposition patterns of the different morphotypes also differed. Where the swollen conidia and germ tubes are completely covered in C3b, the activation of complement on the dormant conidia appears to be located in certain high-density C3b spots on the conidial surface. This could mean that the density of C3b deposition is more important than the total C3b deposition for efficient phagocytosis. A spotted surface localization on the dormant conidia was previously reported when the binding of complement inhibitor factor H was studied [26].

Previous reports, studying only the effect of HI serum on the dormant conidia, observed a decrease in phagocytosis in the presence of the HI serum [27–29]. We show that heat inactivation of serum decreases the phagocytosis of all the morphotypes to background levels. As heat inactivation does not interfere with antibody opsonization, FcγR-mediated phagocytosis of *Aspergillus* morphotypes by neutrophils does not play any role. In addition, opsonization with purified IgG only does not result in efficient phagocytosis (data not shown) [27]. Mice neutrophils lacking FcγR do not show attenuation of neutrophil oxidase activity upon hyphal contact compared to wild-type neutrophils [11]. In contrast, other fungal and bacterial pathogens like *Candida albicans*, *Staphylococcus aureus* and *Streptococcus pneumoniae* do show residual neutrophil-dependent phagocytosis and killing when incubated with HI serum, which is completely inhibited by FcγR-blocking antibodies or staphylococcal FcγR inhibitors [30–32]. Of interest, zymosan, a yeast cell wall fraction composed mainly of polysaccharides, acts in a manner that is similar to *A. fumigatus* and does not induce FcγR-mediated phagocytosis [32, 33]. These dissimilar observations could be explained by the differences in surface pathogen-associated molecular patterns between the pathogens, resulting in pathogen-specific neutrophil activation.

Neutrophils can also recognize *A. fumigatus* independent of opsonization by pattern recognition receptors like dectin-1, Toll-like receptor (TLR) 2, TLR4 and CR3. TLR4 is responsible for neutrophil phagocytosis and the subsequent killing of conidia, while the CD18 subunit of CR3 is responsible for the production of ROS upon contact with hyphae, and it induces hyphal killing by neutrophils in the absence of active complement [8, 11, 34, 35]. TLR2 could play a minor role in the killing of conidia, although the in vivo data does not support any significant role for TLR2 in anti-*Aspergillus* immunity [34, 35]. Dectin-1 detects β-glucans in the fungal cell wall and is important for ROS production and killing by neutrophils of unopsonized swollen conidia [36], but play no role/a minor role in neutrophil oxidase activity and killing after engagement with unopsonized hyphae [8, 11]. Although fungal immune recognition by neutrophils is dependent on multiple receptors, we did not observe residual phagocytosis of the different *Aspergillus* morphotypes in the absence of serum in our experimental setup.

We show that complement is mainly activated via the classical pathway on the swollen conidia and germ tubes. In classical pathway activation, the serine proteases C1s and C1r bind C1q in a calcium-dependent manner to form the C1 complex that recognizes antibody-antigen complexes [13, 14]. Subsequently, C4 and C2 are processed by C1s, leading to the formation of the C2-dependent C3 convertase. Earlier study showed that chelating calcium with MgEGTA, thereby inhibiting classical pathway activation, does not lead to a decreased C3b deposition on swollen conidia and hyphae. However, kinetic experiments with purified alternative pathway proteins showed a slower C3b deposition than with NHS, suggesting a role for complement factors other than the proteins in the alternative pathway for initial complement activation [24]. The discrepancy between these observations and ours can be explained by differences in the serum concentrations used. The alternative pathway plays a more pivotal role in initial complement activation in high concentrations of serum (40%), used in the study of Kozel et al. [24], compared to the ≤10% used in our study [20]. Since complement factors are present in lower concentrations in the lung tissue than in blood, it is physiologically

more relevant to study complement deposition and phagocytosis in low concentrations of serum [37]. The importance of C1q and the classical complement pathway is stressed in a nonimmunocompromised mouse infection model. C1q-deficient mice are more susceptible to *A. fumigatus* infection, while MBL-deficient mice are affected the same as wild-type mice [38, 39].

Complement activation also results in the generation of the chemoattractants C3a and C5a. These peptides are essential for neutrophil recruitment and are important for neutrophil priming. The consequence of lacking C5 was underscored in studies comparing the susceptibility of different mouse strains to infection with *A. fumigatus*. Mice known to carry a loss-of-function mutation in C5 are more susceptible to *Aspergillus* infection [40]. We showed that the generation of C5a is dependent on the initiation of the classical pathway and the activity of the amplification loop of the alternative pathway. High concentrations of C3b are necessary for potent C5 convertase activity [41]. Since the alternative complement pathway acts as an amplification loop to enhance the deposition and increase the concentration of C3b molecules on the surface, this pathway is probably responsible for the assembling of C5 convertase on all *Aspergillus* morphotypes. The classical pathway is necessary for the initial activation of the complement system, as was shown by the lack of C3b deposition in C1q-deficient serum.

In this study, assays were performed with pooled sera from different donors to investigate the general phenotype. It would be of interest to study the consequences of the presence or absence of specific anti-*Aspergillus* antibodies recognizing surface epitopes in healthy individuals or patients at risk for *A. fumigatus* infection. A recent study observed no association between disease and the presence of antibodies recognizing polysaccharide galactosaminogalactan [42]. Although surface-bound antibodies do not cause FcγR-mediated phagocytosis, they are essential for classical pathway complement activation, and therefore antibodies play an important role in host defense against *A. fumigatus*, linking innate and adaptive antifungal immunity. More knowledge about epitopes on the fungal surface can lead to the development of new antifungal prevention and treatment strategies to conquer *Aspergillus* infections.

In view of these results, we conclude that opsonization, especially via classical pathway complement activation, is crucial in the innate host defense against *A. fumigatus*.

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References

1 Stevens DA, Melikian GL: Aspergillosis in the 'nonimmunocompromised' host. Immunol Invest 2011;40:751–766.
2 O'Gorman CM, Fuller HT: Prevalence of culturable airborne spores of selected allergenic and pathogenic fungi in outdoor air. Atmospher Environ 2008;42:4355–4368.
3 Mircescu MM, Lipuma L, van Rooijen N, Pamer EG, Hohl TM: Essential role for neutrophils but not alveolar macrophages at early time points following *Aspergillus fumigatus* infection. J Infect Dis 2009;200:647–656.
4 Winkelstein JA, Marino MC, Johnston RB, Boyle J, Curnutte J, Gallin JI, et al: Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore) 2000;79:155–169.
5 Young RC, Bennett JE, Vogel CL, Carbone PP, DeVita VT: Aspergillosis. The spectrum of the disease in 98 patients. Medicine (Baltimore) 1970;49:147–173.
6 Gerson SL, Talbot GH, Hurwitz S, Strom BL, Lusk EJ, Cassileth PA: Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. Ann Intern Med 1984;100:345–351.
7 Diamond RD, Clark RA: Damage to *Aspergillus fumigatus* and *Rhizopus oryzae* hyphae by oxidative and nonoxidative microbicidal products of human neutrophils in vitro. Infect Immun 1982;38:487–495.
8 Leal SM, Vareechon C, Cowden S, Cobb BA, Latgé J-P, Momany M, et al: Fungal antioxidant pathways promote survival against neutrophils during infection. J Clin Invest 2012;122:2482–2498.
9 Zarember KA, Sugui JA, Chang YC, Kwon-Chung KJ, Gallin JI: Human polymorphonuclear leukocytes inhibit *Aspergillus fumigatus* conidial growth by lactoferrin-mediated iron depletion. J Immunol 2007;178:6367–6373.
10 Rex JH, Bennett JE, Gallin JI, Malech HL, Melnick DA: Normal and deficient neutrophils can cooperate to damage *Aspergillus fumigatus* hyphae. J Infect Dis 1990;162:523–528.
11 Boyle KB, Gyori D, Sindrilaru A, Scharffetter-Kochanek K, Taylor PR, Mócsai A, et al: Class IA phosphoinositide 3-kinase β and δ regulate neutrophil oxidase activation in response to *Aspergillus fumigatus* hyphae. J Immunol 2011;186:2978–2989.
12 Steele C, Rapaka RR, Metz A, Pop SM, Williams DL, Gordon S, et al: The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. PLoS Pathog 2005;1:e42.
13 Gaboriaud C, Juanhuix J, Gruez A, Lacroix M, Darnault C, Pignol D, et al: The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. J Biol Chem 2003;278:46974–46982.
14 Girija UV, Gingras AR, Marshall JE, Panchal R, Sheikh MA, Gál P, et al: Structural basis of the C1q/C1s interaction and its central role in assembly of the C1 complex of complement activation. Proc Natl Acad Sci U S A 2013;110:13916–13920.
15 Berends ETM, Kuipers A, Ravesloot MM, Urbanus RT, Rooijakkers SHM: Bacteria under stress by complement and coagulation. FEMS Microbiol Rev 2014;38:1146–1171.

16 Rossi V, Cseh S, Bally I, Thielens NM, Jensenius JC, Arlaud GJ: Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and -2. J Biol Chem 2001;276:40880–40887.
17 Wasylnka JA, Moore MM: Uptake of *Aspergillus fumigatus* conidia by phagocytic and nonphagocytic cells in vitro: quantitation using strains expressing green fluorescent protein. Infect Immun 2002;70:3156–3163.
18 Petrikkou E, Rodriguez-Tudela JL, Cuenca-Estrella M, Gómez A, Molleja A, Mellado E: Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. J Clin Microbiol 2001;39:1345–1347.
19 Rooijakkers SHM, Ruyken M, Roos A, Daha MR, Presanis JS, Sim RB, et al: Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol 2005;6:920–927.
20 Roos A, Bouwman LH, Munoz J, Zuiverloon T, Faber-Krol MC, Fallaux-van den Houten FC, et al: Functional characterization of the lectin pathway of complement in human serum. Mol Immunol 2003;39:655–668.
21 Bestebroer J, Poppelier MJJG, Ulfman LH, Lenting PJ, Denis CV, van Kessel KPM, et al: Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood 2007;109:2936–2943.
22 Kew RR, Peng T, DiMartino SJ, Madhavan D, Weinman SJ, Cheng D, et al: Undifferentiated U937 cells transfected with chemoattractant receptors: a model system to investigate chemotactic mechanisms and receptor structure/function relationships. J Leukoc Biol 1997;61:329–337.
23 Waldorf AR, Diamond RD: Neutrophil chemotactic responses induced by fresh and swollen *Rhizopus oryzae* spores and *Aspergillus fumigatus* conidia. Infect Immun 1985;48:458–463.
24 Kozel TR, Wilson MA, Farrell TP, Levitz SM: Activation of C3 and binding to *Aspergillus fumigatus* conidia and hyphae. Infect Immun 1989;57:3412–3417.
25 Dumestre-Pérard C, Lamy B, Aldebert D, Lemaire-Vieille C, Grillot R, Brion J-P, et al: *Aspergillus* conidia activate the complement by the mannan-binding lectin C2 bypass mechanism. J Immunol 2008;181:7100–7105.
26 Behnsen J, Hartmann A, Schmaler J, Gehrke A, Brakhage AA, Zipfel PF: The opportunistic human pathogenic fungus *Aspergillus fumigatus* evades the host complement system. Infect Immun 2008;76:820–827.
27 Sturtevant J, Latgé J-P: Participation of complement in the phagocytosis of the conidia of *Aspergillus fumigatus* by human polymorphonuclear cells. J Infect Dis 1992;166:580–586.
28 Levitz SM, Farrell TP: Human neutrophil degranulation stimulated by *Aspergillus fumigatus*. J Leukoc Biol 1990;47:170–175.
29 Lehrer RI, Jan RG: Interaction of *Aspergillus fumigatus* spores with human leukocytes and serum. Infect Immun 1970;1:345–350.
30 Kozel TR, MacGill RS, Percival A, Zhou Q: Biological activities of naturally occurring antibodies reactive with *Candida albicans* mannan. Infect Immun 2004;72:209–218.
31 Stemerding AM, Köhl J, Pandey MK, Kuipers A, Leusen JH, Boross P, et al: *Staphylococcus aureus* formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcγR antagonists that inhibit IgG-mediated effector functions. J Immunol 2013;191:353–362.
32 Brouwer N, Dolman KM, van Houdt M, Sta M, Roos D, Kuijpers TW: Mannose-binding lectin (MBL) facilitates opsonophagocytosis of yeasts but not of bacteria despite MBL binding. J Immunol 2008;180:4124–4132.
33 Kemp AS, Turner MW: The role of opsonins in vacuolar sealing and the ingestion of zymosan by human neutrophils. Immunology 1986;59:69–74.
34 Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, et al: The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J Immunol 2004;172:3059–3069.
35 Leal SM, Cowden S, Hsia Y-C, Ghannoum MA, Momany M, Pearlman E: Distinct roles for dectin-1 and TLR4 in the pathogenesis of *Aspergillus fumigatus* keratitis. PLoS Pathog 2010;6:e1000976.
36 Werner JL, Metz AE, Horn D, Schoeb TR, Hewitt MM, Schwiebert LM, et al: Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against *Aspergillus fumigatus*. J Immunol 2009;182:4938–4946.
37 Watford WT, Ghio AJ, Wright JR: Complement-mediated host defense in the lung. Am J Physiol Lung Cell Mol Physiol 2000;279:L790–L798.
38 Clemons KV, Martinez M, Tong A-J, Stevens DA: Resistance of MBL gene-knockout mice to experimental systemic aspergillosis. Immunol Lett 2010;128:105–107.
39 Garlanda C, Hirsch E, Bozza S, Salustri A, De Acetis M, Nota R, et al: Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. Nature 2002;420:182–186.
40 Hector RF, Yee E, Collins MS: Use of DBA/2N mice in models of systemic candidiasis and pulmonary and systemic aspergillosis. Infect Immun 1990;58:1476–1478.
41 Rawal N, Pangburn M: Formation of high-affinity C5 convertases of the alternative pathway of complement. J Immunol 2001;166:2635–2642.
42 Fontaine T, Delangle A, Simenel C, Coddeville B, van Vliet SJ, van Kooyk Y, et al: Galactosaminogalactan, a new immunosuppressive polysaccharide of *Aspergillus fumigatus*. PLoS Pathog 2011;7:e1002372.