The role of opsonins in *Aspergillus fumigatus* host defense

Steven Braem

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Scanning electron micrograph of the head of a conidiophore (a stalk producing conidia) of *Aspergillus fumigatus* by Iris Brinkman and Steven Braem

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The role of opsonins in *Aspergillus fumigatus* host defense

De rol van opsoninen in de afweer tegen *Aspergillus fumigatus*
(met een samenvatting in het Nederlands)

Proefschrift

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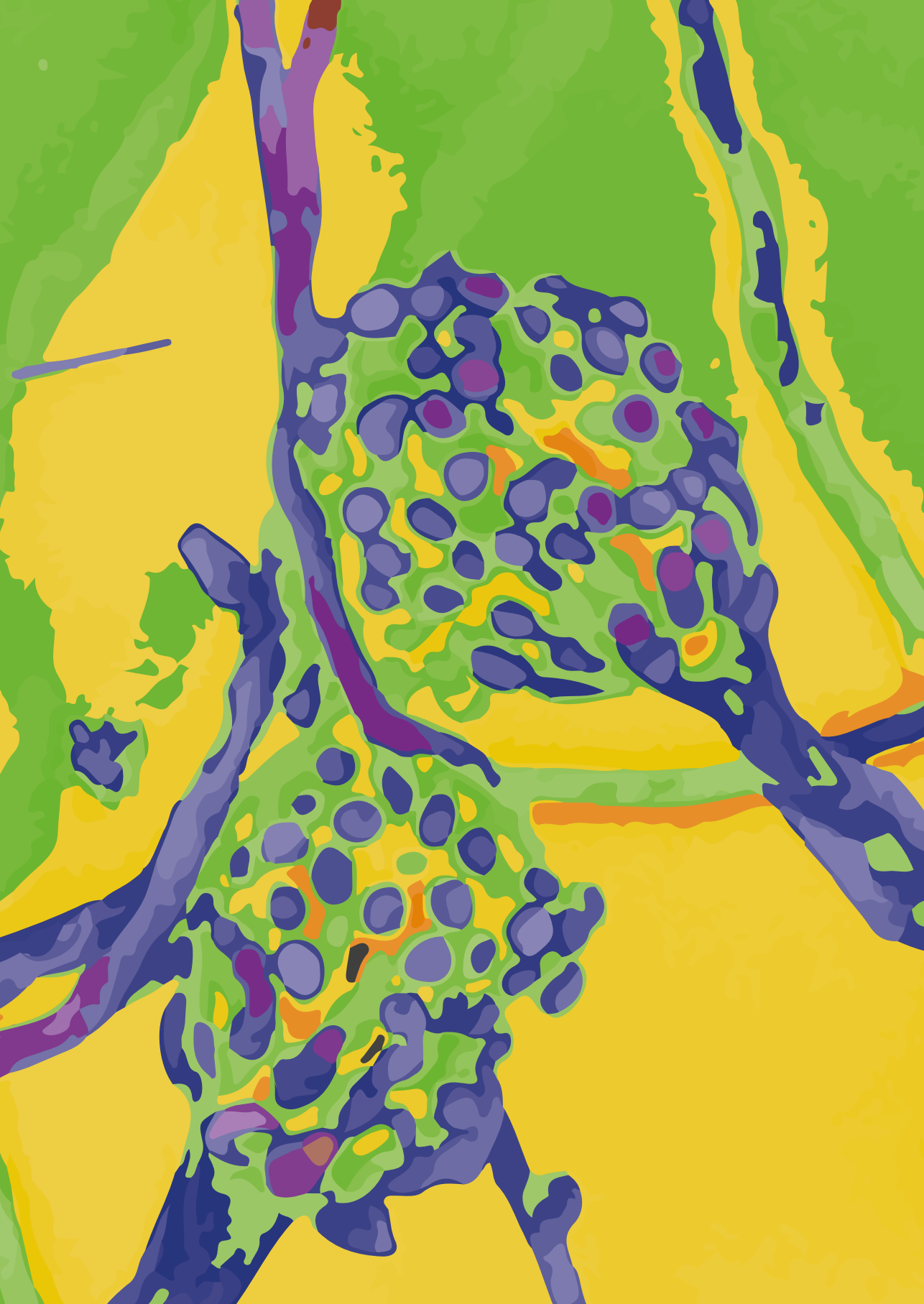

General introduction

Steven GE Braem

INTRODUCTION

Aspergillus fumigatus

Aspergillus species are filamentous fungi, which mainly grow on dead organic material. The genus *Aspergillus* includes over 200 different species. Several *Aspergillus* species are commonly used in the food and pharmaceutical industry, e.g. *A. niger* and *A. oryzae*. A small number of *Aspergillus* species are associated with human and animal diseases. More than 90% of these diseases are caused by *A. fumigatus*, while the remaining 10% is caused by *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans,* and occasionally other *Aspergillus* species[1]. Infection with *A. fumigatus* can lead to a variety of diseases, including allergic bronchopulmonary aspergillosis, non-invasive aspergilloma and invasive aspergillosis. The severity of the infection mainly depends on the immune status of the host. Invasive aspergillosis is the most severe and life-threatening condition, and is associated with an immunosuppressed state of the patient. The incidence of invasive aspergillosis is 7% to 14% in patients with hematological malignancies and 1% to 15% in solid organ transplant recipients[2,3]. The survival ratio of patients with invasive aspergillosis is related to the underlying disease. Overall, 36% of the patients diagnosed with invasive aspergillosis do not survive the first three months post therapy[4]. Therefore, *A. fumigatus* is one of the most important lethal fungal pathogens infecting humans.

A. fumigatus morphotypes

Although a sexual reproduction cycle has been described, *A. fumigatus* mainly reproduces by the production of asexual conidia[5]. These dormant conidia are produced in the phialides of conidiophores and dispersed into the atmosphere. *A. fumigatus* produces small (2–3 µm) and highly hydrophobic conidia allowing for easy air dispersal. Concentrations of conidia in the air are dependent on the season and are generally lower than 10 CFU/m^3, but can reach up to 400 CFU/m^3 [6]. Dormant conidia are metabolic quiescent. Under favorable conditions, conidia become active and start to swell (figure 1). During this process, the cell wall remodels and looses the outermost hydrophobic layer and melanin layer. Subsequently, the swollen conidia start to germinate and produce germ tubes. Elongation of germ tubes results in the generation of a hyphal network, which again can produce new conidiophores for asexual reproduction.

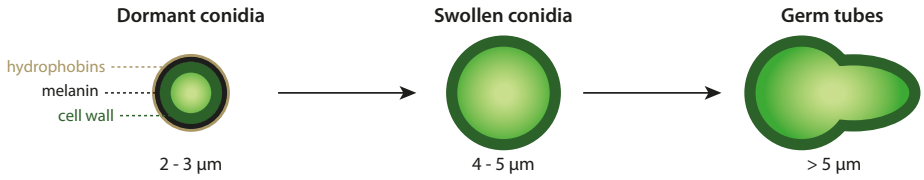

Figure 1 • *A. fumigatus* morphotypes
The human body encounters different morphotypes of *A. fumigatus*. Dormant conidia are 2 - 3 µm in size and covered by a hydrophobin layer and a melanin layer. Under favorable conditions, conidia start to swell and loose their melanin and hydrophobin layer. Swollen conidia are metabolically active and germinate followed by the elongation of germ tubes to produce hyphae.

A. fumigatus cell wall

The cell wall of *A. fumigatus* is composed of several polysaccharides. β-(1,3)-glucan is the main component and comprises 50% of the cell wall[7]. Another component is α-(1,3)-glucan, which induces the agglutination of conidia[8]. Other polysaccharides are chitin, a polymer of N-acetylglucosamine, and galactomannan, which are covalently linked to the β-(1,3)-glucan layer. The cell wall is a dynamic structure and harbors proteins that are responsible for cell wall remodeling and adhesion. The structure and composition of the cell wall of conidia and hyphae differs. Conidia are covered by an outer cell wall layer, which is composed of a hydrophobin layer and a melanin layer (figure 1)[9,10]. During germination, the outer cell wall layer is lost, and a new polysaccharide galactosaminogalactan is produced, secreted and partly retains to the fungal cell wall[11].

Recognition of *A. fumigatus*

The fungal cell wall is essential for existence and growth. However, the components of the cell wall are also the primary ligands for host immune recognition. Humans express several soluble and membrane-bound pattern recognition receptors that recognize the polysaccharides of the *A. fumigatus* cell wall (figure 2).

The dominant polysaccharide β-(1,3)-glucan is recognized by complement receptor 3 (CR3, CD11b/CD18), dectin-1, and ficolin-2. CR3 is broadly expressed on immune cells and recognizes β-(1,3)-glucan and the complement component iC3b. The recognition of hyphae via CR3 induces the production of reactive oxygen species (ROS) by neutrophils and results in the killing of *A. fumigatus*[12,13]. Dectin-1 is a C-type lectin receptor and primarily expressed on myeloid cells[14]. The recognition of *A. fumigatus* by dectin-1 leads to the production of cytokines by macrophages, which induces the recruitment of neutrophils to the site of infection[15-17]. Ficolin-2 is a soluble pattern recognition receptor present in serum that recognizes β-(1,3)-glucan and activates the lectin complement pathway[18]. Ficolin-2 binds conidia in serum-free conditions and enhances the phagocytosis and killing of *A. fumigatus* by macrophages and neutrophils primarily in a more acidic environment[19,20].

The polysaccharide galactomannan is particular found in *Aspergillus* and *Penicillium* species and not in pathogenic yeasts. Galactomannan is recognized by several pattern recognition receptors with different affinities. Surfactant protein A (SP-A) and surfactant protein D (SP-D) are soluble collectin receptors produced in the lung, which bind to mannose, the backbone of galactomannan[21,22]. Both SP-A and SP-D bind via the lectin domains to *A. fumigatus* conidia and induce aggregation, phagocytosis and killing by macrophages and neutrophils[23]. Mannose binding lectin (MBL) is a structural homolog of SP-A and a soluble serum collectin that recognizes conidia[24,25]. MBL binds mannan, resulting in the induction of the lectin complement pathway[26,27]. In serum-free conditions and MBL-deficient serum, the presence of MBL enhances phagocytosis, ROS production and neutrophil killing of conidia and hyphae[28]. The long pentraxin 3 (PTX3) binds *A. fumigatus* conidia via the recognition of galactomannan[29]. PTX3 is produced and released by phagocytes upon activation by conidia, resulting in more efficient phagocytosis and killing[29-31]. In addition, PTX3 synergizes the ficolin-2 induced complement activation on conidia[19]. Dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin (DC-SIGN) is a C-type lectin

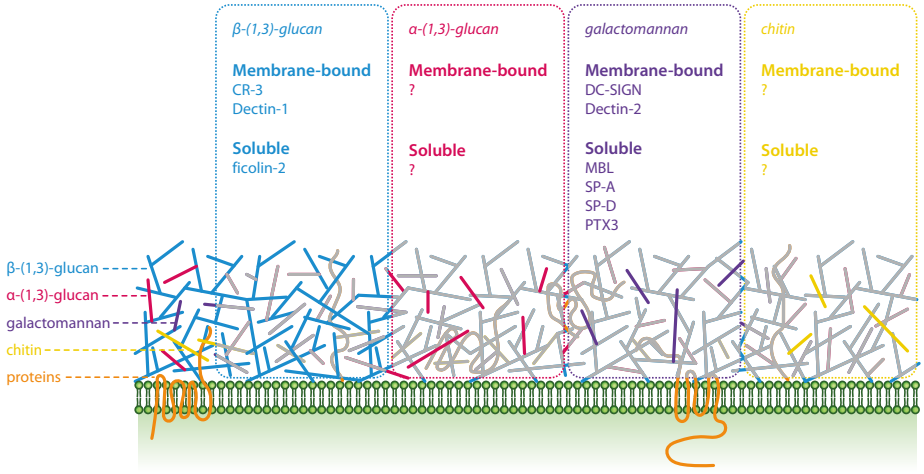

Figure 2 • Recognition of cell wall polysaccharides by pattern recognition receptors
The *A. fumigatus* cell wall is composed of β-(1,3)-glucan, α-(1,3)-glucan, galactomannan and chitin. The cell wall polysaccharides β-(1,3)-glucan and galactomannan are recognized by various soluble and membrane-bound pattern recognition receptors. No pattern recognition receptors have been identified for α-(1,3)-glucan and chitin.

that is primarily expressed on dendritic cells and on interleukin-4 (IL-4)-stimulated macrophages[32,33]. Both dendritic cells and macrophages bind and internalize conidia via the recognition of galactomannan. Conidia induce the maturation of dendritic cells and the production of an anti-inflammatory cytokine IL-10[33]. Dectin-2 is a C-type lectin that is predominantly expressed on myeloid cells[34]. Dectin-2 expressing plasmacytoid dendritic cells recognizes mannan of *A. fumigatus* hyphae and induce killing by the chelation of divalent cations by calprotectin[35].

Although chitin-coated beads are internalized by macrophages, no specific chitin receptor has been identified[36,37]. For α-(1,3)-glucan, no pattern recognition receptors have been identified as well. Interestingly, the pathogenic yeast *Histoplasma capsulatum* produces an outer layer of α-(1,3)-glucans to prevent its β-(1,3)-glucans from recognition by dectin-1[38]. In this way, *H. capsulatum* is able to hide from the innate immune system, suggesting that no α-(1,3)-glucan receptor exists.

A. fumigatus conidia are covered by a hydrophobin layer, which prevents the recognition of the cell wall polysaccharides (figure 1)[39]. The hydrophobin layer is composed of hydrophobin proteins RodA and RodB[10]. RodA does not activate macrophages and dendritic cells[39]. Moreover, RodA reduces the formation of neutrophil extracellular traps, the killing of *A.* fumigatus conidia by macrophages in vitro, and the fungal clearance in vivo[10,40,41]. RodB has been studied, but its function currently remains unclear[10].

Immune response to *A. fumigatus*

Since *A. fumigatus* conidia are present in high concentrations in the air, humans inhale conidia daily[6]. Most of the conidia are immediately cleared by the ciliated mucus membranes in the airway. Some conidia are not effectively cleared and reach the lung

alveoli. To prevent colonization and invasive growth, activation of the innate immune system is necessary. Together with lung pattern recognition receptors and antimicrobial peptides, alveolar leukocytes like macrophages and dendritic cells form the first line of defense against *A. fumigatus* infection. The additional recruitment of neutrophils to the site of infection is essential in the clearance of *A. fumigatus*[42]. Patients with chronic granulomatous disease (CGD), a genetic disease causing neutrophil dysfunction, and neutropenic patients are predisposed to *A. fumigatus* infections[43-45].

Neutrophils recognize *A. fumigatus* directly via CR3 and dectin-1[12,13,16]. Additionally, neutrophils are able to recognize pathogens indirectly via opsonization with antibodies and proteins of the complement system. In general, these humoral factors are important to establish effective neutrophil phagocytosis and intracellular killing of pathogens. The complement cascade is initiated via three distinct pathways (figure 3). The classical pathway is generally initiated after the binding of C1q to antibody-opsonized microbes[46]. Alternatively, C1q can bind microbes directly or via pentraxins to initiate the classical pathway independent of opsonic antibodies[47]. For the initiation of the lectin pathway, MBL, ficolins and collectin-11 bind to carbohydrates on the microbial surface[48,49]. Activation of the classical and the lectin pathway result in the proteolytic cleavage of C4 and subsequently C2, to establish a C3 convertase C4b2a[48,50]. The alternative pathway functions mainly as an amplification loop of the classical and the lectin pathway. Next to the low spontaneous hydrolysis of C3, the alternative pathway can be activated by properdin resulting in a C3 convertase C3bBb[51]. The C3 convertases cleave soluble C3 to massively label the microbe with the potent opsonin C3b. C3b and its inactivated form iC3b are preferentially ligands of CR1 and CR3, respectively. Recognition of C3b by complement receptors on the neutrophil surface induces phagocytosis and killing of the pathogen. Complement activation also leads to the formation of chemoattractants C3a and C5a. C3a and C5a induce neutrophil priming and recruitment to the site of infection. Of interest, C5 deficient mice are more

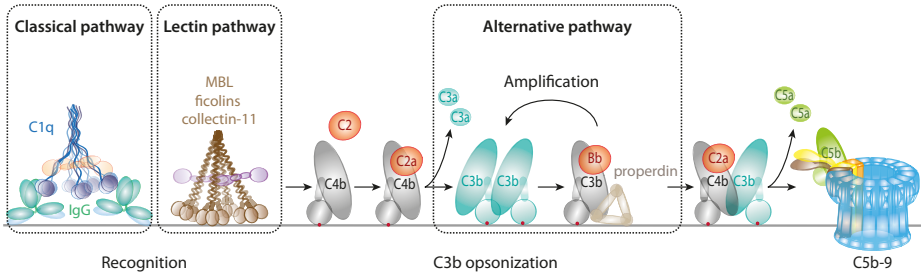

Figure 3 • Activation of the complement system
The complement system can be activated via three pathways. The classical pathway is induced upon binding of C1q to antibody-antigen complexes. The lectin pathway is activated upon the binding of mannose-binding lectin (MBL), ficolins and collectin-11 to sugar moieties on the fungal cell wall. Both pathways result in the cleavage of C4 and subsequently C2 to form a C3 convertase C4b2a. The alternative pathway mainly acts as an amplification loop of the classical and the lectin pathway, but can also be initiated via the direct binding of properdin to the microbial surface. Activation of the alternative pathway results in the formation of a C3 convertase C3bBb. Both C3 convertases cleave C3 to label the surface with the potent opsonin C3b. Activation of the complement system also leads to generation of soluble chemoattractants C3a and C5a, and the formation of C5b-9 on the surface. This figure was adapted from Berends *et al.*[47].

susceptible to *A. fumigatus* infection, indicating an important role for the chemotactic effect of C5a in the clearance of *A. fumigatus* infection[52]. The final stage of the activation of the complement pathway is the formation of the membrane attack complex C5b-9. Although C5b-9 is deposited on conidia and hyphae, *A. fumigatus* is protected for direct lysis by C5b-9 probably due to its dense cell wall.

AIM OF THIS THESIS

A. fumigatus is a common cause of invasive fungal infections in humans. Every year over 200.000 individuals are infected with *A. fumigatus*[53]. The number of patients at risk for the development of invasive aspergillosis is increasing, due to the growing number of immunocompromised patients. This is mainly caused by the use of therapies in patients with hematologic malignancies or a solid organ transplant, which leads to suppression of the immune system. Despite the use of diagnostic tools, it remains difficult to diagnose invasive aspergillosis in an early stage of disease. Moreover, anti-fungal therapies are expensive and inadequate, leading to a suboptimal treatment and a high mortality. Research into the immune response against *A. fumigatus* is essential to better understand the pathogenicity of *A. fumigatus* and its interactions with the host. This knowledge is needed to enable the development of novel treatment strategies.

In this thesis we try to unravel the mechanism of immune recognition of *A. fumigatus*. We investigate opsonins present in the lung and serum and explore their role in immunity against *A. fumigatus*. Since neutrophils are the effector cells in *A. fumigatus* clearance, we examine the influence of opsonins in neutrophil recognition and anti-fungal defense. We try to identify *A. fumigatus* ligands or receptors that bind to known fungal pattern recognition molecules and could initiate immune evasion by *A. fumigatus*. Finally, we investigate the effect of glucocorticoids on *A. fumigatus* growth and gene expression, and correlate changes to possible enhanced pathogenicity. In sum, we aim to better understand the host-pathogen interaction between humans and *A. fumigatus*.

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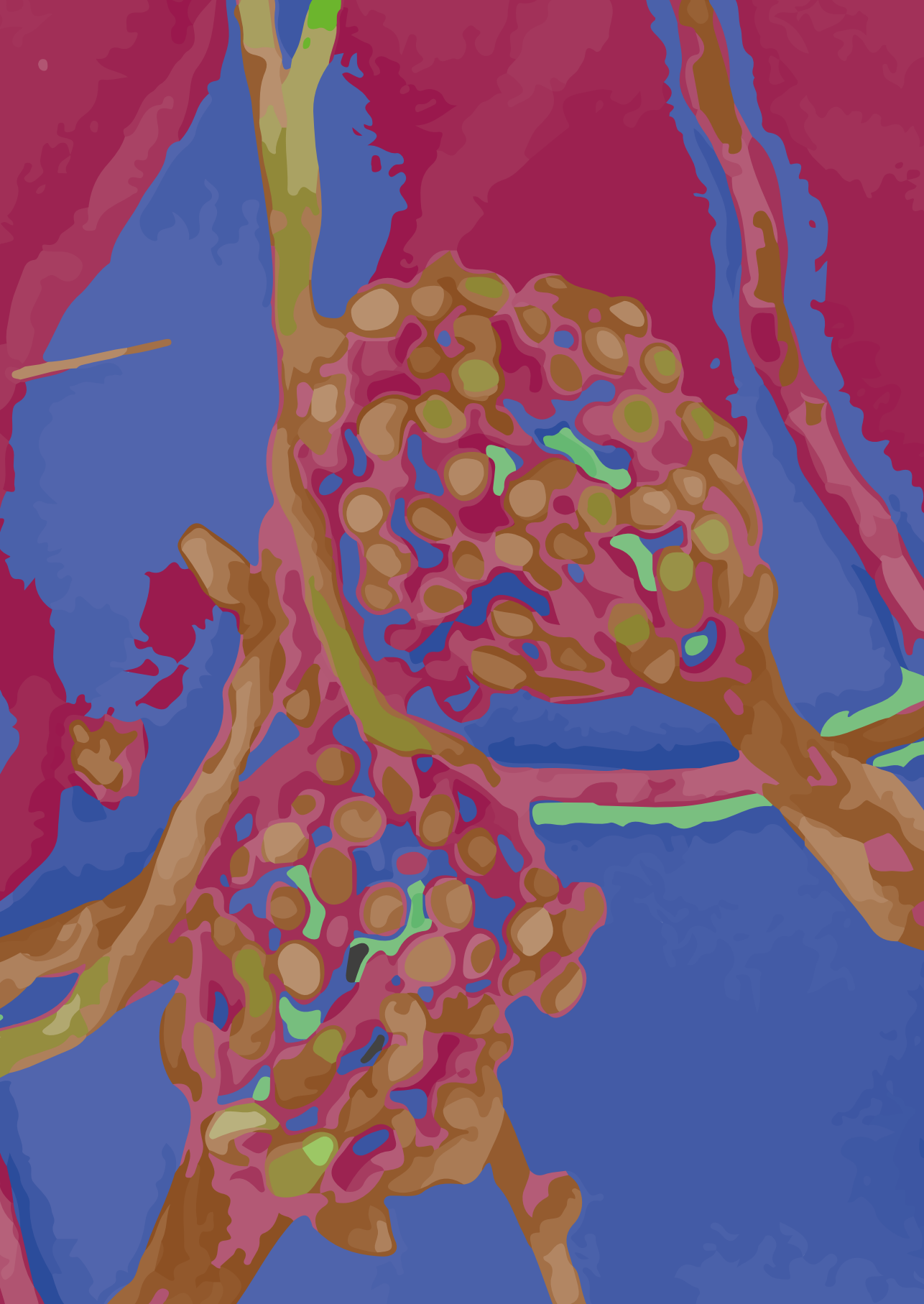

Effective neutrophil phagocytosis of *Aspergillus fumigatus* is mediated by classical pathway complement activation

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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 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 (Supplemental figure 1). 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

result 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 independent 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 2a). Similar observations were made when the MFI of mRFP1 was plotted (Figure 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 (Figure 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 (Figure 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

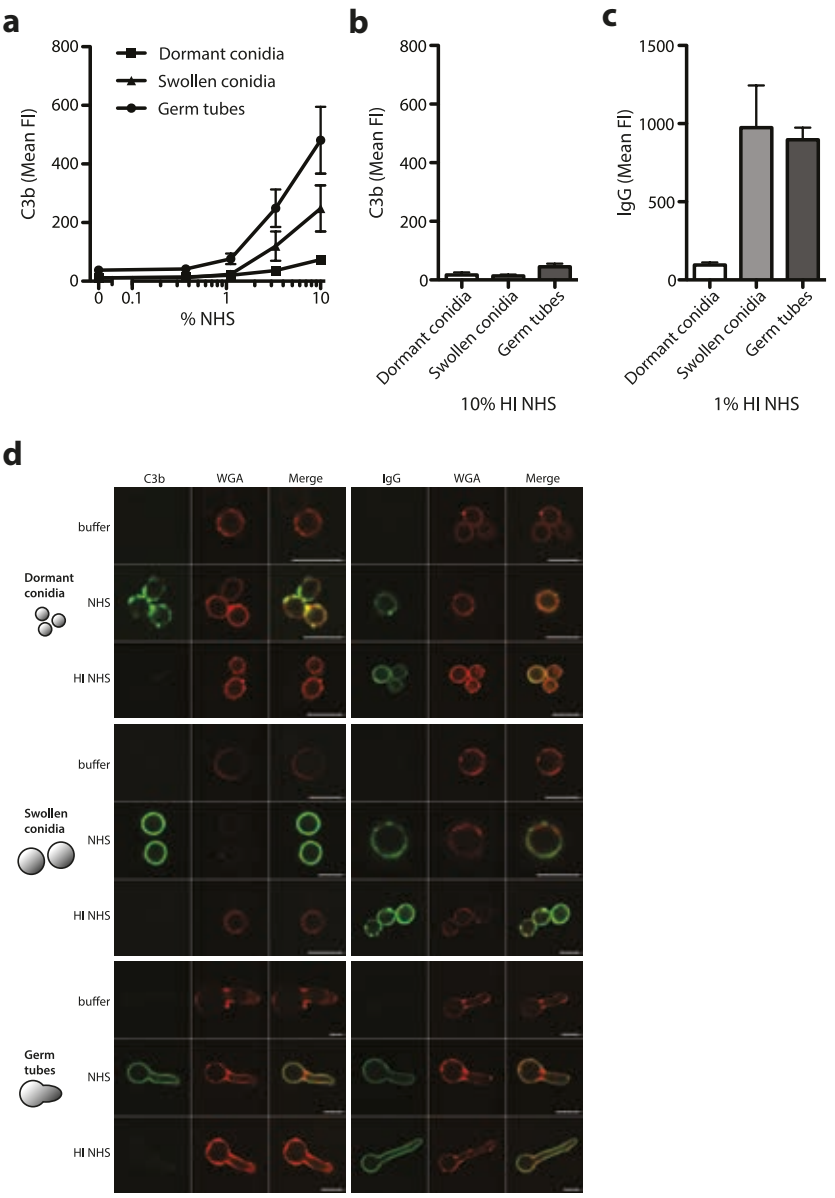

Figure 1 • C3b and IgG deposition on different Aspergillus morphotypes
(**a**) Different morphotypes (dormant conidia, swollen conidia and germ tubes) were incubated with NHS, and C3b deposition was detected by flow cytometry. (**b**) The different morphotypes were incubated with 10% HI NHS and C3b deposition 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.

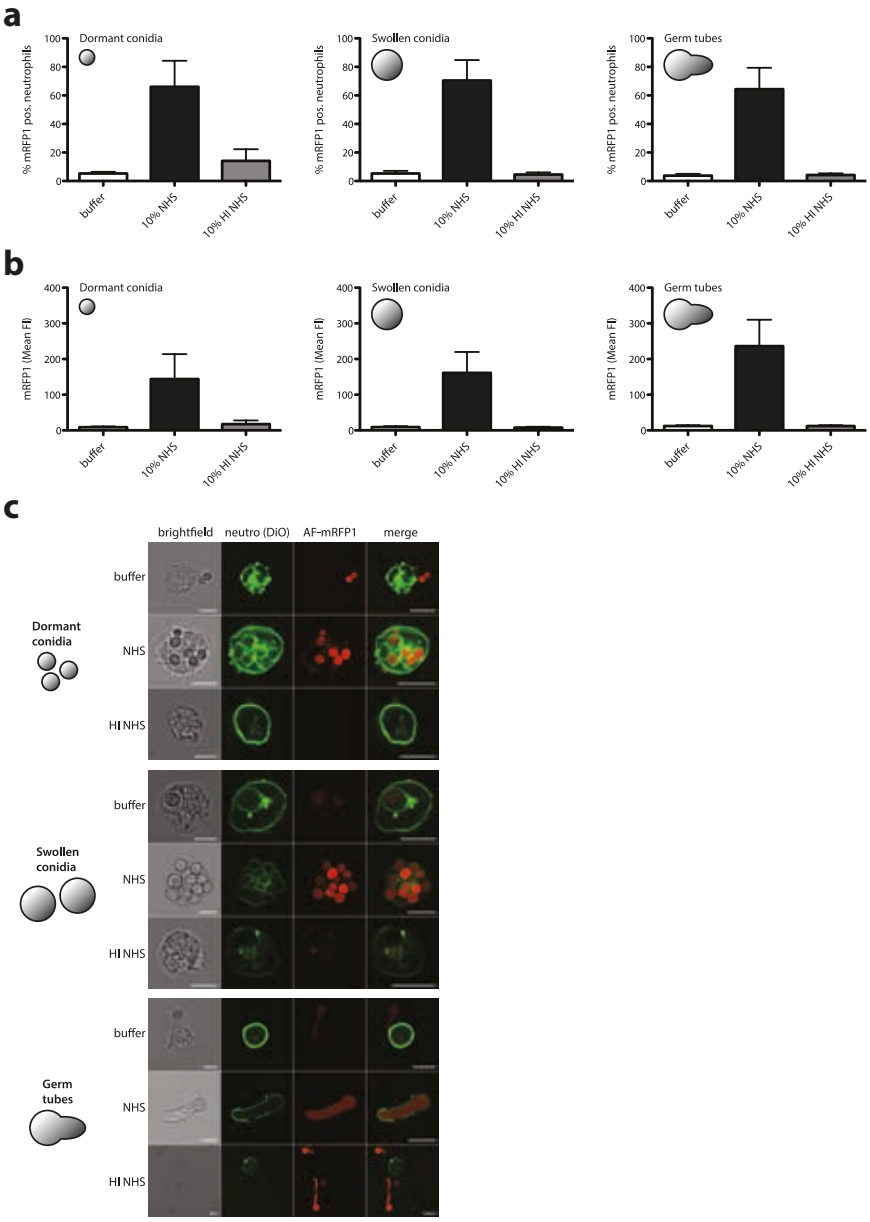

Figure 2 • Complement induces phagocytosis of *A. fumigatus*
(**a-c**) Different morphotypes (dormant conidia, swollen conidia and germ tubes) of a red fluorescent strain Af-mRFP1 were preopsonized with buffer, 10% NHS and 10% HI NHS. DiO-labeled neutrophils (neutro) were added in an effector-to-target 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.

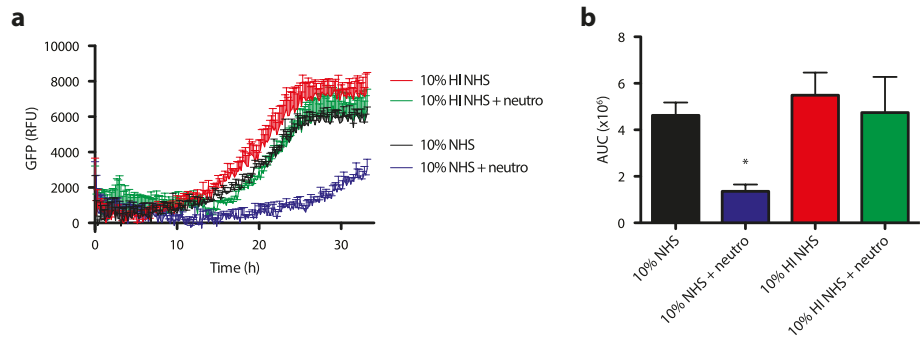

Figure 3 • Complement induces the neutrophil-dependent killing of *A. fumigatus*
(**a, b**) GFP-expressing dormant conidia were incubated with 10% NHS or 10% HI NHS with or without the addition of neutrophils (neutro) in an effector-to-target ratio of 3:1. GFP fluorescence was measured every 10 min for 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**). * $p < 0.05$.

the calculation of the AUC during the complete incubation time, showing a significant delay in growth rebound (Figure 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 (Figure 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 (Supplemental figure 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 (Figure 4d), suggesting that both the alternative and the classical complement pathways are important for the opsonization and phagocytosis of the dormant conidia. The swollen conidia and germ tubes wre also examined for C3b binding and phagocytosis. C3b deposition was not significantly reduced on the swollen

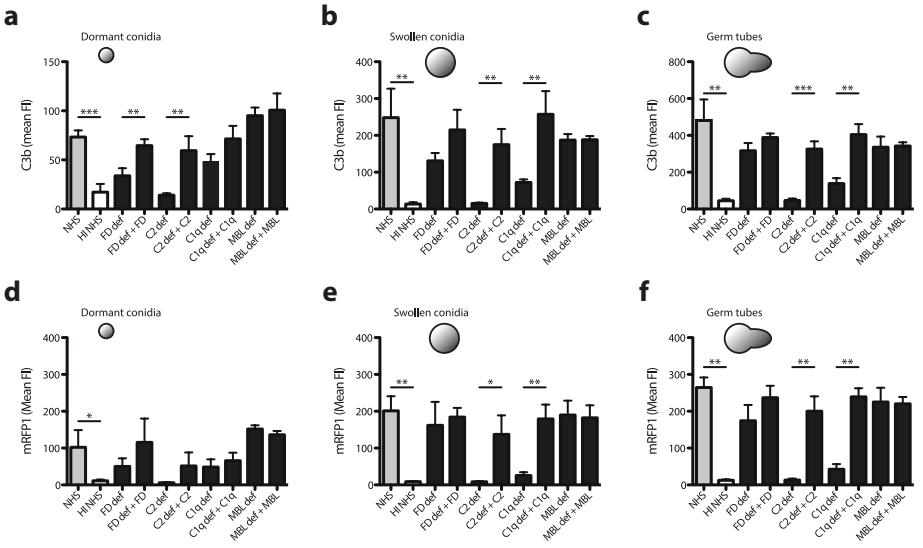

Figure 4 • Opsonization and phagocytosis of *Aspergillus* morphotypes in complement-deficient sera

(**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 (def) 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. * $p < 0.05$, * $p < 0.01$, *** $p < 0.001$.

conidia or germ tubes incubated with FD-deficient serum (Figure 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 (Figure 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

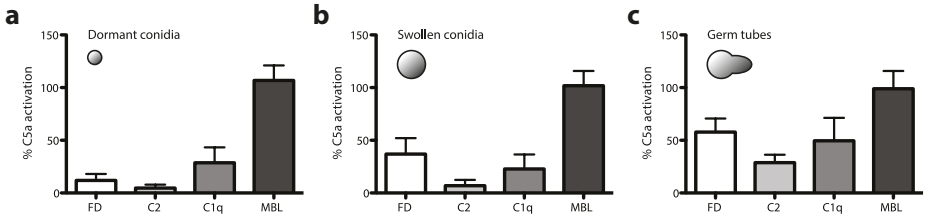

Figure 5 • Generation of chemoattractant C5a
(**a-c**) Different morphotypes, i.e. dormant conidia (**a**), swollen conidia (**b**) and germ tubes (**c**) were incubated with complement-deficient serum with or without reconstitution of complement protein. Supernatant was examined on the presence of C5a 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.

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 (Figure 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 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*.

ACKNOWLEDGEMENTS

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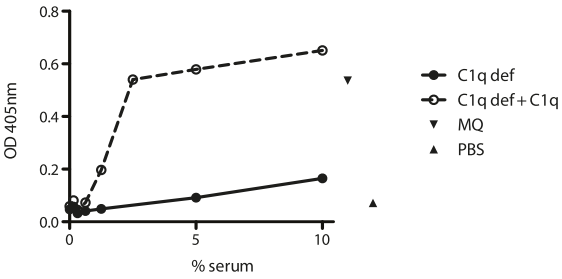
SUPPLEMENTAL FIGURES



Supplemental Figure 1 • Hemolytic CH50 assay with C1q deficient serum

To measure residual C1q activity in C1q deficient serum a standard CH50 hemolytic assay was performed as previously described[1]. We observed some residual classical complement activation in C1q deficient serum. Complete classical complement activation in C1q deficient serum was restored by reconstitution of C1q.

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Ch3

Neutrophil mediated defense against *Aspergillus fumigatus*: the role of antibodies

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ABSTRACT

Immunocompromised patients have increased risk of developing invasive infections with *Aspergillus fumigatus*. Neutrophils are essential in the clearance of *A. fumigatus* and neutropenia is a major risk factor for developing invasive aspergillosis. Neutrophil killing is dependent on complement opsonization of *A. fumigatus*. On *Aspergillus* swollen conidia, complement is activated in an antibody-dependent manner. Here, we studied the role of opsonic antibodies in neutrophil host defense against *A. fumigatus*. Antibody levels against surface antigens of swollen conidia were measured in sera of healthy individuals. Sera with different levels of opsonic antibodies were used to study the role of antibodies in the neutrophil response to *A. fumigatus*. Opsonic antibodies were detected in all healthy individuals but levels varied. High antibody levels resulted in more C3b opsonization, increased phagocytosis and efficient fungal killing by neutrophils. Opsonic antibody levels were determined in sera of patients with a hematological malignancy prior to therapy. Low antibody levels in patient sera were related to increased risk of developing invasive aspergillosis within 40 days after neutropenia. Opsonic antibodies directed against surface antigens of *A. fumigatus* are essential for efficient neutrophil killing and crucial for an effective immune defense against *A. fumigatus*.

INTRODUCTION

Aspergillus fumigatus is an important fungal pathogen and causes a variety of diseases ranging from allergic pulmonary aspergillosis to invasive fungal infections. The type and severity of infection mainly depends on the immune status of the host[1]. Especially immunocompromised patients are at risk for developing invasive aspergillosis (IA). In the University Medical Center Utrecht 7,2% to 14,3% of the patients with hematological malignancies, treated with hematopoietic stem cell transplantation (HSCT) or chemotherapy, are diagnosed with invasive fungal infections[2]. The major risk factor for IA is a prolonged period of neutropenia, as a result of therapy. Other risk factors for IA are neutrophil dysfunction and the use of immunosuppressive drugs.

The innate immune system of the lung is essential in the defense against *A. fumigatus*. Inhaled conidia are trapped in mucus and removed from the lung by the movement of ciliated mucus membranes. However, the size of *A. fumigatus* conidia is ideal for reaching the lung alveoli where they are not cleared by the ciliary motion. Phagocytes, such as alveolar macrophages and neutrophils, clear the residual conidia that reach the alveoli. Neutrophils are the main effector cells and thus are crucial in *A. fumigatus* clearance[3].

Recently, we have shown that opsonization by complement activation is essential for efficient phagocytosis and killing of *A. fumigatus* by neutrophils[4]. Three different pathways can induce complement activation: the classical pathway, the lectin pathway and the alternative pathway. The classical pathway is activated via the binding of antibodies to antigens on the microbial surface. The lectin pathway acts via the binding of mannose-binding lectin (MBL) and ficolins to sugar moieties on the microbe. The alternative pathway functions mainly as amplification loop of complement activation, but can be activated via low levels of spontaneous cleavage of C3. We have shown that the classical complement pathway is the main pathway in the opsonization and phagocytosis of *A. fumigatus*[4]. Antibodies are deposited on the outer surface of *A. fumigatus* resulting in complement activation and effective C3b opsonization. These C3b molecules are preferred ligands for complement receptors and induce efficient neutrophil phagocytosis of *A. fumigatus*.

Neutrophils are also able to recognize antibody-opsonized pathogens directly. Fcγ receptors (FcγRs) present on the neutrophil surface bind to antigen-antibody complexes and induce phagocytosis in the absence of complement activation and C3b opsonization. Fungal and bacterial pathogens like *Candida albicans*, *Staphylococcus aureus* and *Streptococcus pneumoniae* are phagocytosed by neutrophils when opsonized with antibodies[5-7]. In the absence of complement, antibodies do bind *A. fumigatus*, but do not induce any phagocytosis by neutrophils[4,8]. So, antibody opsonization of *A. fumigatus* does not result in FcγR-mediated phagocytosis, but enhances neutrophil phagocytosis by activation of the classical complement pathway.

In literature, the role of antibodies in survival after *A. fumigatus* challenge has been described in several mice studies showing contradicting results. Administration of immune serum containing anti-*Aspergillus* antibodies, does not increase the survival of mice subjected to systemic infection with *A. fumigatus*[9]. However, administration of monoclonal antibodies against a cell wall glycoprotein or an oligosaccharide, or vaccination with a glyco-conjugate vaccine of β-glucan results in enhanced protection in

systemic infection models in mice[10-12]. In contrast, B-cell deficient mice that are unable to produce antibodies are as susceptible to intratracheal infection with *A. fumigatus* as wild type mice. Though administration of normal mouse serum to these B-cell deficient mice reduces fungal growth[13].

In humans, the presence of antibodies in sera from patients with IA has been investigated as a potential predictive infection marker[14-16]. A minority of patients diagnosed with IA has antibodies against *A. fumigatus* antigens. Between patients, antibodies are directed against different sets of antigens. Only one study observed a relation between the presence of antibodies against recombinant proteins of *A.* fumigatus prior to HSCT or chemotherapy and the development of IA within 40 days after treatment[16].

The role of antibodies in *A. fumigatus* host defense is illustrated in patients with hypogammaglobulinemia. Patients with antibody levels lower than 400 mg/dl IgG after lung transplantation developed invasive aspergillosis more often than lung transplant patients with normal antibody levels[17].

Despite previous research it remains unclear how antibody levels are related to the innate immune defense against *A. fumigatus*. Antibody-dependent activation of the complement system is essential for effective phagocytosis and killing of *A. fumigatus* by neutrophils[4]. Therefore, it is likely that antibody levels play a role in the host defense against infections with *A. fumigatus*. In this study, we described the relation between opsonic antibody levels in sera of healthy individuals against surface antigens of *A. fumigatus* and neutrophil function. We showed that high antibody levels were related to more C3b deposition, increased phagocytosis and better killing by neutrophils. We measured antibody levels in sera from patients with hematological malignancies before the start of treatment and found that low levels of opsonic antibodies were related to an early development of IA.

MATERIALS AND METHODS

Fungal strains and culture conditions

Af293 was used to perform antibody and complement deposition assays. A green fluorescent protein (GPF) expressing strain A1258 was used to perform phagocytosis and growth studies[18]. All strains were cultured as described earlier and same protocol was used to obtain swollen conidia[4]. In short, freshly isolated conidia were inoculated in minimal medium supplemented with yeast extract, casamino acids and 2% glucose and grown at 37°C for 4 h. Swollen conidia were harvested, washed twice and gently sonicated for 2 minutes to break up clumps. Concentration was determined by microscopic counting. Swollen conidia were stored at -20°C until further use.

Sera from healthy individuals

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). Sera were collected from 20 healthy individuals. Four sera were selected on the differences in specific antibody levels, and assigned as donor 1 to 4, having high, medium and two times low antibody levels, respectively. Complement inactive serum was obtained by

heating at 56°C for 30 minutes.

Total IgG isolation

Total IgG was isolated from donor 1 to 4 with a HiTrap Protein G HP column (GE Healthcare Life Sciences) following manufactures protocol. Purity was checked by SDS-PAGE and concentration was determined by optical density (1,3 OD_{280nm} = 1 mg/ml).

Hemolytic assays

A standard AP50 and CH50 hemolytic assay with sera of donor 1 to 4 was performed as previously described[19].

IgG and C3b deposition assay

To study IgG deposition, swollen conidia were incubated with different concentrations of complement inactive serum or isolated total IgG in PBS supplemented with 1% BSA on a shaking platform (1000 rpm) at 4°C for 30 minutes. After washing, swollen conidia were stained with goat-anti-human-IgG-FITC (5 µg/ml, 2040-02, SouthernBiotech) in 1% BSA-PBS at 4°C for 30 minutes. After washing, swollen conidia were fixed in 1% paraformaldehyde in PBS and fluorescence was measured by flow cytometry (FACS Verse, BD). To study C3b deposition, swollen conidia were incubated with different concentrations of serum or serum spiked with isolated IgG from donor 1 in RPMI supplemented with 0,05% human serum albumine (RPMI-HSA) at 37°C for 25 minutes. Isolated IgG was spiked in a concentration equivalent to the concentration of IgG present in normal human serum (1,25 mg/ml IgG was spiked in 10% of serum). Swollen conidia were washed and stained with mouse-anti-human-C3 (1 µg/ml, WM-1, ATCC) in 1% BSA-PBS at 37°C for 30 minutes. After washing, swollen conidia were stained with goat-anti-mouse-FITC (1 µg/ml, F0479, DAKO) in 1% BSA-PBS at 4°C for 30 minutes. Swollen conidia were washed and fixed. Fluorescence was measured by flow cytometry (FACS Verse, BD).

Phagocytosis assay

Fresh neutrophils were isolated from healthy volunteers as earlier described[20] and membranes were stained with DiD (1 ul to $4x10^6$ neutrophils/ml, V-22887, Invitrogen) at 4°C for 10 min and washed 3 times with cold RPMI-HSA. GFP expressing A1258 swollen conidia were pre-incubated with different concentrations of serum in the absence or presence of additional total IgG of donor 1, as described above. After 10 minutes, DiD-labelled neutrophils were added in an effector-to-target ratio of 1:5 and incubated on a shaking platform (700 rpm) at 37°C for 15 minutes. Cells were fixed and measured by flow cytometry (FACS Verse, BD). Phagocytosis efficiency was expressed as percentage of GFP positive neutrophils within the total neutrophil population.

Growth rebound

Freshly isolated dormant conidia of GFP expressing strain A1258 were isolated and incubated with RPMI-HSA for 4 hours to form swollen conidia. Sera (3%) in the absence or presence of additional IgG of donor 1 were added. Neutrophils were added in an effector-to-target ratio of 20:1 and incubated for 25 hours at 37°C. GFP fluorescence

(Ex 485 nm, Em 520 nm) was measured every 10 minutes with a FLUOstar Omega plate reader (BMG Labtech). Per measurement, each condition was performed in triplicate and controlled for blank wells containing medium only.

Patient samples

To study antibody levels in patients with hematological malignancies, sera sent for pre-treatment routine serological diagnostics from hematological patients admitted between January 2007 and December 2013, were collected and subsequently heat-inactivated before use. Only sera that were isolated before treatments and administration of blood products were included the study. Diagnosis of proven and probable IA was based on criteria of the European Organization for Research and Treatment of Cancer (EORTC)[21].

Statistical analyses

Statistical analysis on the results of complement deposition, neutrophil phagocytosis and neutrophils killing was performed using an unpaired Student's t test. Mann-Whitney U test was performed to compare baseline antibody levels of patients diagnosed with or without subsequent IA. The relative risk of developing IA within 40 days after start of neutropenia was estimated comparing patients having antibody levels lower than 4300 fluorescence intensity (FI) as opposed to greater than this threshold and groups were compared by Fisher's Exact test. Differences were considered statistically significant if $p \leq 0.05$. All analyses were performed by Graphpad Prism v6.

RESULTS

Antibody levels in sera of healthy individuals

We studied the levels of antibodies in sera from healthy individuals against surface antigens of *A. fumigatus*. Dormant and swollen conidia were incubated with complement inactive serum and the amount of surface bound immunoglobulin G (IgG)

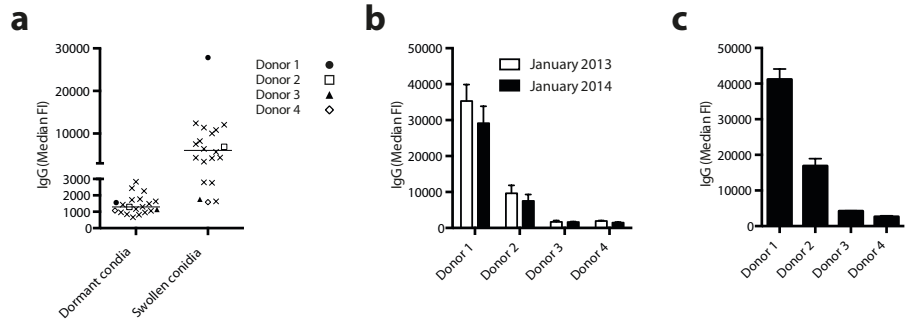

Figure 1 • Differences in antibody levels in healthy individuals
(**a**) Dormant and swollen conidia were incubated with 0,3% complement inactive serum of 20 healthy individuals. (**b**) Swollen conidia were incubated with 0,3% complement inactive sera of four healthy individuals collected in January 2013 and January 2014. (**c**) Swollen conidia were incubated with 125 ug/ml total IgG of donor 1 to 4. In all assays IgG deposition was detected by flow cytometry. The assays were performed three times and the mean ± SEM is shown.

was measured. All sera contained antibodies against surface antigens of dormant and swollen conidia (Figure 1a). Antibody levels differed between individuals and the most antibodies were directed against epitopes present on swollen conidia. We selected four individuals on their different antibody levels and assigned them as donor 1 (highest level) to 4 (lowest level). Sera of these donors were used to study the relation between antibody levels, complement activation and neutrophil function. Antibody levels

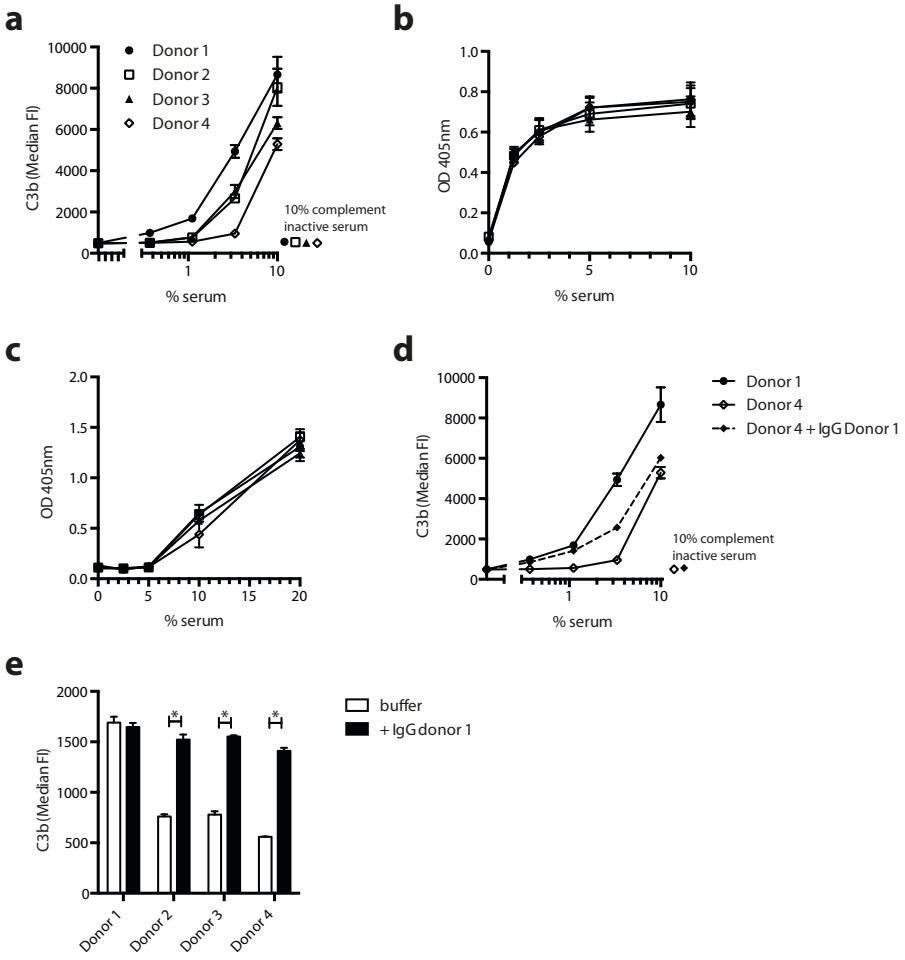

Figure 2 • Antibody levels relate to complement activation
(**a**) Swollen conidia were incubated with different concentrations of serum or 10% of complement inactive serum of donor 1 to 4. C3b deposition was detected by flow cytometry. (**b**) Classical complement activation in sera of donor 1 to 4 was measured by a hemolytic CH50 assay. (**c**) Alternative complement activation in sera of donor 1 to 4 was measured by a hemolytic AP50 assay. (**d**) Swollen conidia were incubated with different concentrations of serum or 10% of complement inactive serum of donor 1 to 4 with or without the addition of total IgG from donor 1. C3b deposition was detected by flow cytometry. (**e**) Swollen conidia were incubated with 1% of complement inactive serum with or without the addition of total IgG from donor 1. C3b deposition was detected by flow cytometry. The assays were performed three times and the mean ± SEM is shown. *$p < 0.05$.

against swollen conidia were 4 times higher in donor 1 than in donor 2. Donor 3 and 4 had the lowest antibody levels of all sera tested, 15.8 and 17.5 times lower than donor 1, respectively. We observed no difference within donors between antibody levels in sera collected in January 2013 and in January 2014 (Figure 1b). Total IgG was isolated from sera of donor 1 to 4 and antibody binding to swollen conidia was measured. Antibody levels against swollen conidia in total IgG of donor 4 resulted in a fluorescence intensity (FI) of 2628. Antibody levels in total IgG of donor 1, 2 and 3 were 15.7, 6.4 and 1.6 times higher, respectively, than in total IgG of donor 4 (Figure 1c).

Complement activation is related to antibody levels

To study the relation between antibody levels and the amount of complement activation, C3b deposition on swollen conidia was examined. Sera of all four donors induced C3b deposition on swollen conidia (Figure 2a). Incubation with sera with the highest antibody levels (donor 1) resulted in the most potent C3b deposition. C3b deposition in sera with lower antibody levels (donor 2, 3 and 4) was significantly reduced compared to serum of donor 1 (Figure 2a, e). Hemolytic assays were performed to investigate the general activation of the complement system in the donor sera. All four donors were equally capable in the activation of both the classical complement pathway and the alternative complement pathway (Figure 2b, c). This indicated that the reduction of C3b deposition in donor 2, 3 and 4 was not caused by deficiencies in complement activation, but was due to decreased antibody levels. Total IgG of donor 1 was used to reconstitute sera with low antibody levels. C3b deposition in sera of donor 2, 3 and 4 was significantly improved after the addition of total IgG of donor 1 (Figure 2d, e, and supplemental figure 1b, c). No increase in the amount of C3b deposition was observed in serum of donor 1 supplemented with its own total IgG (Supplemental figure 1a). These observations showed that opsonic antibody levels are related to the amount of complement activation.

Neutrophil phagocytosis is related to antibody levels

To study the relation between antibody levels and neutrophil function, neutrophil phagocytosis was examined. Neutrophils were incubated with swollen conidia that were pre-opsonized in serum of donor 1 to 4. All donor sera resulted in phagocytosis by neutrophils (Figure 3a). Serum with the highest antibody levels (donor 1) showed the most efficient phagocytosis. Phagocytosis in sera with lower antibody levels (donor 2, 3 and 4) was significantly less efficient compared to serum of donor 1 (Figure 3a, c). As shown before, complement inactive serum was unable to induce any phagocytosis (Figure 3a)[4]. Total IgG of donor 1 was used to reconstitute sera with low antibody levels and the effect on neutrophil phagocytosis was investigated. Addition of total IgG of donor 1 to serum with the lowest antibody levels (donor 4) resulted in significantly enhanced phagocytosis (Figure 3b, c). Phagocytosis efficiency was also significantly increased in sera of donor 2 and 3 supplemented with total IgG of donor 1 (Figure 3c and supplemental figure 2b, c). No improvement of phagocytosis was observed in sera of donor 1 after the addition of its own total IgG (Supplemental figure 2a). These findings illustrated a relation between the opsonic antibody levels and efficient phagocytosis by neutrophils.

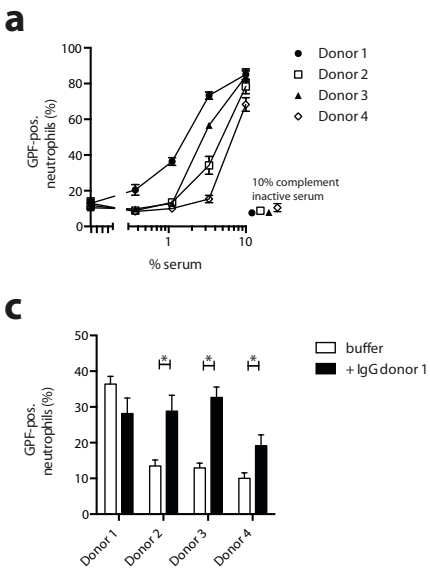

Figure 3 • Antibody levels relate to phagocytosis efficiency
(**a-c**) GFP expressing swollen conidia were pre-incubated with different concentrations of serum or 10% of complement inactive serum of donor 1 to 4 with or without the addition of total IgG from donor 1. Neutrophils were added in an effector-to-target ratio of 1:5. After 15 minutes, cells were fixed and the percentage of GFP-positive neutrophils was measured by flow cytometry. (**c**) Results of phagocytosis assay performed in 1% serum. The assays were performed three times and the mean ± SD is shown. *p < 0.05.

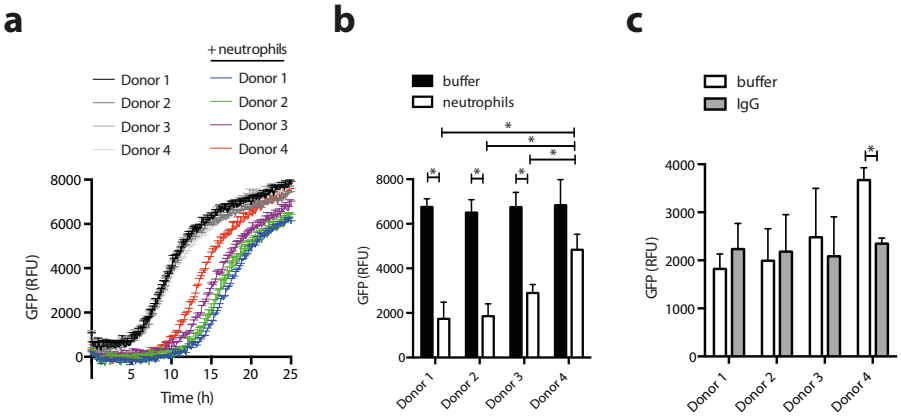

Figure 4 • Antibody levels relate to neutrophil killing
(**a**) Freshly isolated dormant conidia from the GFP expressing strain were incubated to form swollen conidia. After 4 hours, 3% serum of donor 1 to 4 was added. Subsequently, neutrophils were added in an effector-to-target ratio of 20:1 and GFP expression was measured every 10 minutes for 25 hours. GFP expression was plotted as relative fluorescent units (RFU). The assay was performed three independent times in triplicate and a representative plot with mean ± SEM is shown. (**b**) The GFP expression at 15 h after the addition of neutrophils was calculated and the mean ± SD of three independent experiments is shown. (**c**) Similar assay was performed as in A, but then total IgG of donor 1 was added to serum of donor 1 to 4. GFP expression at 20 h after the addition of neutrophils was calculated and the mean ± SD of three independent experiments is shown. *p < 0.05.

Fungal killing is related to antibody levels

Since opsonic antibody levels influenced the phagocytosis efficiency, we investigated the killing of swollen conidia by neutrophils. Dormant conidia were grown until swollen conidia. Serum of donor 1 to 4 was added and growth was measured as the amount of GFP expression. All donor sera resulted in equal growth in the absence of neutrophils (Figure 4a). The addition of neutrophils reduced the fungal growth in all donor sera (Figure 4a, b). Serum with high antibody levels (donor 1 and 2) showed the most effective killing by neutrophils. Fungal killing was reduced when incubated with sera of donor 3. Serum with the lowest antibody levels (donor 4) resulted in significantly less killing. Reconstitution with total IgG of donor 1 in sera with low antibody levels was examined. The neutrophil killing in sera of donor 4 was strongly enhanced and almost restored to the level of donor 1, when total IgG of donor 1 was reconstituted in the serum of donor 4 (Figure 4c). No improvement in killing was observed in sera of donor 1, 2 and 3 supplemented with total IgG of donor 1. These results supported a relationship between the opsonic antibody levels and fungal killing by neutrophils.

Antibody levels in patient sera

We showed a relation between the presence of specific antibodies and enhanced opsonization, phagocytosis and killing. To investigate a relationship between antibody levels and incidence of invasive aspergillosis, we measured the level of opsonic antibodies in sera of 148 hematological patients at risk of developing IA. Of the 148 patients, 26 patients were diagnosed with IA at any time during subsequent follow-up. No difference was observed in the opsonic antibodies levels between the infected and non-infected patients (Figure 5a). Development of early IA within 40 days of treatment has been

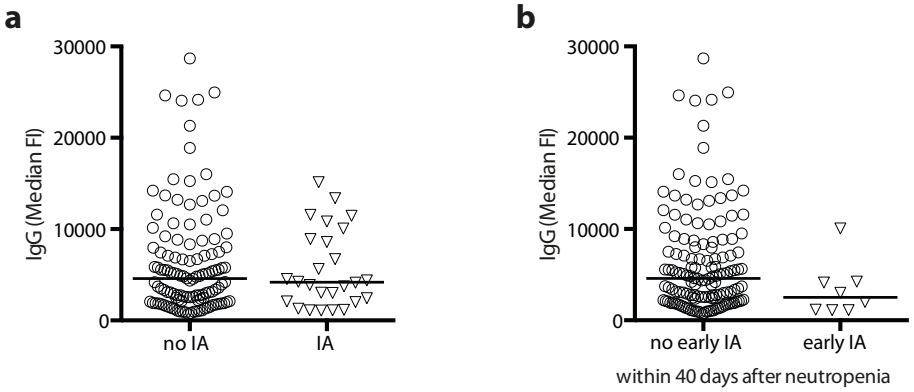

Figure 5 • Antibody levels in patients with hematological malignancies
(**a**) Swollen conidia were incubated with 0,3% complement inactive sera or plasma from patients with hematological malignancies. Samples were measured three times and the mean is shown. Antibody levels from patients diagnosed with proven or probable IA were compared with patients with no invasive *Aspergillus* infection. Opsonic antibody levels between infected and non-infected patients were compared with the Mann-Whitney U test and the difference was not statistically significant. (**b**) Antibody levels from patients diagnosed with IA within 40 days after neutropenia were compared with patients with no *Aspergillus* infection within 40 days. The difference in opsonic antibody levels between infected and non-infected patients was not statistically significant (Mann-Whitney U test).

associated with different risk factors than cases developing in a later time period[22]. Therefore, we additionally compared baseline opsonic antibody levels in patients diagnosed with early onset IA (within 40 days after start of neutropenia, n = 8) to levels in patients not diagnosed with early IA (n = 140). No significant difference was observed between these populations (p = 0.08) (Figure 5b). We observed that almost all patients diagnosed with early onset IA have antibody levels lower than 4300 FI. When applying this threshold, 7 of 73 patients (9.6%) with low antibody levels (<4300 FI) developed early IA as opposed to 1 of 75 patients (1.3%) of patients with higher antibody levels (≥ 4300 FI). Relative risk of developing IA within 40 days after start of neutropenia was 7.2 (95%CI 0.91 – 57.0, $p < 0.05$ (Fisher's Exact)) if the opsonic antibody levels were lower than 4300 FI.

DISCUSSION

In this study we report that opsonic antibody levels against *A. fumigatus* are important in anti-fungal immunity. The levels of antibodies correlate to an effective opsonization, phagocytosis and fungal killing by neutrophils. Several studies investigated the presence of antibodies in patient sera against secreted proteins, cell wall proteins, intracellular proteins and cell wall polysaccharides of *A. fumigatus*[14-16]. We measured antibody levels that are directed against the surface epitopes of intact swollen conidia of *A. fumigatus*. These antibodies recognize multiple antigens that are present on the surface of the fungal particle, like proteins, glycoproteins and polysaccharides. The binding of these antibodies is of high relevance, since it results in functional opsonization and immune recognition of *A. fumigatus*.

We found that all healthy individuals have detectable serum antibodies against *A. fumigatus*. This is in concordance with a previous study which showed the presence of antibodies in sera of healthy individuals directed against a secreted polysaccharide of *A. fumigatus*[23]. Antibodies in sera of healthy individuals were primarily directed against swollen conidia and not against dormant conidia. This difference can be explained by the remodeling of the cell wall during the transition from dormant conidia to swollen conidia. Dormant conidia are covered by a hydrophobic rodlet layer composed of non-immunogenic proteins[24]. During swelling, the conidium becomes metabolically active and releases its rodlet layer. Polysaccharides and proteins of the underlying cell wall will be exposed and are epitopes for immune recognition. The antibody levels varied between individuals, however the individual levels appear to be stable over a one-year period. The absence of dynamics in the antibody levels indicates that high antibody levels were not caused by a recent *Aspergillus* infection. High concentration of *Aspergillus* conidia can be measured in air[25]. Inhalation of several hundred conidia per day leads to a constant exposure of *A. fumigatus* and triggers an immune response. Most likely the differences in antibody levels between the individuals are caused by differences in exposure to *A. fumigatus* conidia over time. Small differences in observed antibody levels can also occur when the antibodies of the studied individuals are directed against a different set of antigens.

We showed that high surface antibody levels led to more C3b deposition, phagocytosis and fungal killing by neutrophils. The differences in opsonization and neutrophil function between individuals are related to the antibody levels, since

complement activation was equal between all individuals studied. However, there is no direct correlation between antibody levels and opsonization and neutrophil function. The antibody level in donor 2 is 4 times higher than in donor 3, while C3b deposition, phagocytosis and killing in both donors is similar. Also, the antibody level in donor 3 and 4 only differ 1.1 times, while donor 4 is clearly less efficient in C3b deposition, phagocytosis and killing compared to donor 3. A possible explanation would be that the opsonic antibodies differ between individuals. The response to antibody binding depends on the epitope that is recognized, its affinity and its subtype. The antibody subtypes vary in the capacity of complement activation and in its recognition by FcγRs[26-28]. Since no FcγR-mediated phagocytosis was observed, the influence of antibody subtypes will be mainly on the level of complement activation.

The incidence of IA in hematological patients is not equally distributed over the risk period[29]. Patients with hematological malignancies developing IA within 40 days after HSCT (early onset) have different risk factors than patients developing IA after 40 days after HSCT (late onset)[22]. We observed that patients with opsonic antibody levels lower than 4300 FI before treatment have an increased risk in developing IA within 40 days after start of neutropenia compared to those with higher baseline antibody levels. A previous study observed an association between high antibody levels and an increased development of early onset of IA[16]. This discrepancy could be caused by a difference in the subset of the antibody levels identified. Du et al. measured antibody levels against 6 recombinant proteins of *A. fumigatus*. Two of the recombinant proteins are cell wall proteins and thereby potentially can induce opsonic antibodies, while the others are secreted or intracellular proteins. We looked at all surface antigen recognizing antibodies that play a key role in *Aspergillus* immune response. A low level of opsonic antibodies in early IA patients may result in the inability to clear subclinical *Aspergillus* colonization before treatment, increasing the risk of invasive infection upon further immunosuppression. We did not observe a relation between opsonic antibody levels and late onset IA. Perhaps that in cases of late development of IA, factors unrelated to antibodies are of increased importance, such as (induced) neutropenia, graft-versus-host disease and *Cytomegalovirus* reactivation or treatment effects of steroids lowering total IgG levels in serum[22,30].

Although it is tempting to directly correlate the absence of anti-*Aspergillus* antibodies to the risk of invasive disease, the increased risk in patients with low antibody levels should be interpreted with caution. There are several factors that may confound the association by affecting both the antibody levels and the risk of IA, including the type of hematological malignancy, underlying disease, therapy and blood transfusions. The small sample size and the difficulty in establishing an IA diagnosis make it hard to investigate relationships[31]. This is illustrated by the patient with early onset IA and an antibody level of 10,000 FI who was diagnosed probable IA in accordance with the EORTC criteria based on multiple intra and extra pulmonary nodules on high resolution computed tomography. These nodules, however, may have been indicative for metastases instead of fungal burdens, since the size and amount of nodules improved over the next couple of weeks of anti cancer therapy despite undetectable serum levels of the antifungal agent. Nevertheless, our observations support the presence of a relation between low opsonic antibodies levels and the development of early IA. Future follow-up studies are needed to further quantify the (additional) prognostic value of

these antibody levels in predicting early IA and assess the potential benefit of directed prophylactic therapy.

In conclusion, levels of opsonic antibodies are essential for an effective immune response against *A. fumigatus*. High antibody levels results in more effective fungal killing by neutrophils. Hematological patients with low levels of opsonic antibodies before treatment have an increased risk in the early development of IA. Antibodies together with neutrophils are crucial in innate host defense against *A. fumigatus*.

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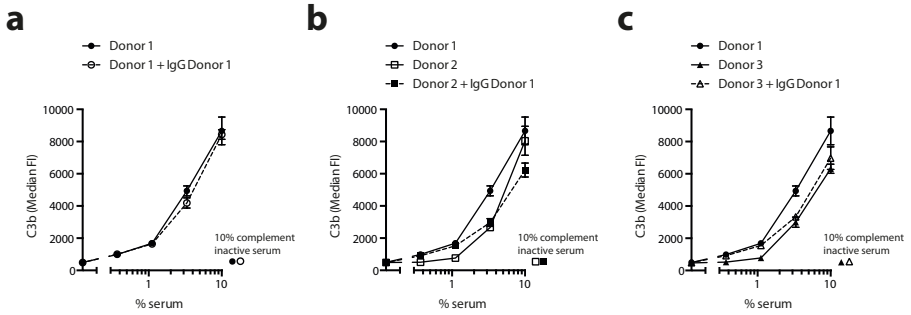
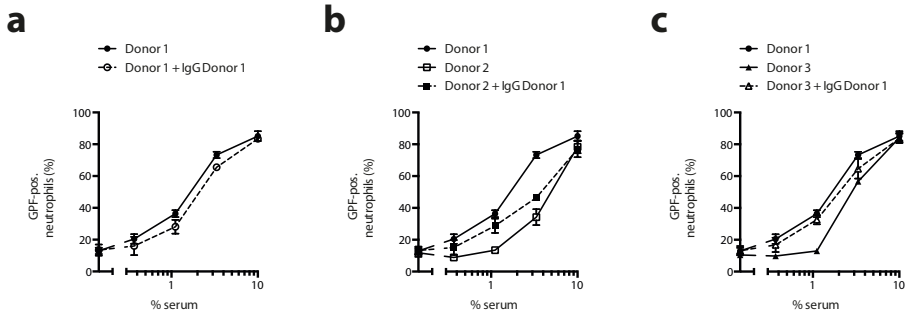
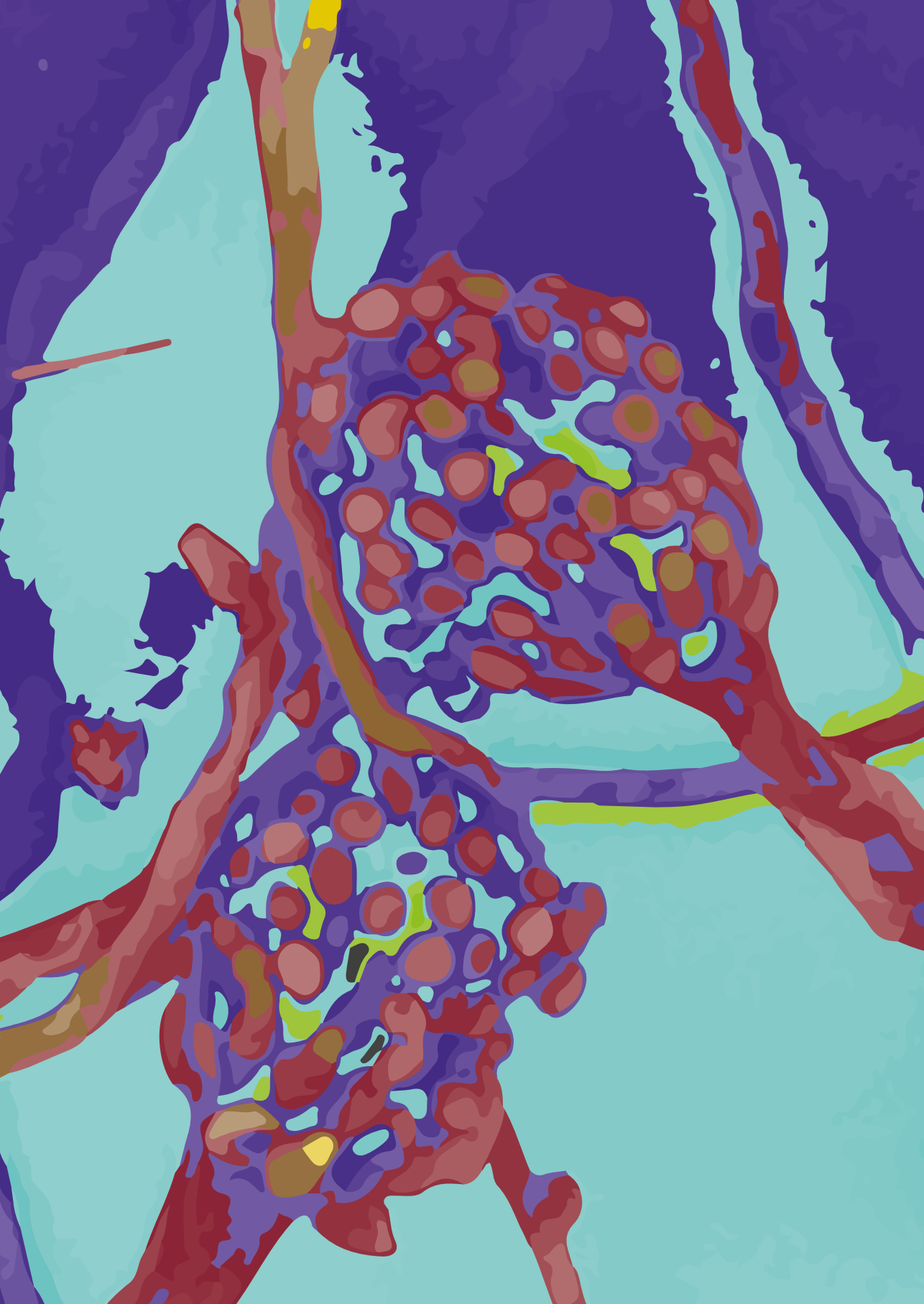
SUPPLEMENTAL FIGURES

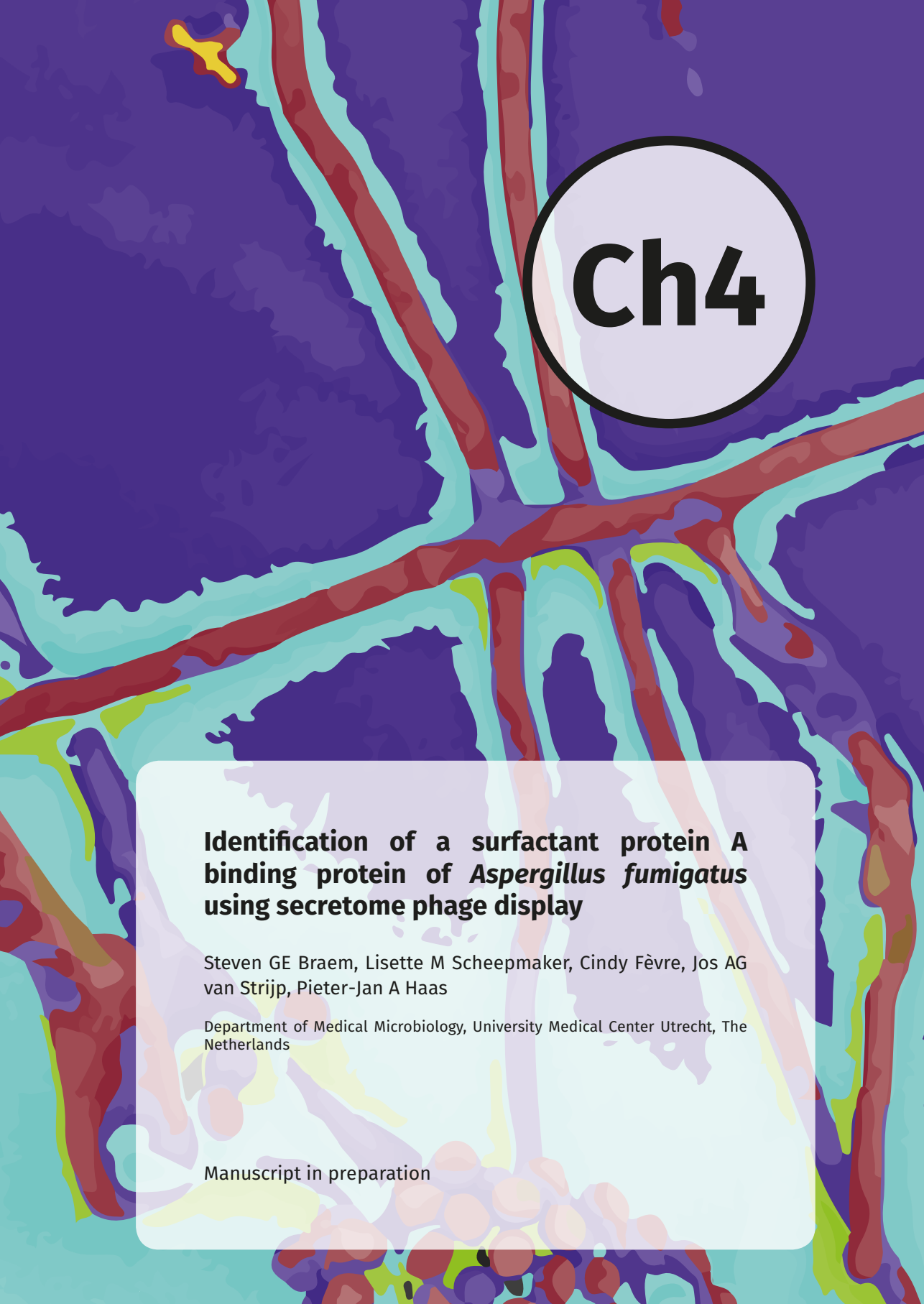


Supplemental figure 1 • Antibody levels relate to complement activation
(**a-c**) Swollen conidia were incubated with different concentrations of serum or 10% of complement inactive serum of donor 1 (**a**), 2 (**b**) and 3 (**c**) with or without the addition of total IgG from donor 1. C3b deposition was detected by flow cytometry. The assays were performed three times and the mean ± SEM is shown.



Supplemental figure 2 • Antibody levels relate to phagocytosis efficiency
(**a-c**) GFP expressing swollen conidia were pre-incubated with different concentrations of serum donor 1 (**a**), donor 2 (**b**) and donor 3 (**c**) with or without the addition of total IgG from donor 1. Neutrophils were added in an effector-to-target ratio of 1:5. After 15 minutes, cells were fixed and the percentage of GFP-positive neutrophils was measured by flow cytometry. The assays were performed three times and the mean ± SD is shown.





Identification of a surfactant protein A binding protein of *Aspergillus fumigatus* using secretome phage display

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Manuscript in preparation

ABSTRACT

Aspergillus fumigatus is an important airborne fungal pathogen. Conidia are dispersed into the atmosphere and high concentrations of conidia are daily inhaled by humans. The lung defense clears most conidia by ciliated movement of epithelial cells. The remaining conidia are opsonized with surfactant proteins, complement proteins and antibodies. This opsonization leads to increased recognition and efficient killing of *A. fumigatus* by phagocytes. In this study we identified a novel ligand XP_748409 of *A. fumigatus* that is recognized by surfactant protein A using a secretome phage display approach. XP_748409 is a cell wall anchored protein that is predicted to be important in adherence. Functional analyses were performed using a soluble recombinant form of XP_748409. The recombinant protein binds to surfactant protein A and not to a structural homologous protein Mannose Binding Lectin. Binding of surfactant protein A to conidia was inhibited in the presence of the recombinant protein. Probably, XP_748409 is a ligand for SP-A that may induce phagocytos or could serve as an adhesin to promote *A. fumigatus* infection.

INTRODUCTION

The *Aspergillus* genus contains more than 200 different species. Although many *Aspergillus* species are commonly found in the environment, only a few of them can cause disease in humans. *Aspergillus* infections lead to a diverse panel of diseases like allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis, and invasive aspergillosis. These infections are primarily caused by *A. fumigatus*, making it one of the most important human fungal pathogen.

We are constantly exposed to dormant conidia of *A. fumigatus*. Dormant conidia are present in the air in high concentrations (10-400 CFU/m^3) and therefore we inhale over hundred conidia per day[1]. Due to their small size (2-3 um), dormant conidia can easily reach the lung alveoli. Epithelial cells will clear most conidia from the lung by ciliated movement. Conidia that are not cleared will be initially opsonized with surfactant proteins. Then antibodies and complement components bind to the conidia and induce recognition by alveolar macrophages. Finally, additional recruitment of neutrophils results in the clearance of *A. fumigatus*[2].

Surfactant proteins are components of the lung innate immune defense. Surfactant protein A (SP-A) and D (SP-D) can bind to microbes resulting in aggregation and opsonization of the microbe. SP-A and SP-D bind to *A. fumigatus* conidia and induce aggregation, opsonization and killing by neutrophils and macrophages[3]. Although both surfactant proteins seem to enhance fungal clearance in vitro, SP-A knockout mice are more resistant than wild type mice and SP-D knockout mice are as suceptible as wild type mice after intranasal infections[4].

The secretome of *A. fumigatus* consist of secreted proteins, cell wall anchored proteins and membrane proteins. These extracellular proteins are important for initial adhesion to lung epithelium, invasion and destruction of tissue, and modulation of the host immune defense. Several studies indicate the presence of adhesion proteins on the conidial surface[5-7]. However, the identity of these proteins is not elucidated. Only one cell wall protein, AfCalAp, is described to be an adhesin that binds to the extracellular matrix component laminin[8]. Proteases are believed to be important factors in fungal invasion. A secreted protease Alp1 induces detachment of cells and causes changes in actin skeleton[9,10]. Alp1 also degrades factors of the complement system[11]. However, disruption of the gene in *A. fumigatus* does not alter virulence[11-13]. Other secreted proteases, Mep and Pep, are also dispensable for virulence[14,15]. In addition, deletion of prtT, which regulates the major secreted proteases, Alp1, Mep, Pep, SedB, DPPIV and DPPV, does not change virulence[16]. *A. fumigatus* also produces extracellular proteins to modulate and hamper the immune defense. Conidia and hyphae of *A. fumigatus* produce catalases to protect them from H_2O_2 challenges. Again, gene deletions do not attenuate its virulence[17-19]. In contrast, a pacC mutant strain, which lacks a pH responsive transcription factor, decreases invasive growth and is attenuated in virulence[20]. PacC regulates 75 secreted proteins, which are still uncharacterized.

Although some secreted proteins are described to be important in establishing invasive *Aspergillus* infection, lots of them are currently unexplored. Proteomic strategies are necessary to find novel proteins of *A. fumigatus* that interact with the human host. Several studies show the identification of proteins present on the conidial surface, in total hyphal homogenate, culture supernatant and a phospholipase C

digested fraction of hyphal surface proteins by the use of 2D gel electrophoresis[21-24]. Difference in protein expression profiles between planktonic and biofilm growth of *A. fumigatus*, and the oxidative challenge of hyphae in the presence or absence of H_2O_2 have been investigated by 2D gel electrophoresis using difference of gel electrophorese (DIGE) labeling technique[25,26]. Another study shows the immunogenic secreted proteins by immunoblotting with patient sera after 2D gel electrophoresis[27]. These proteomic strategies are time consuming and the need for a high-throughput method to characterize secreted proteins that interact with the host immune system is required.

A high-throughput strategy to investigate protein-protein interaction is the phage display approach[28]. Proteins are displayed as fusions to coat proteins on the surface of filamentous bacteriophages and selected on their desired properties via in vitro selection procedures. Since the phage genome directly corresponds to the displayed protein, identification of the protein is simple assessed by sequencing. Screening of large phage libraries, displaying a heterogeneous population of proteins, allow for rapid identification of novel interaction partners. The phage display approach results in the selection of proteins that bind to a specific target with high affinity.

The most commonly used coat protein for phage display is the minor coat protein pIII. This protein allows the display of large fusion proteins fused to the N-terminal end of pIII. The development of eukaryotic phage display expression libraries is challenging. Eukaryotic DNA contains introns and exons that need to be spliced before translation can take place. Therefore eukaryotic phage display libraries are based upon mRNA-derived cDNA. The presence of a stop codon interferes with the N-terminal display of the fusion protein requiring a more complex phage display library design. C-terminal display of fusion proteins has been performed using the minor coat protein pVI[29]. Crameri *et al.* described the construction of a phage display library for *A. fumigatus*[30]. This library is based on a c-fos and c-jun leucine zipper that allows for the display of cDNA products on a capsid protein of the phage.

In this study we describe the design of a secretome phage display library that only displays secreted proteins of *A. fumigatus*. We adapted a genomic-based bacterial secretome phage display library to make the technique applicable for a cDNA library of *A. fumigatus*[31]. We panned the newly designed *A. fumigatus* phage library against several immune targets and identified cell wall protein XP_748409 that interacts with SP-A. SP-A did bind to dormant conidia, which was inhibited by a recombinant form of XP_748409. Here, we identified a new ligand for SP-A expressed by *A. fumigatus.*

RESULTS AND DISCUSSION

Eukaryotic phage display design

To identify novel secreted virulence factors of *A. fumigatus* in a high throughput manner, we applied a phage display strategy that is comparable to a recent described *S. aureus* secretome phage display approach[31]. In this strategy the bacterial genomic DNA was sheared and the DNA fragments were ligated into a phagemid vector resulting in a random genomic library. Only genes that were in frame with the C-terminus of pIII and contained a signal sequence will be displayed on the phage. *A. fumigatus* is an eukaryote and its genomic DNA contains introns. To circumvent that introns will prevent the display of native proteins on the phage, generation of a cDNA-based phage

library is required.

The minor coat protein pIII is the most commonly used coat protein for phage display and its N-terminal part allows the display of large fusion proteins. The presence of a stop codon and a 3' untranslated region (UTR) at the end of cDNA makes it difficult to display cDNA libraries on the N-terminal part of pIII. Several strategies are used to overcome these problems, like cDNA fragmentation, indirect fusion via a c-fos and c-jun leucine zipper, or direct fusion to the C-terminus of pVI[29,30,32]. The construction of a library for indirect fusion needs cDNA digestion by two common restriction enzymes, which results in the generation of similar DNA fragments and the loss of randomness. Moreover, *E. coli* clones can produce phages without the need of a displayed protein, resulting in overestimation of the diversity of the library. A drawback of direct fusion to the C terminus of pVI is the lack of displaying large proteins on this phage capsid protein. Moreover, the use of these strategies leads to the display of all proteins and not only secreted proteins. Here, we described the construction of an *A. fumigatus* secretome phage display library (Figure 1).

We used random hexamer primers to synthesize cDNA, resulting in a cDNA library containing fragments with different lengths. This approach reduced the amount of stop codons and 3' UTRs. The majority of cDNA constructs will contain the start of an open reading frame, because the reverse transcription reaction is directed towards the 5' site of the mRNA. This method allows the expression of secreted proteins with their native signal sequence. The cDNA library was cloned into a phagemid vector and

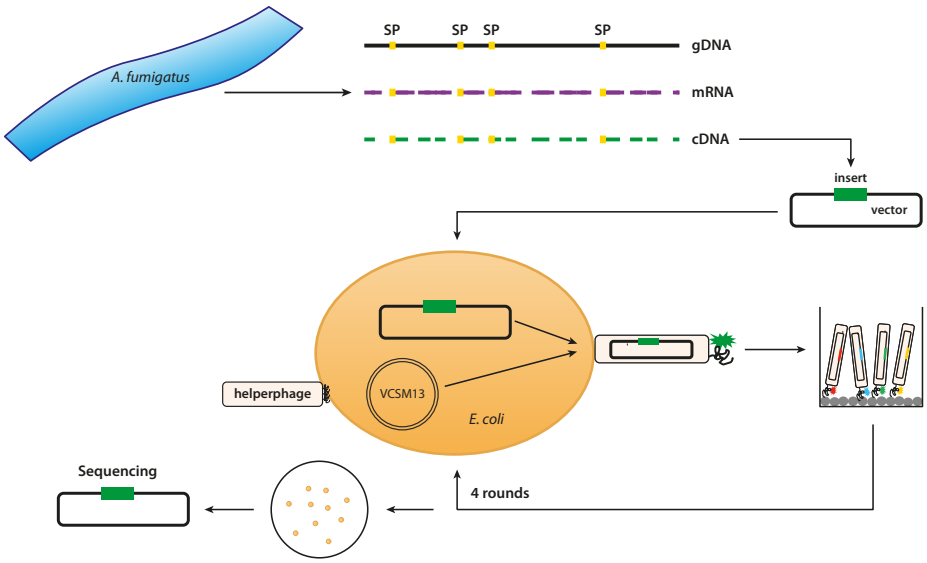

Figure 1 • The *A. fumigatus* secretome phage display library

The construction of the *A. fumigatus* secretome phage display library and the panning procedure. *A. fumigatus* RNA was isolated and mRNA was purified. cDNA was synthesized using random hexamer primers. Some cDNA fragments harbor signal sequences (SP) for native secretion and will be displayed on the phage. The cDNA library is cloned into a phagemid vector and transformed into *E. coli*. Helperphage infection resulted in the production of phages displaying cDNA-encoded proteins. Phages were panned against targets of interest for 4 rounds. After the last round, clones were selected and sequenced for the identification of the displayed protein.

transformed into *E. coli*.

We obtained a bacterial library with a diversity of 2,1 x 10^8 different clones. The majority of the clones contained inserts that originate from ribosomal RNA (rRNA) (51%) (Figure 2a). Although we isolated mRNA before we synthesized cDNA, remaining rRNA was present during the cDNA synthesis. The presence of rRNA after mRNA isolation is probably caused by the high quantity of rRNA present in total isolated RNA. However, the presence of clones originating from rRNA will only decrease the diversity of the library two fold to 1 x 10^8 different clones. The bacterial library contained 39% clones harboring protein-coding DNA. The protein-coding DNA originated from all 8 chromosomes and was equally distributed over the genome (Figure 2b). The remaining clones contained non-coding DNA (8,1%) or no insert was present (2,3%) (Figure 2a).

To evaluate the display of secreted proteins on the phage, we panned the phage library against an anti-Myc antibody. After two rounds, the displayed protein of 240 phages was determined by sequencing. In total 174 phages displayed a protein. The other phages harbored inserts that did not encode for genes or did encode for genes without a signal sequence, that were out of frame with the C-terminus of pIII, or were in the wrong orientation. On these 174 phages, we found 20 different displayed proteins (Table 1). The displayed proteins were secreted, cell wall or cell membrane proteins. In accordance with predictions programs, all proteins, except for XP_751286, contained a signal sequence. XP_751286 was predicted to be a transmembrane protein crossing the membrane six times without having a signal sequence. The fact that we found three different phages displaying different parts of XP_751286, suggested that this protein is secreted but lacks a typical hydrophobic leader sequence. Some proteins were displayed more often and also in different sizes. The GPI anchored protein XP_7522487 was even displayed as full-length protein. Altogether, these observations showed that the phage display library is a divers library displaying only secreted *A. fumigatus* proteins.

The phage display technique based on cDNA libraries has some limitations. Genomic libraries contain randomized fragments of genomic DNA. In contrast, our

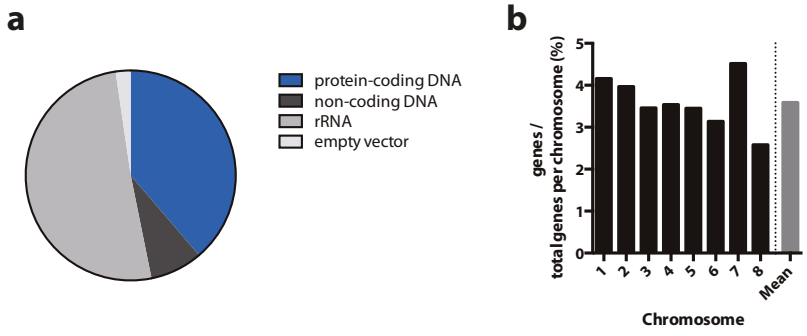

Figure 2 • The *A. fumigatus* bacterial library
(**a**) The bacterial library has a diversity of 2,1 x 10^8 different clones. Of the 173 sequenced clones, 51%, 39% and 8,1% of the clones harbored inserts that originated from rRNA, protein-coding DNA and non-coding DNA, respectively. No insert was present in 2,3% of the clones. (**b**) Inserts encoding a protein were equally distributed over the *A. fumigatus* genome. The amount of genes found in the bacterial library were divided by the total amount of genes on a chromosome (**Chromosome 1 - 8**)[49]. All genes found in the bacterial library were divided by the total amount of genes on the genome (**Mean**).

Table 1 • Proteins displayed on phages (after two rounds of selection with the anti-Myc-tag Ab)

Protein	Protein annotation	Gene	Display (AA)	Protein size (AA)	SignalP	TOPCONS
XP_001481458	conserved threonine rich protein	AFUA_6G07855	1-38	168	+	+
XP_731479	extracellular arabinanase	AFUA_6G00770	92-153 93-139	416	-	+
XP_746510	cell wall serine-threonine-rich galactomannoprotein Mp1	AFUA_4G03240	1-45	284	+	+
XP_746523	GPI anchored protein	AFUA_4G03360	1-26 1-27 1-28 1-30 1-37 1-39 1-41 1-42 1-44 1-49 1-52 1-71 1-78 1-84 1-102 1-108 1-123 1-139 2-31 2-102	198	+	+
XP_746892	conserved hypothetical protein	AFUA_7G00370	1-39 1-42 1-46 1-56 1-63 1-65 1-70 1-75	198	+	+
XP_746961	conserved hypothetical protein	AFUA_8G02450	13-118 15-109 15-115 15-129 15-130 17-74 19-105 19-111 19-136 19-143 20-108 20-109 21-62 21-84 31-84	216	+	+
XP_747307	beta-N-acetylhexosaminidase NagA	AFUA_8G05020	1-18 1-26	600	+	+
XP_748109	major allergen and cytotoxin AspF1	AFUA_5G02330	1-144	176	+	+
XP_748150	GPI anchored protein	AFUA_5G01920	1-58 1-70 1-71 1-76 1-78 1-79 1-81 1-83 1-85 1-86 1-93 1-96 1-97 1-99 1-101 1-103 1-105 1-108 1-109 1-111 1-115 1-117 1-174	205	+	+
XP_748193	hypothetical protein	AFUA_5G01490	57-108	192	-	+
XP_749250	GPI anchored cell wall protein (Dan4)	AFUA_2G01140	1-53	278	+	+
XP_750162	FG-GAP repeat protein	AFUA_1G04130	1-121	307	+	+
XP_750329	GPI anchored serine-rich protein	AFUA_1G05790	1-56 1-82	192	+	+
XP_751286	conserved hypothetical protein	AFUA_6G14120	1-42 1-78 11-63	373	-	-
XP_752487	GPI anchored protein	AFUA_1G11220	1-43 1-85 1-149*	149	+	+
XP_753093	conidial hydrophobin RodB	AFUA_1G17250	1-64	140	+	+
XP_753818	cell wall protein	AFUA_5G08180	1-126	300	+	+
XP_754130	hypothetical protein	AFUA_3G14940	1-47	87	+	+
XP_754900	WSC domain protein	AFUA_3G07050	1-29	235	+	+
XP_755297	RING finger domain protein	AFUA_2G09640	14-63	544	+	+

Phage library was panned two rounds against anti-Myc antibody. Of the 240 selected phages, 174 phages displayed in total 20 different proteins. The portion of the displayed protein is specified in amino acids (Display). When different portions were displayed, these portions are depicted after each other. XP_752487 was displayed as full-length protein (*). The size of the full-length protein is indicated in amino acids (Protein size). The presence of a signal sequence in the protein was estimated using algorithms of SignalP and TOPCONS (does (+) or does not (-) contain a signal sequence).

cDNA library is build from mRNA. The expression level of a gene determines the amount of its mRNA products. Therefore some genes will be more represented in the phage library than others. The isolation of RNA from different growth conditions could enhance the diversity within the library. Another limitation is the lack of the machinery for post-translational modifications in *E. coli*. Some eukaryotic proteins need post-translation modifications for their correct folding and stability. These proteins will probably be less efficiently displayed on a phage. To optimize the expression the post-translation machinery of *C. jejuni* could be expressed in *E. coli*[33]. However, the glycosylation of proteins differs enormously between *A. fumigatus* and *C. jejuni* and it is questionable if this approach can solve the glycosylation issue.

Extracellular conserved serine-rich protein interacts with immune molecules

We panned the phage library against a mixture of immune molecules, i.e. dectin-1, dectin-2, SP-A, SP-D and Toll-like receptor 4 in complex with MD-2 (TLR4/MD-2), important in host recognition of *A. fumigatus*. After 4 round of selection, we sequenced 47 colonies and observed enrichment of four proteins (Figure 3a). Two proteins (XP_748409 and XP_7429922) were predicted to have a native signal sequence. The other two proteins (XP_752756 and XP_753354) did not have a predicted signal sequence and

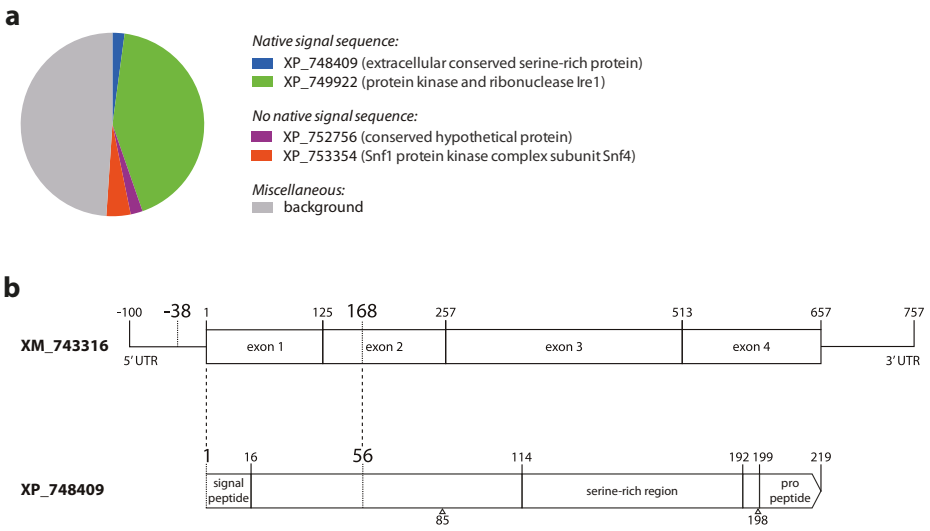

Figure 3 • Phage library panning against a mix of dectin-1, dectin-2, SP-A, SP-D and TLR4/MD-2
(**a**) After 4 rounds of panning, 47 clones were selected and sequenced. Of the 47 clones, 1, 20, 1 and 2 clones encoded protein XP_748409, XP_749922, XP_752756 and SP_753354, respectively. The full-length proteins were predicted to have or have not a signal sequence by algorithms of SignalP and TOPCONS. The remaining clones were considered background, because they harbored inserts from rRNA, non-coding DNA or protein-coding DNA that was not in frame with the C terminus of pIII. (**b**) Schematic diagrams of the mRNA of XP_748409 (XM_743316) and the protein itself. The protein-coding portion including 100 nucleotides of 5' and 3' UTR of XM_743316 is shown. The individual exons are indicated and marked with nucleotide positions. The insert, present in the phage DNA, is indicated with dotted lines (nucleotide -38 - 168). The numbers of XM_743316 indicate nucleotides. The full-length protein of XP_748409 is shown. The position of the signal peptide, the potential N-glysolation (AA 85), the serine-rich region, the lipidation site (AA 198) and the propeptide are indicated. The portion of the displayed protein on the phage is depicted by the dashed lines (amino acid 1 – 56). The numbers of XP_748409 indicate amino acids.

were excluded as potential interaction partner of the immune molecules. XP_749922 is annotated as protein kinase and ribonuclease Ire1. XP_749922 was predicted to span the membrane two times and probably has its function in the endoplasmic reticulum and/or nucleus. This information discarded XP_749922 as interesting protein for further research. One phage displayed XP_748409, which is annotated as extracellular conserved serine-rich protein. Although only one phage was found to display XP_748409, this phage was never found in the anti-Myc antibody panning. This suggested that this phage was not found because of high abundance in the phage library, but was selected during the panning procedure with immune molecules. XP_748409 has a predicted signal sequence, contains no transmembrane domains and has a predicted N-glycosylation site at position 85 (Figure 3b). The first 56 amino acids of XP_748409 were displayed on the selected phage. Of interest, the phagemid harbored an insert from position -38 (5' UTR) till position 168 of the mRNA of XP_748409 and lacked DNA originating from the intron present between exon 1 and exon 2. The remaining colonies harbored inserts originating from rRNA, non-coding DNA, or protein-coding DNA that were not in frame with the C-terminus of pIII or had the wrong orientation and were considered as background.

XP_748409 is identified as a glycosylphosphatidylinositol (GPI)-anchored protein present on the hyphal membrane of *A. fumigatus*[22]. Cleavage by endogenous phospholipase C results in the release of XP_748409 from the membrane. XP_748409 is also predicted to be an important adhesin[8]. Expression of the XP_748409 gene (AFUA_3G00880) is regulated by MedA, a protein that regulates fungal development, adhesion and damage to pulmonary epithelial cells[34]. The MedA knockout strain, exhibiting lower expression of the XP_748409 encoding gene, is attenuated in adhesion and virulence. These studies verify that XP_748409 is a secreted protein and underline its importance as potential adhesin.

Extracellular conserved serine-rich protein binds to surfactant protein A

To validate the binding of XP_748409 to one of the immune molecules, we expressed and isolated the full-length recombinant His-tagged XP_748409 (rXP_748409 17-198)

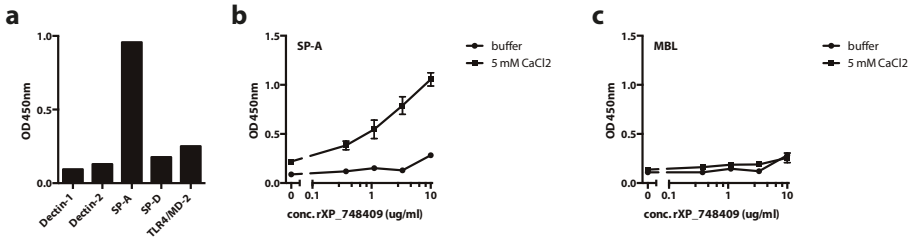

Figure 4 • XP_748409 binds SP-A

(**a**) An ELISA was performed to validate the interaction between selected phage and one of the immune molecules. Dectin-1, dectin-2, SP-A, SP-D and TLR4/MD-2 were coated on an ELISA plate. Binding of recombinant protein rXP_748409 17-198 (10 ug/ml) in the presence of 5 mM $CaCl_2$ was investigated and detected by an anti-His antibody. A representative graph is shown. (**b, c**) SP-A or MBL was coated on an ELISA plate. Binding of different concentrations of rXP_748409 17-198 was investigated in the absence or presence of 5 mM $CaCl_2$. Binding was detected by an anti-His antibody. The graphs show the mean ± SEM of 3 independent experiments.

and tested it for binding to the immune molecules. rXP_748409 17-198 did interact with SP-A, but not with dectin-1, dectin-2, SP-D or TLR4/MD-2 (Figure 4a). The binding of rXP_748409 17-198 to SP-A was dependent on the presence of calcium (Figure 4b). As SP-A and SP-D, Mannose Binding Lectin (MBL), belongs to the family of collectins. MBL is structural homologous to SP-A and also contains a carbohydrate recognition domain (CRD), hence the name collectin. MBL recognizes sugar moieties on the microbial surface via its CRD in a calcium-dependent manner and induce activation of the lectin complement pathway[35]. Since MBL has a similar structure and a comparable recognition of substrates as SP-A, XP_748409 binding to MBL was investigated. No binding of rXP_748409 17-198 was observed to MBL (Figure 4c). These results showed that the binding of XP_748409 to SP-A is specific.

Extracellular conserved serine-rich protein compete with SP-A binding to conidia

To elucidate the function of XP_748409, we investigated the binding of SP-A to *A. fumigatus*. SP-A did bind to dormant conidia in a dose-dependent manner (Figure 5). This is in accordance with literature, where SP-A binds dormant conidia in a calcium dependent manner[3] SP-A binding results in enhanced phagocytosis and killing of *A. fumigatus* conidia by phagocytes. Here, we found an *A. fumigatus* protein that does bind SP-A in the presence of calcium. This suggests that the protein bind SP-A via its CRD. SP-A is known to bind primarily mono- and oligosaccharides via its CRD[36]. However, SP-A also recognizes protein ligands of respiratory syncytial virus and *Haemophilus influenza* in a calcium dependent manner[37,38]. To examine if the extracellular conserved serine-rich protein interferes with SP-A binding to dormant conidia, a soluble form of XP_748409 (rXP_748409 17-109) was expressed and isolated and investigated for the inhibition of SP-A binding to dormant conidia. Addition of rXP_748408 17-109 to dormant conidia reduced the amount of SP-A deposition on dormant conidia (Figure 5). This indicated that XP_748409 could be a new ligand for SP-A mediated phagocytosis.

SP-A deficient mice are used to study the importance of SP-A in microbial clearance. SP-A deficient mice have normal lung function and similar levels of SP-D compared to wild type mice[39]. SP-A deficient mice are more susceptible to several viral, bacterial and fungal pathogens[40-48]. In contrast, immunosuppressed SP-A deficient mice

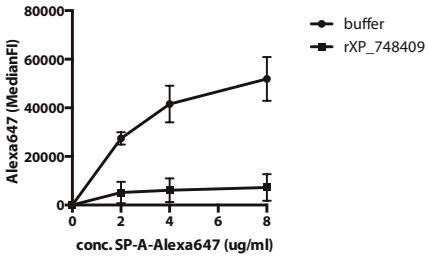

Figure 5 • XP_748409 inhibits binding of SP-A to dormant conidia
Dormant conidia were incubated with Alexa647-labeled SP-A in the absence or presence of 20 ug/ml rXP_748409 17-109. Binding of Alexa647-labeled SP-A was detected by flow cytometry. The mean ± SEM of three independent experiments was shown.

show reduced mortality after intranasal challenge with *A. fumigatus* than wild type mice[4]. This suggests that the presence of SP-A in the lung is beneficial for *A. fumigatus* invasion. It could be that *A. fumigatus* utilize SP-A for adherence to lung epithelium and thereby increase its potency to establish invasive growth. In that case, XP_748409 would act as an adhesion molecule that binds to SP-A and thereby promotes *A. fumigatus* infection.

Here, we identified a SP-A binding protein XP_748409 of *A. fumigatus* via a phage display approach. XP_748409 interferes with SP-A binding on dormant conidia. Further research needs to be performed to elucidate if XP_748409 is a SP-A ligand inducing phagocytosis or an adhesion molecule facilitating *A. fumigatus* infection.

METHODS

Fungal strains and culture conditions

The genome sequenced *A. fumigatus* strain Af293 was used to construct the secretome phage display library[49]. Af293 was cultured on minimal medium agar plates supplemented with 1% of glucose at 37°C. After 2 days, dormant conidia were isolated with PBS-Tween 0.05%. To obtain mycelium for RNA isolation, fresh isolated conidia were inoculated in RPMI supplemented with 0.5% glucose and grown on a shaking platform (250 rpm) at 37°C for 24 and 48 hours. Mycelium was harvested, washed with 0.9% NaCl solution, and frozen with liquid nitrogen and stored at -80°C until further use.

Construction of the secretome phage display library

Mycelium was ground using a Tissue Lyzer (Qiagen) and total RNA was isolated with TRIzol reagent following manufacturer's protocol (15596-018, Invitrogen). Messenger RNA (mRNA) was purified from total RNA using a Nucleotrap mRNA kit (740655, Macherey-Nagel). Purified mRNA was pooled and concentrated using an ethanol precipitation. First and second strand complement DNA (cDNA) was synthesized using RevertAid H Minus First Strand cDNA synthesis kit (K1631, Thermo Scientific). To obtain DNA fragments containing particularly 5' parts of the mRNA, cDNA synthesis was performed with random hexamer primers. After purification of double stranded DNA (dsDNA) with QIAquick PCR Purification kit (28106, Qiagen), small DNA fragments were excluded with ChromaSpin-400 columns (636076, Clontech). DNA fragments were phosphorylated with T4 polynucleotide kinsase (EK0031, Thermo Scientific) to allow efficient ligation. Ligation of the DNA inserts (7,2 ug) into the phagemid vector pDJ01 (1ug) and transformation of the ligation products into TG1 *E. coli* was performed as previously described[31]. After an overnight incubation, 173 clones were selected for colony PCR and sequencing. Identification of the inserts was performed as described[31]. The presence of a signal sequence was predicted using SignalP 4.1 and TOPCONS 2.0[50,51]. To construct a phage library from the cDNA library, phage production was performed as described[31].

Panning procedures

The phage library was panned against an anti-Myc-tag antibody (ab9106, Abcam) and a mix of dectin-1, dectin-2, SP-A, SP-D and the extracellular domain of Toll-like receptor 4 in complex with MD-2. Recombinant human dectin-1 (1859-DC-050, R&D systems)

and dectin-2 (3114-DC-050, R&D systems) were commercially purchased. Human SP-A and SP-D were isolated from patients with alveolar proteinosis and were a kind gift of Henk Haagsman[36]. TLR4/MD-2 was a kind gift of Toshiyuki Shimizu. Five wells of an ELISA plate (Maxisorb, Nunc) were coated with 5 ug/ml anti-Myc-tag antibody or a mix of proteins with 10 ug/ml of each protein in a 0,1 M Na_2CO_3 buffer and incubated at 4°C overnight. The panning procedure was performed as described earlier with minor adaptations[31]. In the case of the protein mix, the phage library was supplemented with 5 mM $CaCl_2$ during the bindings process. After incubation, bound phages were eluted with 0,2 M glycine-HCl pH 2.2 for 15 minutes and neutralized with 1 M Tris pH9.1. To identify displayed proteins, the phage library was panned against anti-Myc-tag antibody for 2 rounds. After panning, 174 clones were selected and identification of the insert was done by sequencing as described above. To identify proteins interacting with dectin-1, dectin-2, SP-A, SP-D and TLR4/MD-2, the phage library was panned against the protein mix for 4 rounds. After panning, 48 clones were selected for sequencing.

Cloning and expression of recombinant XP_748409

A full-length recombinant XP_748409 (assigned as rXP_748409 17-198), lacking the signal sequence (first 16 amino acids) and the propeptide (amino acid 199 - 219) was expressed in *E. coli*. However, the recombinant protein had some solubility issues, probably caused by the absence of the predicted O-glycosylation sites at the serine-rich region (using NetOglyc 4.0 Server[52]). The serine-rich region was thought to anchor XP-748409 into the cell wall after phospholipase C cleavage of the GPI anchor and to be not important for SP-A binding. Therefore, we also expressed a recombinant XP_748409 from amino acid 17 – 109 (assigned as rXP_748409 17-109), lacking the signal sequence, the serine-rich region (amino acid 114 - 192) and the propeptide. XM_743316 was amplified by PCR using Phusion High-Fidelity DNA polymerase (F530, Thermo Scientific) using a forward primer 5'-ATAT*AGATCT*GACGATGACGATAAGCTGTCCATCACCTCTCCCAAGAAG containing an enterokinase site (underlined) and a reverse primer 5'-ATAT*GCGGCCGC*CTAGTTGGGGGTGGT GGTGGG (for rXP_748409 17-198) or 5'-ATAT*GCGGCCGC*CTACTGCTGCGACTGGGCGAGG (for rXP_748409 17-109) on cDNA of *A. fumigatus*. PCR fragments were isolated from 1% agarose gel, purified with GeneJET Gel Extraction Kit (K0692, Thermo Scientific) and digested with BglII and NotI (restriction sites indicated in primer sequence in italics). Digested PCR fragments were cloned into a slightly modified pRSETB vector (Invitrogen), encoding a N-terminal six-residue histidine tag, using T4 DNA ligase (10481220001, Roche) and transformed into TOP10F' *E. coli*. Clones were selected and the correct sequence was verified. The expression vectors pRSETB-rXP_748409 17-198 and pRSETB-rXP_748409 17-109 were transformed into Rosetta-Gami(DE3)pLysS *E. coli* (71057, Novagen). Expression of the recombinant proteins was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (11411446001, Roche) for 4 hours. The recombinant proteins were isolated under native conditions using a Ni^{2+}-charged HiTrap Chelating HP column (17-0408-01, GE Healthcare). Bound protein was eluted by a 200 mM imidazole gradient and dialyzed against PBS. Purity of the recombinant proteins was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and concentration was measured by optical density at 280 nm. Proteins were stored at -20°C.

ELISA

To validate the binding of the phage displayed protein to the mix of immune proteins, rXP_748409 binding was assessed by ELISA. ELISA plates (Maxisorb, Nunc) were coated with dectin-1, dectin-2, SP-A, SP-D, TLR4/MD-2 and MBL (5 ug/ml) in a 0,1 M Na_2CO_3 buffer and incubated at 4°C overnight. Plates were washed with PBS-T 0,05% and blocked with 4% BSA-PBS-T at 37°C for 1 hour. After washing, plates were incubated with different concentrations of rXP_748409 17-198 in the presence or absence of 5 mM $CaCl_2$ in 1% BSA-PBS-T at 37°C for 1 hour. After washing, plates were incubated with 1 ug/ml monoclonal mouse anti-His6-Tag antibody (5H1, Hytest) at 37°C for 1 hour, washed, and incubated with 1 ug/ml goat-anti-mouse-HRP at 37°C for 1 hour. After washing, peroxidase activity was detected by the addition of 100 µg/ml tetramethylbenzidine and 60 µg/ml ureum peroxide in 100 mM sodium acetate buffer at pH 6.0 and the reaction was stopped with H_2SO_4. Optical density at 450 nm was measured using a microplate reader (Bio-Rad).

SP-A labeling and binding

To measure SP-A binding to dormant conidia by flow cytometry, SP-A was buffer exchanged from a 5 mM Tris-HCl pH 7.0 solution to a 0,1 M Na_2CO_3 solution using buffer exchange spin columns and was subsequently labeled with an Alexa Fluor 647 Monoclonal Antibody Labeling Kit following manufacturer's procedure (A20186, Invitrogen). After labeling, Alexa647-labeled SP-A was buffer exchanged to 5 mM Tris-HCl pH 7.0. Alexa647-labeled SP-A was incubated with 5 x 10^6 conidia/ml in a 5 mM Tris buffer supplemented with 5 mM $CaCl_2$ in the presence or absence of 20 ug/ml rXP_748409 17-109 shaking (1000 rpm) at 4°C for 1 hour. Alexa647-labeled SP-A binding was measured by flow cytometry (BD FACSVerse).

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Ch5

Glucocorticoids exposure enhances growth and alters *Aspergillus fumigatus* gene expression

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ABSTRACT

Background

The use of glucocorticoids in hematological patients is associated with increased risk of invasive aspergillosis. This has generally been attributed to the immunosuppressive effects of glucocorticoids. Glucocorticoids exposure has also direct effects on the growth of *Aspergillus fumigatus*. We studied the effect of dexamethasone, methylprednisolone and hydrocortisone on different *Aspergilli* species.

Results

Methylprednisolone and hydrocortisone exposure resulted in an enhanced growth of *A. fumigatus*. In contrast, the growth of *A. niger* and *A. tubingensis* was reduced upon methylprednisolone and hydrocortisone exposure. Methylprednisolone exposure resulted in the *A. fumigatus* colony with browner pigmentation. To identify the genes that were responsible for the observed phenotypic changes in *A. fumigatus* after methylprednisolone exposure, we performed a transcriptome analysis by RNA sequencing. The expression of 400 genes and 502 genes was ≥ 2 fold up-regulated and down-regulated, respectively, upon exposure to methylprednisolone. Ten genes encoding proteins that modify the fungal cell wall carbohydrates were differentially expressed. Methylprednisolone exposure altered the expression of genes that are associated with virulence. The expression of four hydrophobin genes, including *rodA* and *rodB,* was induced upon methylprednisolone exposure. The expression of melanin synthesis genes was altered confirming the change of colony pigmentation. Also the expression of genes responsible for secondary metabolite production, like a siderophore biosynthesis gene and gliotoxin biosynthesis genes, were differentially expressed.

Conclusion

Methylprednisolone and hydrocortisone enhances the growth of *A. fumigatus*, but reduces the growth of *A. niger* and *A. tubingensis*. The expression of several genes, which are involved in virulence, is altered upon methylprednisolone exposure. The response of *A. fumigatus* on the exposure of methylprednisolone could increase fungal virulence.

BACKGROUND

Aspergillus fumigatus is an airborne fungal pathogen and a major cause of invasive aspergillosis (IA) in immunocompromised patients. The incidence of IA in patients with hematological malignancies, treated with transplantations and/or chemotherapy, reaches up to 14%[1,2]. Since neutrophils are the key effectors cells to clear *A. fumigatus* infections, prolonged neutropenia and neutrophil dysfunction are major risk factors in the development of invasive aspergillosis [3-6]. Some other risk factors are underlying disease, older age, graft-versus-host disease (GVHD), *Cytomegalovirus* reactivation and prolonged glucocorticoids usage. The use of glucocorticoids is particularly associated with the development of IA with a late onset[7-10].

Glucocorticoids have a large impact on the function of the immune system and suppress the immune response against *A. fumigatus*. Glucocorticoids are used to prevent rejection of the transplant and to treat GVHD in hematological patients. Glucocorticoids reduce numbers and functions of monocytes and lymphocytes. Phagocytes, which are crucial in controlling *A. fumigatus* infections, are also affected by glucocorticoids. Glucocorticoids suppress the phagocytosis and killing capacity of phagocytes. Moreover, the production of cytokines and chemotaxis are impaired. Eventually, the immune suppressive effects of glucocorticoids do result in enhanced susceptibility to fungal infections[11,12].

A previous study suggests that the immune suppressive effects initiated by glucocorticoids use are not the only explanation for the increased risk of IA. Hydrocortisone enhanced the growth of *A. fumigatus* and not that of *A. oryzae* or *A. niger*[13]. Although this finding has potential clinical value for glucocorticoid-treated patients, the effect of glucocorticoid exposure on fungal gene expression was not investigated.

Here, we studied the phenotypic response of *Aspergilli* species on the exposure to dexamethasone, hydrocortisone and methylprednisolone. The exposure of *A. fumigatus* Af293 to methylprednisolone resulted in the greatest effect on growth. We performed transcriptome analysis to investigate the influence of methylprednisolone exposure on fungal gene expression. Methylprednisolone exposure resulted in alterations in the expression of genes related to cell wall morphogenesis, melanin and gliotoxin biosynthesis. Especially, the expression of hydrophobin genes was up-regulated. Since hydrophobins, melanin and gliotoxin are associated with virulence[14], the glucocorticoid-induced alterations in their gene expression could influence *A. fumigatus* pathogenesis.

RESULTS AND DISCUSSION

Effects of glucocorticoids on *Aspergillus* micro-colony size

To study the effect of glucocorticoid treatment on the growth of *Aspergilli*, several *Aspergillus* strains were grown in the presence of dexamethasone, hydrocortisone or methylprednisolone. Incubation with hydrocortisone or methylprednisolone resulted in significantly larger micro-colonies of *A. fumigatus* Af293 compared to the untreated control (Figure 1a). Although dexamethasone has increased anti-inflammatory and immunosuppressive properties there appears to be no effect on fungal growth of Af293 at concentrations tested. The size of micro-colonies of *A. fumigatus* T33439 was increased in the presence of all three glucocorticoids tested (Figure 1b). The size

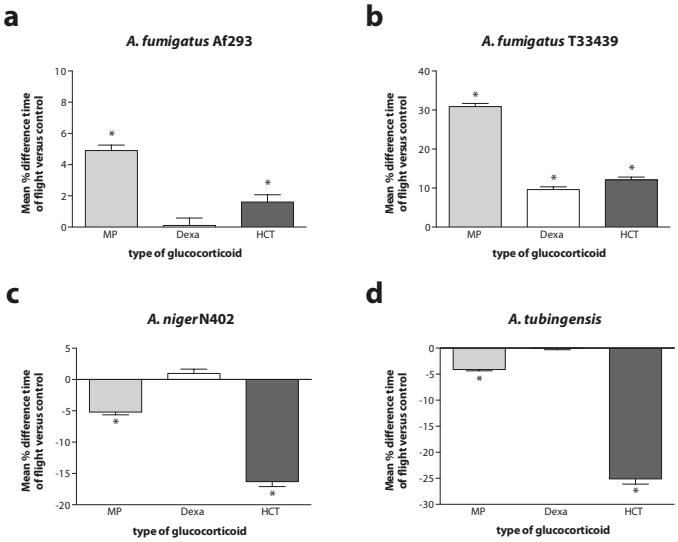

Figure 1 • Micro-colony size of Aspergilli species after glucocorticoid exposure
Freshly isolated conidia from *A. fumigatus* Af293 (**a**), *A. fumigatus* T33439 (**b**), *A. niger* N402 (**c**) and *A. tubingensis* (**d**) were grown in a liquid shaken culture in the absence or presence of dexamethasone (Dexa), hydrocortisone (HCT) and methylprednisolone (MP) for 16 hours. Micro-colonies were isolated and the time of flight was measured by COPAS. Experiments were performed at least three times and the data was pooled. The mean difference between the time of flight of glucocorticoid-exposed micro-colonies and the time of flight of non-exposed micro-colonies is expressed in percentage ± SEM. * $p < 0.05$.

of micro-colonies of *A. niger* and *A. tubingensis* was decreased when incubated with hydrocortisone and methylprednisolone (Figure 1c, d). Incubation with dexamethasone did not have any effect on *A. niger* nor on *A. tubingensis*. The enhanced growth of *A. fumigatus* upon hydrocortisone exposure was in line with a previous study, where the growth rate of single *A. fumigatus* hyphae was increased[13]. In contrast to our observations, Ng *et al.* observed no difference in growth rate of *A. niger* exposed to hydrocortisone. This discrepancy could be explained by the difference in the used *A. niger* strain or the experimental procedure. Of interest, the effects of glucocorticoids were inverted between strains belonging to the *Aspergillus* section *Fumigati* (both *A. fumigatus* strains) and strains belonging to the *Aspergillus* section *Nigri* (*A. niger* and *A. tubingensis*). Where hydrocortisone and methylprednisolone enhanced the growth of both *A. fumigatus* strains, both glucocorticoids reduced the growth of *A. niger* and *A. tubingensis*. The enhanced growth of *A. fumigatus* on glucocorticoids exposure could be one of the reasons of the increased virulence of *A. fumigatus* compared to other *Aspergilli* species.

A. fumigatus transcript profile during methylprednisolone treatment

Since we observed phenotypic changes and the greatest effect on growth of *A. fumigatus* Af293 after methylprednisolone treatment, we investigated the effects of methylprednisolone on the expression of genes of *A. fumigatus* Af293 by RNA sequencing. In total 9030 out of 9887 genes were expressed in untreated and/or

methylprednisolone treated micro-colonies. Of these genes, 221 were solely expressed in the condition with methylprednisolone, while 237 genes were exclusively expressed in the untreated micro-colonies. In total 400 genes were significantly ≥ 2-fold up-regulated in methylprednisolone-treated micro-colonies compared to untreated colonies (Supplemental table 1). A total of 502 genes were found to be ≥ 2-fold down-regulated in methylprednisolone-treated micro-colonies compared to untreated colonies (Supplemental table 2).

Changes in the expression of cell wall associated genes

The *Aspergillus* fungal cell wall is composed of several carbohydrates, e.g. α-(1,3)-glucan, β-(1,3)-glucan, chitin and galactomannan. These carbohydrates are linked to each other to form a rigid and flexible cell wall structure. Synthesis and modification of the cell wall are essential for growth of the fungus. Several enzymes are involved in these processes. Since we observed the enlargement of the micro-colonies upon treatment with methylprednisolone, we expected that genes related to cell wall morphogenesis would be differentially expressed. The expression of most of the genes was not changed (Supplemental table 3). Four genes encoding for proteins that are responsible for α-(1,3)-glucan modifications were differentially expressed (Figure 2). The expression of two β-(1,3)-glucanase genes was up-regulated, while glucanosyltransferase gene *gel6*, responsible for the elongation of β-(1,3)-glucan side chains, was down-regulated. Transcripts of a chitinase gene were less abundant and the expression of two chitinase genes was lost upon methylprednisolone treatment. No changes were observed in the expression of genes related to the synthesis of galactomannan or the cross-linking of the different carbohydrates.

To interpret the dataset obtained after the RNA sequencing, over-represented Gene Ontology (GO) terms with differential expressed genes were determined (Supplemental table 4). GO analysis of cellular components showed that the up-regulated genes were over-represented with GO term intrinsic to membrane (Table 1).

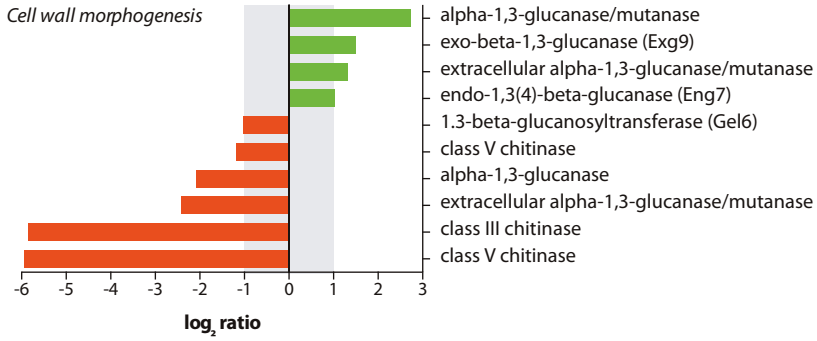

Figure 2 • Differential expression of genes related to cell wall morphogenesis
List of all genes that are related to the cell wall morphogenesis and are differentially expressed upon methylprednisolone are shown. Gene expression was considered differential if the expression between the groups has a $\log_2$ ratio of ≤ -1 (red, down-regulated upon methylprednisolone exposure) or ≥ 1 (green, up-regulated upon methylprednisolone exposure) and a false discovery rate (FDR) of ≤ 0,001. The protein annotation and/or protein name are plotted on the y-axis.

Table 1 • Over-represented GO terms of cellular components amongst genes with increased gene expression in methylprednisolone-treated samples

Accession	GO term	List hits	List total	Population hits	Population total	p-value
GO:0031224	intrinsic to membrane	26	48	618	2482	1,70E-04
GO:0016020	membrane	28	48	761	2482	8,90E-04
GO:0044425	membrane part	26	48	678	2482	1,04E-03
GO:0005618	cell wall	3	48	9	2482	8,41E-03
GO:0030312	external encapsulating structure	3	48	15	2482	4,20E-02

The population total illustrates the amount of genes that are mapped to any term in the cellular component, which are present in the *A. fumigatus* Af293 genome. The population hits illustrates the amount of genes that are mapped to the particular GO term, which are present in the *A. fumigatus* Af293 genome. The list total depicts the amount of genes that are mapped to any term in the cellular component, which have increased expression upon methylprednisolone treatment. The list hits illustrates the amount of genes that are mapped to the particular GO term, which have increased expression upon methylprednisolone treatment.

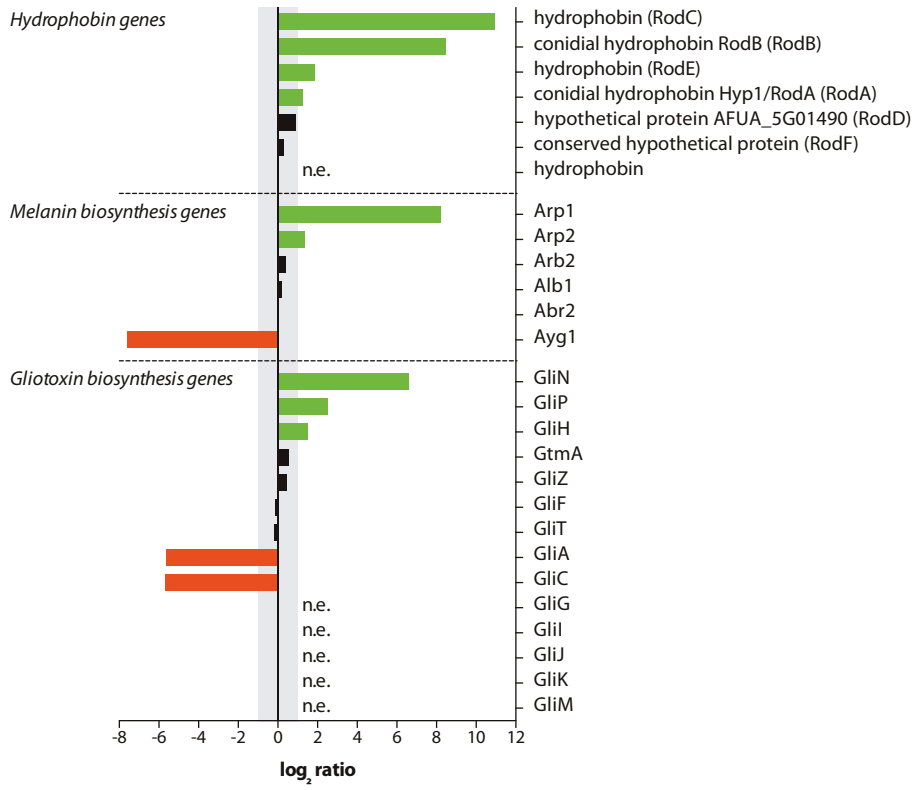

Figure 3 • Differential expression of genes related to hydrophobins, melanin biosynthesis and gliotoxin biosynthesis

List of all genes that are related to hydophobins, melanin biosynthesis and gliotoxin biosynthesis are shown. Gene expression was considered differential if the expression between the groups has a $\log_2$ ratio of ≤ -1 (red, down-regulated upon methylprednisolone exposure) or ≥ 1 (green, up-regulated upon methylprednisolone exposure) and a false discovery rate (FDR) of ≤ 0,001. Also genes that were not differentially expressed between the conditions (black) or not expressed in both conditions (n.e.) are shown. The protein annotation and/or protein name are plotted on the y-axis.

Most of the genes were related to metabolism of the fungus, like sugar transporters and amino acid permeasas (Supplemental table 5). GO analysis also revealed three genes annotated to the cell wall that were up-regulated. Of interest, these genes encode for hydrophobin proteins. Hydrophobins are small amphipathic molecules, which can form amyloid-like structures. The immunological inert hydrophobin RodA covers the conidia cell wall and thereby prevents recognition and killing of *A. fumigatus* by macrophages and dendritic cells[15-17]. RodB has been studied, however its function remains unclear[17]. Five other hydrophobin genes have been identified in the genome[18,19]. Expression of four hydrophobin genes, including *rodA* and *rodB*, was up-regulated upon methylprednisolone treatment (Figure 3 and supplemental table 6). The other three hydrophobin genes were not expressed (one gene) or not differentially expressed (two genes). The up-regulation of hydrophobin genes could lead to decreased recognition by the immune system or more resistance against killing by phagocytes.

Melanin is another important cell wall component associated with resistance to host immune response. *A. fumigatus* produces dihydroxynaphthalene (DHN) melanin and pyomelanin[20,21]. DHN melanin is responsible for the bluish green pigmentation of the conidia and reduces complement activation and phagocytosis by neutrophils[22]. DHN melanin protects conidia from damage by hydrogen peroxide[23]. Moreover, *A. fumigatus* strains without pigmentation were less virulent compared to wild type strains in intravenous infection models[22-24]. Pyomelanin is formed by *A. fumigatus* hyphae and secreted in culture supernatant[20]. Although pyomelanin is protective against hydrogen peroxide, an *A. fumigatus* mutant strain that does not produce pyomelanin was not attenuated in virulence[20,25]. In *A. fumigatus,* a cluster of six genes are involved in the biosynthesis of DHN melanin[21]. In our RNA sequencing data, the expression of scytalone dehydratase *arp1* and tetrahydroxynaphthalene reductase *arp2* were up-regulated, while the expression of tetrahydroxynaphthalene hydrolase *ayg*1 was down-regulated upon exposure with methylprednisolone (Figure 3 and supplemental table 7). In line with gene deletion mutants of the melanin biosynthesis gene cluster, the color of the pigment of a colony changed to a browner phenotype when grown in the presence of methylprednisolone (Figure 4)[21]. In contrast to the melanin biosynthesis gene cluster, no differential expressing genes were found in the pyomelanin biosynthesis gene cluster.

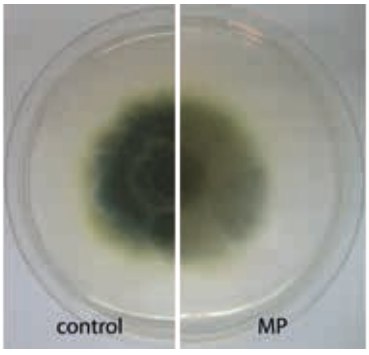

Figure 4 • Change in pigmentation of methylprednisolone exposed *A. fumigatus* colony
A. fumigatus Af293 was grown on minimal medium agar plates supplement without or with 0,5 μM methylprednisolone for 5 days. The assay was performed twice and a representative image is shown.

Changes in the expression of genes related to secondary metabolites

A. fumigatus produces several secondary metabolites, like siderophores and mycotoxins, which are important for virulence. Siderophores are essential in iron uptake from the extracellular milieu and are produced during iron limiting conditions[26]. Four genes, *sidA*, *sidF*, *sidD* and *sidG,* are responsible for the formation of extracellular siderophores [27]. The presence of *sidA*, *sidF* and *sidD* is essential to sustain full virulence, while deletion of *sidG* does not attenuate virulence of *A. fumigatus*[26,27]. Only *sidG* was strongly up-regulated upon exposure with methylprednisolone (Supplemental table 1). The other siderophore genes were not differentially expressed. The change in *sidG* expression could lead to an altered production of extracellular siderophores.

A. fumigatus can produce numerous mycotoxins. Gliotoxin is the most abundant toxin and important in the pathogenesis of *A. fumigatus*. Gliotoxin induces apoptosis in monocytes, macrophages, dendritic cells and T cells, and inhibits the function of mast cells and cytotoxic T cells[28-35]. Moreover, gliotoxin specifically suppress the function of T and B cells by inhibiting NF-κB-dependent signaling[36]. Neutrophils are resistant to gliotoxin-mediated apoptosis, but are inhibited in reactive oxygen species production and phagocytosis activity[37-40]. In the genome of *A. fumigatus*, a cluster of thirteen genes is responsible for the biosynthesis of gliotoxin. GliZ is the regulator of the gliotoxin biosynthesis cluster. Deletion of the *gliZ* gene did abolish gliotoxin production, but did not alter virulence in a neutropenic mouse model[41]. GliP is a nonribosomal peptide synthetase and catalyzes the first step in the gliotoxin biosynthesis[42]. Disruption of the *gliP* gene also eliminated the production of gliotoxin. This mutant was also not altered in virulence in a neutropenic mouse model[42,43]. However, in a corticosteroid immunosuppressed mouse model, not depleted from neutrophils, the *gliP* deletion strain was attenuated in virulence[40]. Methylprednisolone exposure of *A. fumigatus* increased the expression of *gliN*, *gliP* and *gliH*, while *gliA* and *gliC* were down-regulated (Figure 2 and supplemental table 8). Three genes (*gliF*, *gliT* and *gliZ*) were not differential regulated and five genes (*gliG*, *gliI*, *gliJ*, *gliK* and *gliM*) were neither expressed in methylprednisolone-exposed *A. fumigatus* nor in the control condition. Also, the *gtmA* gene, encoding a bis-thiomethyltransferase that diminishes the production of gliotoxin, was not differentially expressed. When gliotoxin will be synthesized, these changes in gliotoxin gene expression could alter the synthesis and levels of gliotoxin.

Glucocorticoids have numerous effects on the host immune system leading to increased risk of the development of invasive aspergillosis[11]. Here, we investigated the direct effect of glucocorticoids on *A. fumigatus* and showed that methylprednisolone induces phenotypic changes of *A. fumigatus*. In contrast, the *A. fumigatus* growth was not altered after dexamethasone exposure. Although the anti-inflammatory potency of dexamethasone is much stronger than hydrocortisone and methylprednisolone, the effect of glucocorticoid exposure to *A. fumigatus* growth is the opposite. Improved growth induced by methylprednisolone exposure was also observed in the plant pathogen and human opportunistic fungus *Exserohilum rostratum*[44]. Moreover, its virulence was enhanced in an immunocompetent fly infection model after methylprednisolone exposure. Although *A. fumigatus* responds on glucocorticoid exposure, a potential glucocorticoid receptor is not yet identified. No protein homologues to the human glucocorticoid receptor have been found in *A. fumigatus*. A glucocorticoid-binding

protein, unrelated to the steroid receptor gene superfamily, was identified in *Candida albicans*. However, its function and importance for virulence it not yet elucidated[45].

CONCLUSIONS

The use of glucocorticoids has been strongly associated with invasive aspergillosis[7-10]. The immune suppressive activity of glucocorticoids will be the major reason for the increased susceptibility. However, our study indicates that glucocorticoids also have their effects on *A. fumigatus* itself. The phenotypic effects, increased expression of hydrophobin genes and altered expression of melanin synthesis genes upon methylprednisolone exposure could influence *A. fumigatus* pathogenesis. Therefore, the direct effects of glucocorticoids on *A. fumigatus*, may contribute to its enhanced virulence compared to other *Aspergilli* species and affect pathogenesis and the development of invasive aspergillosis.

METHODS

Fungal strains and growth conditions

A. fumigatus Af293, *A. fumigatus* T33429, *A. niger* N402 and A. tubingensis[46] were used to study the effects of glucocorticoids on growth. All strains were cultured on minimal medium agar plates supplemented with 2% of glucose. After 2 days, conidia were isolated with PBS-Tween 0,05% and filtered using miracloth (475855, Calbiochem). To study the effects of glucocorticoids on micro-colony size, minimal medium, supplemented with 0.5 % yeast extract, 0.2% casamino acids and 2% glucose, either or not supplemented, with 0.5 μM dexamethasone (18660, Serva Electrophoresis), 1 μM hydrocortisone (331020, Brunschwig Chemie), or 0.5 μM methylprednisolone (sc-205749, Santa Cruz Biotechnology) was inoculated with 8 x 10^8 fresh isolated conidia. Cultures were grown in 100 ml medium in 500 ml Erlenmeyer flasks at 37°C shaking (200 rpm) for 16 hours. The glucocorticoid concentrations represented physiological levels in blood of patients treated with glucocorticoids at high dose[47].

Measurement of micro-colony size

Micro-colony sorting followed the protocol described earlier[48]. At least 500 micro-colonies were investigated for size, measured as time of flight (TOF), using a Complex Object Parametric Analyzer and Sorter (COPAS) Plus (Union Biometrica). Sorting parameters were set based on extinction values to exclude clusters of colonies or debris from the analysis (lower extinction thresholds are: Af293 > 250; T33429 > 500; N402 > 2000; A. tubingensis > 4000). Results of pooled data of at least three biological replicates are presented. Statistical significance was calculated on the pooled data with a t-test using SPSS.

RNA sequencing

To assess the effect of methylprednisolone on gene expression of *A. fumigatus* Af293, conidia were grown as described above. The experiment was performed in three biological replicates for both the non-treated culture and the methylprednisolone-treated culture. Fungal mycelium was harvested after 8 hours, washed with 0.8% NaCl

solution and snap-frozen in liquid nitrogen. Samples were transferred to a 2 ml tube containing two metal bullets and placed in a precooled adapter set of a TissueLyser (Qiagen). Samples were homogenized for 2 minutes at 20 Hz. Homogenized material was taken up in 1 ml TRIzol reagent (15596-018, Invitrogen) and thoroughly vortexed. RNA was purified following the instructions provided by the manufacturer. RNA purity and integrity were checked using an Agilent 2100 bioanalyzer (Agilent Technologies). The preparation of cDNA libraries, sequencing and analyses were performed by Beijing Genomics Institute (BGI) as previously described[49]. The raw Illumina sequencing data are deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/)[50] with accession number GSE46559. Gene expression in the biological replicates had at least a Spearman's correlation coefficient r > 0,96. The gene expression level was calculated using reads per kilo base per million reads (RPKM)[51]. Identification of differentially expressed genes (DEGs) was performed on the pooled data of the three samples treated with methylprednisolone versus the three untreated control samples, with correction for the total number of reads per group. Expression difference was considered differential when the average $\log_2$ ratio was ≤ -1 or ≥ 1 with a false discovery rate (FDR) of ≤ 0.001. Blast2GO was used to map the DEGs to gene ontology (GO) terms and perform GO enrichment analysis. A hypergeometric test with Bonferroni correction was used to find significantly enriched GO terms. GO terms having a corrected p-value ≤ 0,05 were considered to be enriched with DEGs.

Availability of supporting data

The data set supporting the results of this article is available in the NCBI's Gene Expression Omnibus repository, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46559.

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SUPPLEMENTAL TABLES

Supplemental table 1 • Genes with up-regulated gene expression levels after methylprednisolone treatment

log. Ratio	control (RPKM)	MP (RPKM)	p-value	FDR	GI number	Accession	Annotation
10,94	0,001	1,97	0	0	70983957	XP_747504.1	hydrophobin
10,50	0,001	1,45	0	0	70981512	XP_731538.1	conserved hypothetical protein
10,32	0,001	1,28	0	0	70991761	XP_750729.1	conserved hypothetical protein
10,09	0,001	1,09	0	0	70985508	XP_748260.1	conserved hypothetical protein
9,95	0,001	0,99	0	0	70984515	XP_747764.1	hypothetical protein AFUA_6G03120
9,85	0,001	0,92	0	0	70981620	XP_746339.1	hypothetical protein AFUA_4G01180
9,67	0,001	0,82	0	0	70995992	XP_752751.1	hypothetical protein AFUA_1G13810
9,59	0,001	0,77	0	0	71000914	XP_755138.1	hypothetical protein AFUA_2G08030
9,53	0,001	0,74	0	0	70986068	XP_748534.1	ion channel
9,51	0,001	0,73	0	0	70993562	XP_751628.1	hypothetical protein AFUA_4G12030
9,35	0,001	0,65	0	0	70988679	XP_749197.1	conserved hypothetical protein
9,33	0,001	0,64	0	0	70983021	XP_747038.1	hypothetical protein AFUA_8G01680
8,92	0,001	0,48	0	0	71001628	XP_755495.1	pheromone P-factor (Map2)
8,90	0,001	0,48	0	0	70990498	XP_750098.1	conserved hypothetical protein
8,84	0,001	0,46	0	0	146324558	XP_746881.2	hypothetical protein AFUA_7G00490
8,83	0,001	0,45	0	0	70981568	XP_746313.1	glutathione S-transferase family protein
8,80	0,001	0,45	0	0	70999093	XP_754268.1	toxin biosynthesis protein
8,72	0,001	0,42	0	0	70988631	XP_749174.1	GNAT family acetyltransferase
8,71	0,001	0,42	0	0	70985568	XP_748290.1	hypothetical protein AFUA_5G00490
8,64	0,001	0,40	0	0	70985350	XP_748181.1	hypothetical protein AFUA_5G01610
8,61	0,001	0,39	0	0	70997573	XP_753530.1	hypothetical protein AFUA_5G11180
8,50	0,001	0,36	0	0	70993244	XP_751469.1	hypothetical protein AFUA_4G13620
8,47	0,001	0,35	0	0	70996676	XP_753093.1	conidial hydrophobin RodB
8,46	0,001	0,35	0	0	70995259	XP_752391.1	hypothetical protein AFUA_1G10250
8,40	0,001	0,34	0	0	70999952	XP_754693.1	hypothetical protein AFUA_3G09270
8,38	0,001	0,33	0	0	146323968	XP_748244.2	DUF614 domain protein
8,38	0,001	0,33	0	0	71002322	XP_755842.1	O-methyltransferase
8,34	0,001	0,32	0	0	70991527	XP_750612.1	NmrA-like family protein
8,28	0,001	0,31	0	0	70983175	XP_747115.1	conserved hypothetical protein
8,27	0,001	0,31	0	0	70992111	XP_750904.1	hypothetical protein AFUA_6G10150
8,27	0,001	0,31	0	0	146324277	XP_731484.2	LysM domain protein
8,24	0,001	0,30	0	0	146324271	XP_731489.2	extracellular serine-rich protein
8,22	0,001	0,30	0	0	70996354	XP_752932.1	conserved hypothetical protein
8,21	0,001	0,30	0	0	70990306	XP_750002.1	hypothetical protein AFUA_1G02520
8,21	0,001	0,30	0	0	146323237	XP_748667.2	conserved hypothetical protein
8,21	0,001	0,30	0	0	71001440	XP_755401.1	hypothetical protein AFUA_2G10680
8,20	0,001	0,30	0	0	71002824	XP_756093.1	conidial pigment biosynthesis scytalone dehydratase Arp1
8,15	0,001	0,28	0	0	146324269	XP_001481440.1	secreted peptide
8,15	0,001	0,28	0	0	146324568	XP_001481418.1	hypothetical protein AFUA_7G00805
8,13	0,001	0,28	0	0	70982670	XP_746863.1	chitin binding domain protein Peritrophin-A
8,12	0,001	0,28	0	0	70985837	XP_748424.1	RTA1 domain protein
8,09	0,001	0,27	0	0	146324313	XP_747739.2	short-chain dehydrogenase/reductase
8,06	0,001	0,27	0	0	70981604	XP_746331.1	integral membrane protein
8,02	0,001	0,26	0	0	146324639	XP_747149.2	cytochrome P450 oxidoreductase
8,01	0,001	0,26	0	0	71002844	XP_756103.1	conserved hypothetical protein
7,98	0,001	0,25	0	0	70981959	XP_746508.1	amino acid permease
7,96	0,001	0,25	0	0	146323711	XP_752167.2	conserved hypothetical protein
7,96	0,001	0,25	0	0	70983826	XP_747439.1	Ankyrin domain protein
7,94	0,001	0,25	0	0	146322753	XP_001481644.1	cyanovirin-N family protein
7,91	0,001	0,24	0	0	70995960	XP_752735.1	hypothetical protein AFUA_1G13650
7,89	0,001	0,24	0	0	71002252	XP_755807.1	endo-arabinase
7,89	0,001	0,24	0	0	70985626	XP_748319.1	hypothetical protein AFUA_5G00210
7,88	0,001	0,24	0	0	71001184	XP_755273.1	cutinase
7,85	0,001	0,23	0	0	70986328	XP_748658.1	sugar O-acetyltransferase
7,85	0,001	0,23	0	0	70997673	XP_753574.1	conserved hypothetical protein
7,82	0,001	0,23	0	0	71001224	XP_755293.1	UDP-N-acetylglucosamine 1-carboxyvinyltransferase family protein
7,81	0,001	0,22	0	0	70998873	XP_754158.1	MFS transporter
7,79	0,001	0,22	0	0	71001838	XP_755600.1	hypothetical protein AFUA_2G12690
7,76	0,001	0,22	0	0	146323553	XP_746428.2	conserved hypothetical protein
7,74	0,001	0,21	0	0	70992467	XP_751082.1	rhamnosidase
7,73	0,001	0,21	0	0	70983965	XP_747508.1	conserved hypothetical protein
7,70	0,001	0,21	0	0	70983876	XP_747464.1	intermediate filament associated protein
7,64	0,001	0,20	0	0	146322954	XP_755614.2	UDP-glucosyl transferase family protein
7,63	0,001	0,20	0	0	71002842	XP_756102.1	hypothetical protein AFUA_2G17670
7,61	0,001	0,20	0	0	70981714	XP_746386.1	zinc-binding oxidoreductase CipB
7,61	0,001	0,19	0	0	70986294	XP_748641.1	conserved hypothetical protein
7,58	0,001	0,19	0	0	70985973	XP_748490.1	sterigmatocystin biosynthesis cytochrome P450 monooxygenase
7,57	0,001	0,19	0	0	70989874	XP_749786.1	hypothetical protein AFUA_1G00340
7,57	0,001	0,19	0	0	70993264	XP_751479.1	oxidoreductase, short-chain dehydrogenase/reductase family
7,55	0,001	0,19	0	0	70996692	XP_753101.1	dioxygenase
7,54	0,001	0,19	0	0	70983177	XP_747116.1	spherulin 4-like cell surface protein
7,50	0,001	0,18	0	0	70986282	XP_748635.1	glycerol dehydrogenase (GCY1)
7,49	0,001	0,18	0	0	70981438	XP_731501.1	conserved hypothetical protein
7,48	0,001	0,18	0	0	146323217	XP_748602.2	lipase/esterase
7,47	0,001	0,18	0	0	146322771	XP_001481649.1	hypothetical protein AFUA_2G02485
7,45	0,001	0,17	0	0	70981861	XP_746459.1	O-methylsterigmatocystin oxidoreductase
7,44	0,001	0,17	0	0	70994212	XP_751953.1	conserved hypothetical protein
7,43	0,001	0,17	0	0	70993002	XP_751349.1	transferase family protein
7,36	0,001	0,16	0	0	146323119	XP_748351.2	3-hydroxyacyl-CoA dehydrogenase
7,35	0,001	0,16	0	0	146322311	XP_749787.2	metalloreductase
7,34	0,001	0,16	0	0	71002764	XP_756143.1	conserved hypothetical protein
7,33	0,001	0,16	0	0	70986446	XP_748717.1	cytochrome b5 reductase
7,32	0,001	0,16	0	0	146323581	XP_746336.2	conserved hypothetical protein
7,32	0,001	0,16	0	0	146324085	XP_001481500.1	conserved hypothetical protein
7,30	0,001	0,16	0	0	70983267	XP_747161.1	conserved hypothetical protein
7,29	0,001	0,16	0	0	70981971	XP_746514.1	short-chain dehydrogenase/reductase family protein
7,28	0,001	0,16	0	0	70998893	XP_754168.1	dUTPase
7,26	0,001	0,15	0	0	70983253	XP_747154.1	cytochrome P450 oxidoreductase OrdA-like
7,25	0,001	0,15	0	0	70983305	XP_747180.1	cytochrome P450 monooxygenase
7,23	0,001	0,15	0	0	70983203	XP_747129.1	cytochrome P450
7,22	0,001	0,15	0	0	70997802	XP_753633.1	3-demethylubiquinone-9 3-methyltransferase
7,20	0,001	0,15	0	0	70999091	XP_754267.1	conserved hypothetical protein
7,16	0,001	0,14	0	0	70983652	XP_747353.1	integral membrane protein
7,13	0,001	0,14	0	0	70983227	XP_747141.1	glycosyl transferase
7,12	0,001	0,14	0	0	70993024	XP_751360.1	GNAT family acetyltransferase
7,11	0,001	0,14	0	0	70986094	XP_748546.1	conserved hypothetical protein
7,07	0,001	0,13	0	0	70981680	XP_746369.1	antigenic cell wall galactomannoprotein
7,06	0,001	0,13	0	0	146324627	XP_747185.2	cytochrome P450
7,04	0,001	0,13	0	0	71002796	XP_756079.1	2-amino-3-carboxymuconate-6-semialdehyde decarboxylase, putative
7,04	0,001	0,13	0	0	70982935	XP_746995.1	hypothetical protein AFUA_8G02110
7,01	0,001	0,13	0	0	70986082	XP_748540.1	conserved hypothetical protein
6,98	0,001	0,13	0	0	70986358	XP_748673.1	nitroreductase family protein
6,98	0,001	0,13	0	0	70981969	XP_746513.1	epoxide hydrolase
6,94	0,001	0,12	0	0	70982961	XP_747008.1	conserved hypothetical protein
6,92	0,001	0,12	0	0	146323010	XP_755785.2	hydrolase
6,89	0,001	0,12	0	0	70982905	XP_746980.1	neutral amino acid permease
6,88	0,001	0,12	0	0	70983890	XP_747471.1	hypothetical protein AFUA_8G06710
6,86	0,001	0,12	0	0	70987024	XP_748996.1	O-methyltransferase
6,83	0,001	0,11	0	0	70999073	XP_754258.1	Ctr copper transporter family protein
6,81	0,001	0,11	0	0	146322952	XP_755603.2	conserved hypothetical protein
6,80	0,001	0,11	0	0	70999079	XP_754261.1	conserved hypothetical protein

6,79	0,001	0,11	0	0	70983325	XP_747190.1	MFS transporter
6,78	0,001	0,11	0	0	70996957	XP_753233.1	Thi/Pfpl family protein
6,78	0,001	0,11	0	0	70986641	XP_748811.1	fumarylacetoacetate hydrolase family protein
6,76	0,001	0,11	0	0	70984781	XP_747897.1	endoglucanase
6,75	0,001	0,11	0	0	70992075	XP_750886.1	oxidoreductase, short chain dehydrogenase/reductase family
6,74	0,001	0,11	0	0	70988903	XP_749303.1	monooxygenase
6,74	0,001	0,11	0	0	70998845	XP_754144.1	pathogenesis associated protein Pep2
6,73	0,001	0,11	0	0	70986234	XP_748611.1	MFS sugar transporter
6,73	0,001	0,11	0	0	70997788	XP_753627.1	MFS multidrug transporter
6,72	0,001	0,11	0	0	70986382	XP_748685.1	GNAT family acetyltransferase
6,72	0,001	0,11	0	0	70981434	XP_731499.1	hypothetical protein AFUA_6G00560
6,71	0,001	0,10	0	0	146324493	XP_751089.2	nitrilase family protein
6,71	0,001	0,10	0	0	70983239	XP_747147.1	glutathione S-transferase
6,71	0,001	0,10	0	0	71000349	XP_754869.1	conserved hypothetical protein
6,71	0,001	0,10	0	0	70986694	XP_748837.1	endoglucanase
6,69	0,001	0,10	0	0	70992843	XP_751270.1	integral membrane protein
6,67	0,001	0,10	0	0	70989842	XP_749770.1	hypothetical protein AFUA_1G00180
6,67	0,001	0,10	0	0	71002336	XP_755849.1	choline transport protein
6,64	0,001	0,10	0	0	70996506	XP_753008.1	GPI anchored protein
6,64	0,001	0,10	0	0	70992141	XP_750919.1	conserved hypothetical protein
6,60	0,001	0,10	0	0	70992453	XP_751075.1	conserved hypothetical protein
6,60	0,001	0,10	0	0	70992025	XP_750861.1	methyltransferase GlnV
6,60	0,001	0,10	0	0	70987060	XP_749012.1	3-ketoacyl-acyl carrier protein reductase
6,58	0,001	0,10	0	0	146323201	XP_748566.2	hypothetical protein AFUA_3G02430
6,57	0,001	0,09	0	0	70992427	XP_751062.1	RTA1 domain protein
6,56	0,001	0,09	0	0	71002906	XP_756134.1	ergot alkaloid biosynthetic protein A
6,56	0,001	0,09	0	0	70992819	XP_751258.1	conserved hypothetical protein
6,55	0,001	0,09	0	0	146324475	XP_751046.2	FAD-dependent isoamyl alcohol oxidase
6,55	0,001	0,09	0	0	70996897	XP_753203.1	MFS monosaccharide transporter
6,53	0,001	0,09	0	0	146324083	XP_753944.2	HSP70 family protein
6,51	0,001	0,09	0	0	70981875	XP_746466.1	haemolysin-III family protein
6,47	0,001	0,09	0	0	70982027	XP_746542.1	hypothetical protein AFUA_4G03550
6,46	0,001	0,09	0	0	70985390	XP_748201.1	hypothetical protein AFUA_5G01410
6,45	0,001	0,09	0	0	70985422	XP_748217.1	oxidoreductase
6,43	0,001	0,09	0	0	70986199	XP_748594.1	MFS transporter
6,41	0,001	0,09	0	0	146323380	XP_754674.2	alpha/beta fold family hydrolase
6,41	0,001	0,09	0	0	70983277	XP_747166.1	DltD N-terminal domain protein
6,41	0,001	0,08	0	0	146324900	XP_001481436.1	conserved hypothetical protein
6,38	0,001	0,08	0	0	146324789	XP_747467.2	short-chain dehydrogenase/reductase family protein
6,35	0,001	0,08	0	0	70992073	XP_750885.1	conserved hypothetical protein
6,35	0,001	0,08	0	0	146323193	XP_001481591.1	conserved hypothetical protein
6,32	0,001	0,08	0	0	70981490	XP_731527.1	Nmr4-like family protein
6,24	0,001	0,08	0	0	146323145	XP_748441.2	integral membrane protein Pth11-like
6,23	0,001	0,08	0	0	70985745	XP_748378.1	conserved hypothetical protein
6,23	0,001	0,07	0	0	71001854	XP_755608.1	alpha-L-arabinofuranosidase
6,20	0,001	0,07	0	0	70996668	XP_753089.1	conserved hypothetical protein
6,20	0,001	0,07	0	0	70983796	XP_747424.1	hypothetical protein AFUA_8G06230
6,19	0,001	0,07	0	0	70998729	XP_754086.1	telomere-associated RecQ helicase
6,17	0,001	0,07	0	0	70981706	XP_746382.1	hypothetical protein AFUA_4G00740
6,16	0,001	0,07	0	0	70982718	XP_746887.1	hypothetical protein AFUA_7G00420
6,14	0,001	0,07	0	0	70983225	XP_747140.1	LPS glycosyltransferase
6,11	0,001	0,07	0	0	70983255	XP_747155.1	acetate-CoA ligase
6,09	0,001	0,07	0	0	71002626	XP_755994.1	short chain dehydrogenase/reductase
6,09	0,001	0,07	0	0	70982596	XP_746826.1	salicylate hydroxylase
6,07	0,001	0,07	0	0	70993128	XP_754412.1	conserved hypothetical protein
6,06	0,001	0,07	0	0	146324667	XP_747034.2	hypothetical protein AFUA_8G01720
6,06	0,001	0,07	0	0	146322710	XP_749198.2	GDSL-like lipase/acylhydrolase domain protein
6,02	0,001	0,07	0	0	70999902	XP_754668.1	3-oxoacyl-(acyl-carrier-protein) reductase
6,02	0,001	0,06	0	0	146323571	XP_746347.2	C6 and C2H2 transcription factor
6,02	0,001	0,06	0	0	71002852	XP_756107.1	PAF acetylhydrolase family protein
6,00	0,001	0,06	0	0	70999075	XP_754259.1	integral membrane protein
6,00	0,001	0,06	0	0	70992851	XP_751274.1	toxin biosynthesis protein Tri7-like
5,99	0,001	0,06	0	0	70992929	XP_751313.1	extracellular cellulose binding protein (Cip2)
5,99	0,001	0,06	0	0	70981536	XP_746297.1	hypothetical protein AFUA_4G01600
5,97	0,001	0,06	0	0	70997047	XP_753278.1	hypothetical protein AFUA_5G13760
5,92	0,001	0,06	0	0	70988665	XP_749191.1	hypothetical protein AFUA_2G00560
5,87	0,001	0,06	0	0	70981548	XP_746303.1	salicylate hydroxylase
5,85	0,001	0,06	0	0	70982744	XP_746900.1	cytochrome P450 monooxygenase
5,84	0,001	0,06	0	0	70983081	XP_747068.1	NB-ARC and TPR domain protein
5,82	0,001	0,06	0	0	70990110	XP_749904.1	endonuclease/exonuclease/phosphatase family protein
5,75	0,001	0,05	0	0	70986288	XP_748638.1	MFS multidrug transporter
5,74	0,001	0,05	0	0	146323207	XP_748590.2	C6 finger domain protein
5,73	0,001	0,05	0	0	70995488	XP_752499.1	glycosyl hydrolase family 88
5,71	0,001	0,05	0	0	146323583	XP_746335.2	GABA permease
5,70	0,001	0,05	0	0	146323601	XP_746456.2	metalloproteinase
5,67	0,001	0,05	0	0	146323587	XP_746325.2	C6 transcription factor
5,65	0,001	0,05	0	0	71002916	XP_756139.1	O-acetyltransferase
5,64	0,001	0,05	0	0	70984483	XP_747748.1	swollenin
5,63	0,001	0,05	0	0	70982576	XP_746816.1	cytochrome P450 alkane hydroxylase
5,61	0,001	0,05	0	0	70992849	XP_751273.1	acetyltransferase
5,60	0,001	0,05	0	0	70985364	XP_748188.1	MFS aflatoxin efflux pump
5,58	0,001	0,05	0	0	71002914	XP_756138.1	cytochrome P450 monooxygenase
5,58	0,001	0,05	0	0	70983201	XP_747128.1	sugar transporter family protein
5,57	0,001	0,05	0	0	70983832	XP_747442.1	MFS multidrug transporter
5,56	0,001	0,05	0	0	146322333	XP_749876.2	MFS transporter
5,56	0,001	0,05	0	0	70983309	XP_747182.1	cytochrome P450
5,56	0,001	0,05	0	0	146324868	XP_748888.2	serine protease
5,56	0,001	0,05	0	0	70981762	XP_746410.1	chlorohydrolase family protein
5,55	0,001	0,05	0	0	70981590	XP_746324.1	MFS monosaccharide transporter
5,52	0,001	0,05	0	0	70985715	XP_748363.1	high-affinity glucose transporter
5,51	0,001	0,05	0	0	146323245	XP_001481597.1	conserved hypothetical protein
5,41	0,001	0,04	0	0	70982572	XP_746814.1	extracellular lipase
5,38	0,001	0,04	0	0	70986108	XP_748552.1	arylsulfatase
5,31	0,001	0,04	0	0	70998452	XP_753948.1	hypothetical protein AFUA_5G06850
5,30	0,001	0,04	0	0	71002890	XP_756126.1	C6 transcription factor
5,29	0,001	0,04	0	0	70985606	XP_748309.1	flavin-containing monooxygenase
5,27	0,001	0,04	0	0	70992131	XP_750914.1	alkaline serine protease AorO
5,22	0,001	0,04	0	0	70983087	XP_747071.1	hypothetical protein AFUA_8G01350
5,21	0,001	0,04	0	0	70988579	XP_749150.1	FAD monooxygenase
5,21	0,001	0,04	0	0	70985863	XP_748437.1	iron-sulfur cluster-binding protein, rieske family domain protein
5,16	0,001	0,04	0	0	70986680	XP_748830.1	L-amino acid oxidase LaoA
5,15	0,001	0,04	0	0	70985570	XP_748291.1	exoinulinase InuD
5,14	0,001	0,04	0	0	70987089	XP_749026.1	hypothetical protein AFUA_7G04840
5,13	0,001	0,04	0	0	70983704	XP_747379.1	NACHT and Ankyrin domain protein
5,05	0,001	0,03	0	0	70989063	XP_749381.1	conserved hypothetical protein
5,00	0,001	0,03	0	0	70993138	XP_751417.1	Diacylglycerol acyltransferase family
4,97	0,001	0,03	0	0	70985434	XP_748223.1	conserved hypothetical protein
4,85	0,001	0,03	0	0	70996750	XP_753130.1	C6 transcription factor RegA
4,58	0,001	0,02	0	0	146324241	XP_001481522.1	Pfs, NACHT and Ankyrin domain protein
4,27	0,04	0,84	2,12E-13	3,16E-13	70989862	XP_749780.1	hypothetical protein AFUA_1G00280
4,11	0,001	0,02	0	0	70992991	XP_751344.1	telomere-associated RecQ helicase
3,49	0,10	1,07	2,12E-13	3,15E-13	71002196	XP_755779.1	oxidoreductase, short-chain dehydrogenase/reductase family
3,39	0,05	0,55	6,71E-13	9,87E-13	70993202	XP_751448.1	MFS multidrug transporter

3,15	0,14	1,21	5,15E-13	7,59E-13	70981688	XP_746373.1	MFS peptide transporter
2,78	0,12	0,83	0	0	70988683	XP_749199.1	conserved hypothetical protein
2,78	0,06	0,41	0	0	146323032	XP_001481680.1	citrate synthase
2,76	0,05	0,34	6,71E-13	9,82E-13	70983121	XP_747088.1	MFS sugar transporter
2,76	0,19	1,26	0	0	70981963	XP_746510.1	cell wall serine-threonine-rich galactomannoprotein Mp1
2,74	0,05	0,36	6,71E-13	9,87E-13	70989361	XP_749530.1	alpha-1,3-glucanase/mutanase
2,59	0,11	0,65	6,71E-13	9,86E-13	70992955	XP_751326.1	PTR family peptide transporter
2,59	0,15	0,90	0	0	70985286	XP_748149.1	hypothetical protein AFUA_5G01930
2,57	0,05	0,32	6,71E-13	9,86E-13	70996716	XP_753113.1	high affinity nitrate transporter NrtB
2,54	0,10	0,58	6,71E-13	9,85E-13	70985462	XP_748237.1	oxidoreductase, short-chain dehydrogenase/reductase family, putative
2,54	0,14	0,82	0	0	71001956	XP_755659.1	conserved hypothetical protein
2,53	0,08	0,46	2,12E-13	3,16E-13	146323543	XP_746433.2	oxidoreductase, 2OG-Fe(II) oxygenase family
2,49	0,01	0,07	6,71E-13	9,84E-13	70992013	XP_750855.1	nonribosomal peptide synthase GliP
2,43	0,28	1,53	0	0	70981949	XP_746503.1	conserved hypothetical protein
2,36	0,05	0,26	0	0	70993294	XP_751449.1	MFS multidrug transporter
2,30	0,04	0,19	2,12E-13	3,16E-13	70986060	XP_748530.1	short-chain dehydrogenase/reductase
2,27	0,13	0,62	5,88E-13	8,64E-13	70985486	XP_748249.1	transesterase (LovD)
2,27	0,19	0,90	0	0	146323177	XP_748519.2	DUF636 domain protein
2,26	0,06	0,30	6,71E-13	9,81E-13	70992053	XP_750875.1	hypothetical protein AFUA_6G09865
2,26	0,05	0,26	6,71E-13	9,81E-13	70992335	XP_751016.1	MFS transporter
2,26	0,44	2,12	6,71E-13	9,84E-13	70986715	XP_748847.1	hypothetical protein AFUA_7G06640
2,23	0,06	0,29	6,71E-13	9,85E-13	70985550	XP_748281.1	conserved hypothetical protein
2,22	0,64	2,97	2,12E-13	3,16E-13	70992289	XP_750993.1	conserved hypothetical protein
2,21	0,32	1,48	0	0	70989761	XP_749730.1	alkaline lipase
2,19	1,27	5,82	4,51E-12	6,42E-12	70984910	XP_747961.1	conidiation-specific protein Con-10
2,18	0,05	0,24	1,65E-12	2,39E-12	70997992	XP_753728.1	DEAD box helicase involved in nonsense mediated decay
2,15	0,05	0,20	1,22E-12	1,77E-12	146323444	XP_754409.2	fatty acid oxygenase PpoC
2,09	0,11	0,49	0	0	70994094	XP_751894.1	conserved hypothetical protein
2,06	0,14	0,59	0	0	70993668	XP_751681.1	hypothetical protein AFUA_4G11490
2,01	0,13	0,54	6,71E-13	9,83E-13	70988759	XP_749234.1	conserved hypothetical protein
2,00	0,06	0,26	2,12E-13	3,16E-13	70983840	XP_747446.1	Rieske 2Fe-2S family protein
1,98	0,07	0,28	6,71E-13	9,82E-13	70981436	XP_731500.1	glycosyl transferase family 8 family
1,97	0,80	3,13	1,08E-12	1,57E-12	70990082	XP_749890.1	hypothetical protein AFUA_1G01400
1,97	0,16	0,63	2,12E-13	3,16E-13	70996266	XP_752888.1	hypothetical protein AFUA_1G15190
1,95	0,04	0,15	0	0	146323948	XP_748329.2	squalene-hopene-cyclase
1,95	0,13	0,49	1,31E-14	1,97E-14	146323779	XP_751899.2	conserved hypothetical protein
1,95	0,07	0,28	6,71E-13	9,82E-13	70987152	XP_749056.1	short chain dehydrogenase/reductase family
1,95	0,08	0,29	6,71E-13	9,81E-13	70982945	XP_747000.1	glycan biosynthesis protein (PigL)
1,95	0,08	0,32	6,71E-13	9,84E-13	70983001	XP_747028.1	nitrilase
1,95	0,03	0,10	6,71E-13	9,81E-13	70996987	XP_753248.1	cell-associated beta-galactosidase
1,93	0,05	0,19	2,12E-13	3,16E-13	70996817	XP_753163.1	cell surface metalloreductase (FreA)
1,91	0,05	0,19	6,71E-13	9,85E-13	70992447	XP_751072.1	MFS alpha-glucoside transporter
1,91	0,19	0,70	0	0	146323221	XP_001481594.1	conserved hypothetical protein
1,91	0,21	0,80	6,71E-13	9,82E-13	70998690	XP_754067.1	hypothetical protein AFUA_5G05650
1,91	0,18	0,69	6,71E-13	9,87E-13	71002164	XP_755763.1	DUF636 domain protein
1,91	0,05	0,20	6,71E-13	9,85E-13	70984002	XP_747526.1	conserved hypothetical protein
1,90	0,29	1,06	2,12E-13	3,15E-13	70988735	XP_749223.1	hypothetical protein AFUA_2G00860
1,85	0,73	2,63	1,30E-12	1,88E-12	70983729	XP_747391.1	hydrophobin
1,84	0,29	1,04	0	0	70986672	XP_748826.1	phosphoserine phosphatase
1,79	0,29	1,01	5,88E-13	8,64E-13	70990198	XP_749948.1	IgE binding protein
1,79	1,01	3,47	0	0	70993690	XP_751692.1	hypothetical protein AFUA_4G11370
1,78	0,27	0,93	1,65E-12	2,38E-12	71000341	XP_754865.1	DRAP deaminase
1,76	0,22	0,75	0	0	71002138	XP_755750.1	hypothetical protein AFUA_2G14170
1,75	0,30	1,02	1,08E-12	1,57E-12	70985470	XP_748241.1	oxidoreductase, 2OG-Fe(II) oxygenase family
1,74	0,15	0,50	1,65E-12	2,38E-12	70988821	XP_749264.1	D-mandelate dehydrogenase
1,73	0,19	0,63	1,08E-12	1,57E-12	70988581	XP_749151.1	catecholamine-O-methyltransferase
1,71	0,20	0,64	2,08E-12	3,00E-12	146322751	XP_749273.2	GABA permease
1,68	0,04	0,12	0	0	70988637	XP_749177.1	amidase family protein
1,63	0,10	0,31	0	0	70995420	XP_752466.1	short chain oxidoreductase/dehydrogenase
1,62	0,06	0,19	2,12E-13	3,15E-13	70986558	XP_748770.1	ornithine decarboxylase
1,59	0,06	0,18	6,71E-13	9,83E-13	70983145	XP_747100.1	hypothetical protein AFUA_8G01060
1,59	0,06	0,18	6,71E-13	9,84E-13	71002910	XP_756136.1	dimethylallyl tryptophan synthase FgaPT1
1,59	0,06	0,19	6,71E-13	9,83E-13	70988986	XP_749797.1	N-acetylglucosamine-6-phosphate deacetylase (NagA)
1,59	0,05	0,15	0	0	71002918	XP_756140.1	catalase Cat
1,59	0,35	1,06	0	0	146324673	XP_001481396.1	hypothetical protein AFUA_8G01795
1,57	0,05	0,16	2,12E-13	3,16E-13	70983063	XP_747059.1	flavin containing amine oxidase
1,57	0,17	0,51	2,12E-13	3,15E-13	70985783	XP_748397.1	GNAT family N-acetyltransferase
1,54	0,06	0,19	6,71E-13	9,86E-13	70981865	XP_746461.1	MFS monocarboxylate transporter
1,54	0,13	0,39	1,65E-12	2,39E-12	70985642	XP_748327.1	capsule polysaccharide biosynthesis protein
1,54	0,02	0,05	0	0	70986586	XP_748784.1	conserved hypothetical protein
1,53	0,42	1,23	2,37E-13	3,51E-13	71002748	XP_756055.1	hypothetical protein AFUA_2G17210
1,53	0,12	0,35	5,15E-13	7,59E-13	70998318	XP_753881.1	conserved hypothetical protein
1,53	0,09	0,25	2,12E-13	3,16E-13	70988659	XP_749188.1	conserved hypothetical protein
1,53	0,42	1,21	2,12E-13	3,16E-13	70985472	XP_748242.1	hypothetical protein AFUA_5G00990
1,53	0,94	2,70	0	0	70995390	XP_752452.1	urea hydro-lyase/cyanamide hydratase
1,51	0,13	0,37	1,08E-12	1,57E-12	70992401	XP_751049.1	amidohydrolase
1,51	0,10	0,28	1,08E-12	1,57E-12	70997984	XP_753724.1	amidase
1,49	0,06	0,17	6,71E-13	9,87E-13	70986454	XP_748721.1	MFS transporter
1,49	0,23	0,66	6,71E-13	9,81E-13	70981885	XP_746471.1	hypothetical protein AFUA_4G02850
1,49	0,03	0,09	6,71E-13	9,85E-13	71002786	XP_756074.1	conserved hypothetical protein
1,49	0,04	0,11	6,71E-13	9,84E-13	70983812	XP_747432.1	Patatin-like serine hydrolase
1,49	0,06	0,16	6,71E-13	9,86E-13	70988729	XP_749220.1	short chain dehydrogenase/reductase
1,49	0,03	0,07	6,71E-13	9,86E-13	70988641	XP_749179.1	exo-beta-1,3-glucanase
1,49	0,26	0,73	6,71E-13	9,86E-13	70991359	XP_750528.1	mating alpha-pheromone PpgA
1,49	0,86	2,41	0	0	70989209	XP_749454.1	conserved hypothetical protein
1,49	0,15	0,42	6,71E-13	9,82E-13	146324463	XP_750864.2	conserved hypothetical protein
1,49	0,15	0,42	2,98E-13	4,41E-13	70992469	XP_751083.1	MFS sugar permease
1,49	0,30	0,84	1,08E-12	1,57E-12	70993846	XP_751770.1	conserved hypothetical protein
1,48	0,03	0,09	2,12E-13	3,16E-13	70992035	XP_750866.1	geranylgeranyl diphosphate synthase
1,48	0,04	0,12	2,12E-13	3,15E-13	70985484	XP_748248.1	amino acid permease
1,48	0,11	0,30	2,12E-13	3,15E-13	71002064	XP_755713.1	malate dehydrogenase
1,48	0,08	0,22	2,12E-13	3,16E-13	70986791	XP_748884.1	zinc-containing alcohol dehydrogenase
1,48	0,07	0,19	2,12E-13	3,16E-13	70993200	XP_751447.1	TfdA family oxidoreductase
1,46	0,26	0,73	0	0	70982712	XP_746884.1	conserved hypothetical protein
1,43	0,90	2,43	2,63E-11	3,60E-11	70981658	XP_746358.1	MFS quinate transporter
1,41	0,19	0,51	7,27E-13	1,06E-12	70982837	XP_746946.1	cytochrome P450 monooxygenase
1,41	0,29	0,76	2,98E-13	4,41E-13	146322398	XP_750086.2	conserved hypothetical protein
1,37	0,44	1,14	1,51E-12	2,18E-12	71000307	XP_754848.1	metallo-beta-lactamase superfamily protein
1,36	1,29	3,33	0	0	146322650	XP_001481730.1	DNA repair protein Dds20/MeiS
1,36	0,32	0,81	5,88E-13	8,64E-13	70993988	XP_751841.1	hypothetical protein AFUA_4G09850
1,35	0,30	0,76	0	0	70996807	XP_753158.1	hypothetical protein AFUA_5G14990
1,35	0,29	0,75	4,60E-14	6,89E-14	71002820	XP_756091.1	conidial pigment biosynthesis 1,3,6,8-tetrahydroxynaphthalene reductase Arp2
1,35	0,15	0,39	4,60E-14	6,89E-14	71002586	XP_756124.1	C6 finger domain protein
1,34	0,54	1,38	2,13E-12	3,07E-12	70995261	XP_752392.1	conserved hypothetical protein
1,34	0,72	1,83	6,24E-12	8,82E-12	146323911	XP_001481575.1	cell surface protein
1,33	4,80	12,09	0	0	70983955	XP_747503.1	conserved hypothetical protein
1,32	0,49	1,21	4,17E-12	5,95E-12	70986546	XP_748764.1	extracellular alpha-1,3-glucanase/mutanase
1,31	1,45	3,59	0	0	70986090	XP_748544.1	conserved hypothetical protein
1,27	0,12	0,30	1,31E-14	1,97E-14	70981718	XP_746388.1	aquaglyceroporin
1,26	0,78	1,87	1,83E-11	2,54E-11	70983301	XP_747178.1	F-box domain and ankyrin repeat protein
1,26	5,48	13,14	4,58E-12	6,51E-12	70997898	XP_753681.1	conidial hydrophobin Hyp1/RodA
1,26	0,14	0,34	0	0	71002188	XP_755775.1	cutinase
1,25	0,07	0,17	1,08E-12	1,57E-12	70981562	XP_746310.1	C6 finger domain protein

1,24	0,32	0,74	6,24E-12	8,82E-12	70985622	XP_748317.1	hypothetical protein AFUA_5G00230
1,24	0,30	0,70	2,37E-13	3,51E-13	70995335	XP_752425.1	GPI anchored protein
1,23	0,15	0,36	0	0	70983229	XP_747142.1	conserved hypothetical protein
1,22	0,43	1,00	1,08E-12	1,57E-12	71002170	XP_755766.1	conserved hypothetical protein
1,22	0,24	0,57	1,08E-12	1,57E-12	70990028	XP_749863.1	conserved hypothetical protein
1,22	3,05	7,09	7,63E-12	1,08E-11	70989053	XP_749376.1	hypothetical protein AFUA_2G02410
1,20	0,87	2,00	0	0	70984783	XP_747898.1	conserved hypothetical protein
1,20	0,60	1,37	3,53E-13	5,22E-13	70986244	XP_748616.1	allergen
1,20	0,64	1,46	7,07E-13	1,03E-12	70989397	XP_749548.1	hypothetical protein AFUA_2G04160
1,19	0,74	1,69	1,76E-12	2,54E-12	70991737	XP_750717.1	ankyrin repeat protein
1,18	0,31	0,70	4,69E-12	6,67E-12	146323551	XP_746430.2	MFS transporter
1,18	0,15	0,34	0	0	70982073	XP_746565.1	rhamnogalacturonase B
1,18	0,19	0,43	0	0	70989848	XP_749773.1	hypothetical protein AFUA_1G00210
1,18	0,20	0,45	0	0	70983574	XP_747314.1	MFS transporter
1,18	0,20	0,45	4,60E-14	6,88E-14	70994184	XP_751939.1	conserved hypothetical protein
1,17	0,30	0,68	8,32E-13	1,21E-12	70983943	XP_747497.1	ankyrin repeat protein
1,17	0,58	1,30	3,43E-12	4,92E-12	70986000	XP_748503.1	amino acid permease
1,16	0,53	1,18	5,96E-13	8,76E-13	70996801	XP_753155.1	arsenic methyltransferase Cyt19
1,16	0,45	1,00	5,26E-12	7,46E-12	70991435	XP_750566.1	l-fucose permease
1,15	0,96	2,13	0	0	70986584	XP_748783.1	heat shock transcription factor
1,15	0,14	0,31	0	0	71001188	XP_755275.1	alpha-N-acetylglucosaminidase
1,15	0,31	0,68	2,37E-13	3,51E-13	71001832	XP_755597.1	DUF636 domain protein
1,15	0,86	1,90	1,14E-11	1,60E-11	146324538	XP_751254.2	MFS multidrug transporter
1,14	0,30	0,67	2,53E-13	3,75E-13	70985781	XP_748396.1	short-chain dehydrogenase/reductase
1,14	0,81	1,78	0	0	70986092	XP_748545.1	DUF427 domain protein
1,13	2,03	4,42	1,64E-11	2,29E-11	70984441	XP_747727.1	conserved hypothetical protein
1,12	0,91	1,98	3,24E-12	4,64E-12	70992375	XP_751036.1	DUF636 domain protein
1,11	0,29	0,62	0	0	70981738	XP_746398.1	hypothetical protein AFUA_4G00580
1,10	0,53	1,14	1,47E-13	2,20E-13	70986022	XP_748513.1	conserved hypothetical protein
1,10	0,61	1,31	4,51E-12	6,42E-12	70982873	XP_746964.1	conserved hypothetical protein
1,10	0,24	0,52	3,24E-12	4,64E-12	70987032	XP_749000.1	glucuronyl hydrolase
1,10	0,33	0,70	0	0	70991799	XP_750748.1	protein kinase
1,09	0,43	0,92	0	0	70983027	XP_747041.1	hypothetical protein AFUA_8G01650
1,09	0,52	1,11	0	0	70986362	XP_748675.1	hypothetical protein AFUA_3G03550
1,09	0,44	0,94	4,92E-12	6,99E-12	70984012	XP_747531.1	alpha/beta hydrolase
1,09	1,45	3,09	4,31E-12	6,14E-12	70999049	XP_754246.1	conserved hypothetical protein
1,09	0,28	0,60	3,83E-12	5,48E-12	146324223	XP_753243.2	hypothetical protein AFUA_5G14140
1,08	1,40	2,96	9,67E-12	1,36E-11	70985841	XP_748426.1	conserved hypothetical protein
1,08	0,75	1,58	4,31E-12	6,14E-12	71001932	XP_755647.1	conserved hypothetical protein
1,07	0,23	0,48	1,47E-13	2,20E-13	70992845	XP_751271.1	FAD-dependent monooxygenase (PaxM)
1,07	1,12	2,35	3,07E-12	4,41E-12	70997205	XP_753356.1	hypothetical protein AFUA_5G12970
1,06	2,67	5,57	0	0	70989916	XP_749807.1	conserved hypothetical protein
1,06	0,36	0,74	4,53E-12	6,44E-12	70981430	XP_731497.1	ankyrin repeat protein
1,06	0,25	0,52	2,65E-12	3,82E-12	70998326	XP_753885.1	steroid monooxygenase (CpmA)
1,06	0,86	1,78	4,73E-12	6,71E-12	146324217	XP_001481519.1	glycoside hydrolase
1,05	0,80	1,66	2,28E-11	3,14E-11	70991110	XP_750404.1	MFS transporter
1,05	0,10	0,21	6,71E-13	9,85E-13	71002912	XP_756137.1	short chain dehydrogenase/oxidoreductase CpoX2
1,05	0,09	0,18	6,71E-13	9,83E-13	146324635	XP_747157.2	phytanoyl-CoA dioxygenase family protein
1,05	0,05	0,10	6,71E-13	9,83E-13	70984769	XP_747891.1	MFS lactose permease
1,04	2,39	4,92	7,70E-12	1,09E-11	70995690	XP_752600.1	hypothetical protein AFUA_1G12320
1,03	0,33	0,67	4,33E-12	6,17E-12	70992057	XP_750877.1	MFS monocarboxylate transporter
1,02	0,06	0,13	0	0	70998476	XP_753960.1	protein kinase
1,02	0,05	0,11	0	0	70986769	XP_748873.1	C6 transcription factor
1,02	0,07	0,14	0	0	70998883	XP_754163.1	extracellular dioxygenase
1,02	0,04	0,07	0	0	70983205	XP_747130.1	C2H2 transcription factor
1,02	0,09	0,18	0	0	70986272	XP_748630.1	endo-1,3(4)-beta-glucanase
1,02	0,09	0,19	1,22E-12	1,77E-12	70983173	XP_747114.1	UDP-glucose dehydrogenase Ugd1
1,02	0,27	0,55	0	0	70986468	XP_748728.1	hypothetical protein AFUA_3G04100
1,02	0,09	0,19	0	0	71001848	XP_755605.1	methyltransferase
1,02	0,29	0,59	3,53E-13	5,22E-13	70985937	XP_748473.1	MFS multidrug transporter
1,01	0,21	0,43	0	0	70983914	XP_747483.1	oxidoreductase, short chain dehydrogenase/reductase family
1,01	38,00	76,37	0	0	70988595	XP_749157.1	conserved hypothetical protein
1,00	0,10	0,21	1,31E-14	1,97E-14	70981606	XP_746332.1	mitochondrial chaperone ATPase (Bcs1)

List of all up-regulated genes with a differential gene expression upon methylprednisolone exposure of a $\log_2$ ratio ≥ 1 and a false discovery rate (FDR) of ≤ 0,001. Gene expression was expressed as reads per kilo base per million reads (RPKM) in non-exposed (control) and methylprednisolone exposed micro-colonies (MP). The geninfo identifier (GI) number, accession, annotation and the protein name of the genes are depicted.

Supplemental table 2 • Genes with down-regulated gene expression levels after methylprednisolone treatment

log. Ratio	control (RPKM)	MP (RPKM)	p-value	FDR	GI number	Accession	Annotation
-10,50	1,45	0,001	3,28E-142	7,90E-142	70996865	XP_753187.1	conserved hypothetical protein
-10,39	1,34	0,001	1,05E-286	3,36E-286	71000880	XP_755121.1	hypothetical protein AFUA_2G07860
-10,14	1,13	0,001	0	0	70981965	XP_746511.1	hypothetical protein AFUA_4G03250
-10,09	1,09	0,001	0	0	70990978	XP_750338.1	hypothetical protein AFUA_1G05880
-10,01	1,03	0,001	6,08E-210	1,70E-209	70985130	XP_748071.1	hypothetical protein AFUA_5G02710
-9,85	0,92	0,001	2,83E-73	5,66E-73	71000856	XP_755109.1	conserved hypothetical protein
-9,83	0,91	0,001	0	0	70981905	XP_746481.1	nucleotidase
-9,57	0,76	0,001	3,20E-145	7,82E-145	70988835	XP_749270.1	hypothetical protein AFUA_2G01340
-9,53	0,74	0,001	3,20E-145	7,82E-145	70997361	XP_753429.1	conserved hypothetical protein
-9,50	0,72	0,001	3,71E-214	1,05E-213	71002330	XP_755846.1	hypothetical protein AFUA_2G15120
-9,48	0,72	0,001	4,20E-140	1,00E-139	70987014	XP_748992.1	conserved hypothetical protein
-9,31	0,63	0,001	0	0	146324151	XP_753559.2	conserved hypothetical protein
-9,30	0,63	0,001	4,75E-212	1,33E-211	71000421	XP_754905.1	hypothetical protein AFUA_3G07010
-9,17	0,58	0,001	3,28E-288	1,05E-287	71002168	XP_755765.1	HHE domain protein
-9,13	0,56	0,001	6,89E-282	2,19E-281	70999143	XP_754293.1	hypothetical protein AFUA_3G13290
-9,07	0,54	0,001	2,83E-73	5,61E-73	70989401	XP_749550.1	conserved hypothetical protein
-8,92	0,48	0,001	2,26E-72	4,47E-72	70983065	XP_747060.1	hypothetical protein AFUA_8G01460
-8,92	0,48	0,001	2,56E-144	6,22E-144	70990262	XP_749980.1	conserved hypothetical protein
-8,88	0,47	0,001	2,83E-73	5,63E-73	70999163	XP_754303.1	hypothetical protein AFUA_3G13190
-8,86	0,47	0,001	1,05E-286	3,36E-286	146324870	XP_748885.2	DSBA-like thioredoxin domain protein
-8,77	0,44	0,001	8,01E-146	1,96E-145	70983694	XP_747374.1	beta-glucosidase
-8,76	0,43	0,001	1,05E-286	3,36E-286	70983725	XP_747389.1	2-haloalkanoic acid dehalogenase
-8,65	0,40	0,001	2,90E-70	5,61E-70	146323482	XP_754272.2	hypothetical protein AFUA_3G13500
-8,58	0,38	0,001	1,03E-289	3,30E-289	70994324	XP_751983.1	conserved hypothetical protein
-8,58	0,38	0,001	2,83E-73	5,65E-73	71002378	XP_755870.1	hypothetical protein AFUA_2G15350
-8,56	0,38	0,001	9,07E-218	2,58E-217	70983099	XP_747077.1	hypothetical protein AFUA_8G01290
-8,52	0,37	0,001	3,20E-145	7,81E-145	70983273	XP_747164.1	conserved hypothetical protein
-8,52	0,37	0,001	4,10E-143	9,92E-143	70981684	XP_746371.1	hypothetical protein AFUA_4G00850
-8,51	0,37	0,001	4,20E-140	1,00E-139	70994462	XP_752010.1	hypothetical protein AFUA_4G08160
-8,39	0,33	0,001	2,90E-70	5,62E-70	146324397	XP_001481457.1	conserved hypothetical protein
-8,38	0,33	0,001	4,20E-140	1,00E-139	70999826	XP_754630.1	hypothetical membrane protein
-8,37	0,33	0,001	3,71E-214	1,05E-213	70984016	XP_747533.1	nucleoside-diphosphate-sugar epimerase
-8,32	0,32	0,001	3,20E-145	7,82E-145	146322712	XP_001481639.1	conserved hypothetical protein
-8,32	0,32	0,001	2,83E-73	5,67E-73	146324495	XP_001481471.1	hypothetical protein AFUA_6G12145
-8,31	0,32	0,001	3,20E-145	7,83E-145	70994100	XP_751897.1	conserved hypothetical protein
-8,30	0,32	0,001	2,26E-72	4,43E-72	70996474	XP_752992.1	hypothetical protein AFUA_1G16260
-8,24	0,30	0,001	8,01E-146	1,96E-145	70992451	XP_751074.1	hypothetical protein AFUA_6G11950
-8,24	0,30	0,001	3,63E-217	1,03E-216	70983281	XP_747168.1	NlpC/P60-like cell-wall peptidase
-8,23	0,30	0,001	1,19E-212	3,34E-212	70988865	XP_749285.1	hypothetical protein AFUA_2G01490
-8,22	0,30	0,001	2,26E-72	4,43E-72	70981508	XP_731536.1	conserved hypothetical protein
-8,21	0,30	0,001	3,20E-145	7,82E-145	70986256	XP_748622.1	phosphatidylethanolamine-binding protein
-8,17	0,29	0,001	1,31E-287	4,21E-287	70984451	XP_747732.1	conserved hypothetical protein
-8,13	0,28	0,001	2,26E-72	4,46E-72	70992461	XP_751079.1	hypothetical protein AFUA_6G12000
-8,12	0,28	0,001	1,19E-212	3,34E-212	70983003	XP_747029.1	conserved hypothetical protein
-8,08	0,27	0,001	2,90E-70	5,62E-70	71000405	XP_754897.1	hypothetical protein AFUA_3G07090
-8,06	0,27	0,001	2,56E-144	6,23E-144	146324259	XP_731521.2	hypothetical protein AFUA_6G00340
-8,04	0,26	0,001	2,83E-73	5,64E-73	146324651	XP_747134.2	secreted antimicrobial peptide
-8,04	0,26	0,001	2,83E-73	5,63E-73	70993246	XP_751470.1	hypothetical protein AFUA_4G13610
-8,01	0,26	0,001	2,90E-70	5,58E-70	70993172	XP_751434.1	hypothetical protein AFUA_4G13980
-8,00	0,26	0,001	4,10E-143	9,91E-143	146324255	XP_753150.2	AhpC/TSA family thioredoxin peroxidase
-7,95	0,25	0,001	2,26E-72	4,45E-72	70983159	XP_747107.1	F-box domain protein
-7,91	0,24	0,001	2,56E-144	6,23E-144	71002812	XP_756087.1	GNAT family N-acetyltransferase
-7,90	0,24	0,001	9,07E-218	2,58E-217	71002880	XP_756121.1	3-beta hydroxysteroid dehydrogenase/isomerase
-7,90	0,24	0,001	2,26E-72	4,47E-72	70992419	XP_751058.1	hypothetical protein AFUA_6G11760
-7,85	0,23	0,001	2,83E-73	5,61E-73	70982740	XP_746898.1	hypothetical protein AFUA_7G00310
-7,85	0,23	0,001	2,26E-72	4,46E-72	146324572	XP_001481419.1	conserved hypothetical protein
-7,84	0,23	0,001	4,10E-289	1,32E-288	70982520	XP_746788.1	conserved hypothetical protein
-7,84	0,23	0,001	2,83E-73	5,62E-73	71002902	XP_756132.1	conserved hypothetical protein
-7,77	0,22	0,001	2,83E-73	5,66E-73	70989840	XP_749769.1	DUF614 domain protein
-7,74	0,21	0,001	3,20E-145	7,81E-145	70989832	XP_749765.1	hypothetical protein AFUA_1G00130
-7,73	0,21	0,001	1,16E-215	3,29E-215	146323952	XP_001481479.1	C6 finger domain protein
-7,72	0,21	0,001	2,56E-144	6,23E-144	146323563	XP_746365.2	short chain oxidoreductase (CsgA)
-7,71	0,21	0,001	3,20E-145	7,83E-145	70981648	XP_746353.1	short chain dehydrogenase
-7,71	0,21	0,001	3,20E-145	7,81E-145	71002836	XP_756099.1	conserved hypothetical protein
-7,71	0,21	0,001	3,28E-288	1,05E-287	146323865	XP_751590.2	HMG box protein
-7,69	0,21	0,001	2,26E-72	4,48E-72	70982692	XP_746874.1	hypothetical protein AFUA_7G00560
-7,69	0,21	0,001	2,26E-72	4,43E-72	146323329	XP_001481691.1	conserved hypothetical protein
-7,67	0,20	0,001	1,03E-289	3,30E-289	146324413	XP_750704.2	cytochrome P450 monooxygenase
-7,62	0,20	0,001	2,90E-70	5,59E-70	70984418	XP_747722.1	conserved hypothetical protein
-7,60	0,19	0,001	3,71E-214	1,05E-213	71002818	XP_756090.1	conidial pigment biosynthesis protein Ayg1
-7,57	0,19	0,001	2,26E-72	4,47E-72	70990016	XP_749857.1	conserved hypothetical protein
-7,56	0,19	0,001	3,28E-142	7,89E-142	71001226	XP_755294.1	hypothetical protein AFUA_2G09610
-7,55	0,19	0,001	1,19E-212	3,34E-212	146324487	XP_001481470.1	sodium bile acid symporter family protein
-7,54	0,19	0,001	2,90E-70	5,59E-70	70981778	XP_746418.1	hypothetical protein AFUA_4G00380
-7,51	0,18	0,001	2,83E-73	5,62E-73	70992985	XP_751341.1	hypothetical protein AFUA_6G14670
-7,48	0,18	0,001	4,20E-140	1,00E-139	146323946	XP_748330.2	integral membrane protein
-7,44	0,17	0,001	4,10E-289	1,32E-288	70985904	XP_748457.1	siderochrome-iron transporter
-7,43	0,17	0,001	8,01E-146	1,96E-145	70984018	XP_747534.1	isoflavone reductase family protein (CipA)
-7,37	0,17	0,001	3,71E-214	1,05E-213	70981742	XP_746400.1	NAD binding Rossmann fold oxidoreductase
-7,35	0,16	0,001	2,90E-70	5,61E-70	71002850	XP_756106.1	conserved hypothetical protein
-7,34	0,16	0,001	2,56E-144	6,23E-144	70988663	XP_749190.1	carboxyphosphonoenolpyruvate phosphonomutase
-7,33	0,16	0,001	3,28E-142	7,91E-142	146323127	XP_748377.2	conserved hypothetical protein
-7,32	0,16	0,001	2,26E-72	4,46E-72	70994070	XP_751882.1	carbonic anhydrase
-7,30	0,16	0,001	2,26E-72	4,47E-72	70997264	XP_753383.1	conserved hypothetical protein
-7,22	0,15	0,001	2,26E-72	4,44E-72	70982919	XP_746987.1	hypothetical protein AFUA_8G02190
-7,20	0,15	0,001	2,56E-144	6,24E-144	70990112	XP_749905.1	high affinity zinc ion transporter
-7,17	0,14	0,001	2,83E-73	5,65E-73	71001836	XP_755599.1	conserved hypothetical protein
-7,15	0,14	0,001	2,90E-70	5,62E-70	70983853	XP_748434.1	cell wall protein
-7,14	0,14	0,001	4,75E-212	1,33E-211	70992417	XP_751057.1	C6 transcription factor
-7,11	0,14	0,001	2,90E-70	5,58E-70	70981510	XP_731537.1	hypothetical protein AFUA_6G00193
-7,10	0,14	0,001	2,26E-72	4,43E-72	70998791	XP_754117.1	hypothetical protein AFUA_3G15070
-7,10	0,14	0,001	2,83E-73	5,65E-73	71002834	XP_756098.1	conserved hypothetical protein
-7,09	0,14	0,001	2,26E-72	4,46E-72	70990108	XP_749903.1	GrpB domain protein
-7,08	0,14	0,001	8,01E-146	1,96E-145	70990350	XP_750024.1	polyketide synthase
-7,06	0,13	0,001	2,56E-144	6,22E-144	70988747	XP_749229.1	extracellular glycosyl hydrolasecellulase
-7,05	0,13	0,001	1,19E-212	3,34E-212	70985404	XP_748208.1	lysophospholipase Plb2
-7,05	0,13	0,001	2,56E-144	6,23E-144	70984767	XP_747890.1	conserved hypothetical protein
-7,02	0,13	0,001	3,20E-145	7,81E-145	70983698	XP_747376.1	C6 finger domain protein
-7,02	0,13	0,001	2,26E-72	4,45E-72	146323577	XP_001481530.1	hypothetical protein AFUA_4G01120
-6,96	0,12	0,001	4,10E-143	9,91E-143	70992931	XP_751314.1	conserved hypothetical protein
-6,96	0,12	0,001	2,83E-73	5,66E-73	70985861	XP_748436.1	GPI anchored cell wall protein
-6,96	0,12	0,001	2,83E-73	5,62E-73	70984473	XP_747743.1	conserved hypothetical protein
-6,95	0,12	0,001	4,10E-143	9,92E-143	70983085	XP_747070.1	conserved hypothetical protein
-6,94	0,12	0,001	2,26E-72	4,46E-72	146323213	XP_001481593.1	conserved hypothetical protein
-6,94	0,12	0,001	2,83E-73	5,64E-73	146323209	XP_001481592.1	conserved hypothetical protein
-6,90	0,12	0,001	2,26E-72	4,42E-72	70994464	XP_752011.1	conserved hypothetical protein
-6,88	0,12	0,001	2,26E-72	4,47E-72	70998745	XP_754094.1	amine transporter
-6,83	0,11	0,001	8,01E-146	1,96E-145	70996975	XP_753242.1	lipase
-6,83	0,11	0,001	2,26E-72	4,43E-72	70981630	XP_746344.1	dienelactone hydrolase family protein
-6,81	0,11	0,001	8,01E-146	1,96E-145	70997988	XP_753726.1	neutral amino acid permease
-6,80	0,11	0,001	2,26E-72	4,42E-72	70981710	XP_746384.1	protein phosphatase 2C

-6,78	0,11	0,001	2,56E-144	6,23E-144	70992855	XP_751276.1	conserved hypothetical protein
-6,75	0,11	0,001	3,20E-145	7,82E-145	70989836	XP_749767.1	IBR finger domain protein
-6,74	0,11	0,001	3,28E-142	7,89E-142	70992947	XP_751322.1	alpha-1,3-mannosyltransferase
-6,74	0,11	0,001	2,26E-72	4,42E-72	70985542	XP_748277.1	integral membrane protein
-6,73	0,11	0,001	2,26E-72	4,47E-72	70981454	XP_731509.1	hypothetical protein AFUA_6G00460
-6,73	0,11	0,001	3,20E-145	7,83E-145	146324550	XP_001481477.1	cytochrome P450 monooxygenase
-6,73	0,11	0,001	2,83E-73	5,62E-73	70999077	XP_754260.1	extracellular serine-rich protein
-6,72	0,11	0,001	2,26E-72	4,44E-72	146323273	XP_754990.2	mating locus protein
-6,71	0,11	0,001	3,28E-142	7,90E-142	70990086	XP_749892.1	F-box domain protein
-6,70	0,10	0,001	2,83E-73	5,66E-73	70983904	XP_747478.1	hypothetical protein AFUA_8G06790
-6,70	0,10	0,001	2,90E-70	5,61E-70	146324219	XP_753245.2	toxin biosynthesis ketoreductase
-6,69	0,10	0,001	2,26E-72	4,46E-72	70983101	XP_747078.1	epsilon-lactone hydrolase
-6,64	0,10	0,001	2,90E-70	5,61E-70	146324423	XP_750739.2	phosphoglycerate mutase family protein
-6,63	0,10	0,001	2,83E-73	5,64E-73	146323984	XP_748189.2	short chain dehydrogenase
-6,63	0,10	0,001	3,28E-142	7,90E-142	70986352	XP_748670.1	MFS multidrug transporter
-6,61	0,10	0,001	3,28E-142	7,91E-142	70985968	XP_748488.1	MFS hexose transporter
-6,60	0,10	0,001	2,90E-70	5,59E-70	70986719	XP_748849.1	integral membrane protein Pth11-like
-6,58	0,10	0,001	2,83E-73	5,64E-73	70983223	XP_747139.1	solute transporter
-6,52	0,09	0,001	2,26E-72	4,44E-72	146324485	XP_751063.2	RTA1 domain protein
-6,52	0,09	0,001	2,26E-72	4,42E-72	70983039	XP_747047.1	hypothetical protein AFUA_8G01590
-6,50	0,09	0,001	4,10E-289	1,32E-288	146324393	XP_750596.2	NACHT and Ankyrin domain protein
-6,50	0,09	0,001	2,26E-72	4,44E-72	70986180	XP_748585.1	conserved hypothetical protein
-6,49	0,09	0,001	1,31E-287	4,21E-287	70986144	XP_748568.1	conserved hypothetical protein
-6,49	0,09	0,001	2,83E-73	5,64E-73	70985960	XP_748484.1	RTA1 domain protein
-6,48	0,09	0,001	2,90E-70	5,58E-70	70985490	XP_748251.1	G-protein signalling regulator
-6,47	0,09	0,001	4,10E-143	9,92E-143	70982885	XP_746970.1	TRI7-like toxin biosynthesis protein
-6,46	0,09	0,001	2,83E-73	5,61E-73	146322704	XP_749160.2	conserved hypothetical protein
-6,46	0,09	0,001	2,90E-70	5,60E-70	70988603	XP_749161.1	conserved hypothetical protein
-6,46	0,09	0,001	3,28E-142	7,90E-142	70990462	XP_750080.1	carboxylesterase
-6,46	0,09	0,001	2,26E-72	4,42E-72	146324221	XP_753244.2	NmrA-like family protein
-6,45	0,09	0,001	2,26E-72	4,42E-72	70983702	XP_747378.1	hypothetical protein AFUA_8G05770
-6,44	0,09	0,001	2,56E-144	6,22E-144	70983293	XP_747174.1	conserved hypothetical protein
-6,43	0,09	0,001	2,90E-70	5,58E-70	70981558	XP_746308.1	fumarylacetoacetate hydrolase family protein
-6,41	0,09	0,001	2,26E-72	4,48E-72	70986658	XP_748819.1	NmrA family transcriptional regulator
-6,40	0,08	0,001	2,26E-72	4,48E-72	70985723	XP_748367.1	endo-1,4-beta-xylanase
-6,40	0,08	0,001	2,83E-73	5,65E-73	146323382	XP_754672.2	3-hydroxyisobutyrate dehydrogenase
-6,39	0,08	0,001	2,26E-72	4,46E-72	70982766	XP_746911.1	NAD dependent epimerase/dehydratase
-6,39	0,08	0,001	2,26E-72	4,47E-72	70985554	XP_748283.1	hypothetical protein AFUA_5G00560
-6,39	0,08	0,001	2,26E-72	4,42E-72	70983765	XP_747409.1	hypothetical protein AFUA_8G06070
-6,38	0,08	0,001	2,90E-70	5,61E-70	70985888	XP_748449.1	alpha/beta hydrolase
-6,38	0,08	0,001	2,83E-73	5,61E-73	146324421	XP_750738.2	inorganic diphosphatase
-6,35	0,08	0,001	2,26E-72	4,42E-72	70981867	XP_746462.1	DUF829 domain protein (PaxU)
-6,35	0,08	0,001	2,83E-73	5,63E-73	70985426	XP_748219.1	RTA1 domain protein
-6,34	0,08	0,001	2,83E-73	5,66E-73	70991989	XP_750843.1	endoglucanase
-6,33	0,08	0,001	2,83E-73	5,61E-73	70988661	XP_749189.1	DUF1275 domain protein
-6,32	0,08	0,001	3,20E-145	7,81E-145	70983937	XP_747494.1	hypothetical protein AFUA_8G06960
-6,30	0,08	0,001	2,90E-70	5,58E-70	146323535	XP_746440.2	short-chain dehydrogenase
-6,28	0,08	0,001	2,83E-73	5,62E-73	70982638	XP_746847.1	alpha/beta hydrolase
-6,28	0,08	0,001	2,83E-73	5,62E-73	70985608	XP_748310.1	zinc-binding oxidoreductase
-6,26	0,08	0,001	2,26E-72	4,48E-72	70982676	XP_746866.1	aromatic ring-opening dioxygenase family protein
-6,25	0,08	0,001	3,28E-142	7,89E-142	146324315	XP_747738.2	conserved hypothetical protein
-6,23	0,08	0,001	2,83E-73	5,63E-73	70981552	XP_746305.1	gentisate 1,2-dioxygenase
-6,22	0,07	0,001	2,26E-72	4,45E-72	70985602	XP_748307.1	conserved hypothetical protein
-6,22	0,07	0,001	2,83E-73	5,64E-73	70995424	XP_752467.1	L-arabinitol 4-dehydrogenase
-6,20	0,07	0,001	2,26E-72	4,42E-72	70998763	XP_754103.1	endo-1,4-beta-xylanase
-6,14	0,07	0,001	4,10E-143	9,91E-143	70992465	XP_751081.1	C2H2 finger domain protein
-6,13	0,07	0,001	2,90E-70	5,60E-70	71002452	XP_755907.1	hypothetical protein AFUA_2G15720
-6,09	0,07	0,001	2,26E-72	4,45E-72	70985757	XP_748384.1	conserved hypothetical protein
-6,07	0,07	0,001	2,83E-73	5,66E-73	70990062	XP_749880.1	GPI anchored protein
-6,06	0,07	0,001	2,26E-72	4,47E-72	70982915	XP_746985.1	alpha-ketoglutarate-dependent taurine dioxygenase
-6,03	0,07	0,001	2,26E-72	4,45E-72	146323941	XP_751358.2	hypothetical protein AFUA_4G14751
-6,03	0,07	0,001	2,83E-73	5,62E-73	70983924	XP_747488.1	extracellular exo-polygalacturonase
-6,02	0,07	0,001	2,26E-72	4,45E-72	70982612	XP_746834.1	extracellular cysteine-rich protein
-6,02	0,06	0,001	2,90E-70	5,60E-70	70992433	XP_751065.1	conserved hypothetical protein
-5,97	0,06	0,001	2,26E-72	4,43E-72	70985194	XP_748103.1	Auxin Efflux Carrier superfamily
-5,96	0,06	0,001	2,83E-73	5,61E-73	70985144	XP_748078.1	O-methyltransferase
-5,95	0,06	0,001	2,83E-73	5,63E-73	70983858	XP_747455.1	ornithine decarboxylase
-5,94	0,06	0,001	2,26E-72	4,43E-72	146322309	XP_749783.2	class V chitinase
-5,91	0,06	0,001	2,26E-72	4,48E-72	70988725	XP_749219.1	extracellular GDSL-like lipase/acylhydrolase
-5,90	0,06	0,001	2,90E-70	5,60E-70	70981772	XP_746415.1	hypothetical protein AFUA_4G00410
-5,90	0,06	0,001	2,90E-70	5,60E-70	70984509	XP_747761.1	conserved hypothetical protein
-5,89	0,06	0,001	2,26E-72	4,44E-72	70982656	XP_746856.1	protein kinase
-5,87	0,06	0,001	2,90E-70	5,59E-70	70989613	XP_749656.1	salicylate hydroxylase
-5,87	0,06	0,001	2,83E-73	5,66E-73	70981598	XP_746328.1	UPF0075 family protein
-5,86	0,06	0,001	2,90E-70	5,59E-70	70984543	XP_747778.1	extracellular exo-polygalacturonase
-5,86	0,06	0,001	2,26E-72	4,45E-72	146324089	XP_753942.2	conserved hypothetical protein
-5,84	0,06	0,001	2,90E-70	5,61E-70	70987022	XP_748995.1	class III chitinase
-5,82	0,06	0,001	2,83E-73	5,65E-73	71002200	XP_755781.1	oxidoreductase, FAD-binding
-5,79	0,06	0,001	2,26E-72	4,42E-72	70998797	XP_754120.1	xenobiotic compound monooxygenase, DszA family
-5,78	0,06	0,001	2,90E-70	5,60E-70	70983327	XP_747191.1	hypothetical protein AFUA_8G00130
-5,78	0,05	0,001	2,90E-70	5,59E-70	70982927	XP_746991.1	acetylornithine aminotransferase
-5,77	0,05	0,001	2,26E-72	4,46E-72	70996967	XP_753238.1	beta-glucanase
-5,77	0,05	0,001	2,90E-70	5,62E-70	70997808	XP_753636.1	cytochrome b5 reductase
-5,74	0,05	0,001	2,83E-73	5,64E-73	70985653	XP_748332.1	succinate-semialdehyde dehydrogenase
-5,71	0,05	0,001	2,26E-72	4,46E-72	70985827	XP_748419.1	MFS transporter Lic1/Seo1
-5,71	0,05	0,001	2,90E-70	5,58E-70	146324625	XP_747192.2	hypothetical protein AFUA_8G00120
-5,70	0,05	0,001	2,26E-72	4,45E-72	70988731	XP_749221.1	MFS transporter
-5,69	0,05	0,001	2,83E-73	5,61E-73	146324576	XP_746813.2	MFS transporter
-5,69	0,05	0,001	2,90E-70	5,62E-70	146323597	XP_746307.2	lignostilbene dioxygenase
-5,66	0,05	0,001	2,83E-73	5,63E-73	70985560	XP_748286.1	inulinase
-5,66	0,05	0,001	2,90E-70	5,61E-70	70986046	XP_748524.1	glycosyl hydrolase
-5,65	0,05	0,001	2,26E-72	4,45E-72	71002372	XP_755867.1	MFS efflux transporter
-5,65	0,05	0,001	2,26E-72	4,44E-72	71000890	XP_755126.1	myo-inositol transporter
-5,65	0,05	0,001	2,90E-70	5,59E-70	146324455	XP_750856.2	cytochrome P450 oxidoreductase GliC
-5,63	0,05	0,001	2,83E-73	5,66E-73	70992023	XP_750860.1	MFS gliotoxin efflux transporter GliA
-5,59	0,05	0,001	2,83E-73	5,65E-73	70984493	XP_747753.1	conserved hypothetical protein
-5,59	0,05	0,001	2,26E-72	4,44E-72	70981676	XP_746367.1	conserved hypothetical protein
-5,58	0,05	0,001	2,83E-73	5,65E-73	146322684	XP_753059.2	MFS multidrug transporter
-5,56	0,05	0,001	2,26E-72	4,47E-72	70986448	XP_748718.1	cytochrome P450 monooxygenase
-5,54	0,05	0,001	2,90E-70	5,59E-70	70985424	XP_748218.1	general amidase
-5,54	0,05	0,001	2,90E-70	5,59E-70	70986590	XP_748786.1	vegetative incompatibility WD repeat protein
-5,53	0,05	0,001	2,26E-72	4,44E-72	70986699	XP_748839.1	tannase
-5,52	0,05	0,001	2,83E-73	5,63E-73	70999071	XP_754257.1	siderochrome-iron transporter
-5,51	0,05	0,001	2,26E-72	4,43E-72	70992837	XP_751267.1	adenylate-forming enzyme
-5,50	0,05	0,001	2,83E-73	5,62E-73	70982684	XP_746870.1	hydrolase, CocE/NonD family
-5,49	0,04	0,001	2,90E-70	5,62E-70	70983061	XP_747058.1	potassium channel
-5,46	0,04	0,001	2,26E-72	4,48E-72	146322932	XP_755493.2	alpha-glucosidase/alpha-amylase
-5,44	0,04	0,001	2,26E-72	4,43E-72	70993072	XP_751384.1	extracellular dihydrogeodin oxidase/laccase
-5,40	0,04	0,001	2,83E-73	5,64E-73	70998434	XP_753939.1	dipeptidyl peptidase III
-5,38	0,04	0,001	2,26E-72	4,45E-72	70981768	XP_746413.1	isoamyl alcohol oxidase
-5,35	0,04	0,001	2,83E-73	5,67E-73	70986284	XP_748636.1	flavin-containing monooxygenase
-5,34	0,04	0,001	2,26E-72	4,44E-72	146322602	XP_752496.2	AMP-binding enzyme domain protein
-5,30	0,04	0,001	2,83E-73	5,65E-73	70985540	XP_748276.1	choline dehydrogenase

-5,29	0,04	0,001	2,90E-70	5,60E-70	70983119	XP_747087.1	isoamyl alcohol oxidase
-5,22	0,04	0,001	2,90E-70	5,60E-70	146323903	XP_001481573.1	conserved hypothetical protein
-5,16	0,04	0,001	2,26E-72	4,46E-72	70985552	XP_748282.1	conserved hypothetical protein
-5,15	0,04	0,001	2,26E-72	4,47E-72	70988687	XP_749201.1	beta-N-hexosaminidase
-5,14	0,04	0,001	2,83E-73	5,62E-73	70982901	XP_746978.1	C6 transcription factor
-5,08	0,03	0,001	2,26E-72	4,44E-72	70983137	XP_747096.1	Melibiase subfamily
-5,02	0,03	0,001	2,26E-72	4,43E-72	70985958	XP_748483.1	Ankyrin and HET domain protein
-4,92	0,03	0,001	2,90E-70	5,62E-70	71002932	XP_756147.1	telomere-associated RecQ helicase
-4,88	0,03	0,001	2,83E-73	5,63E-73	71000435	XP_754912.1	C2H2 zinc finger domain protein
-4,81	0,03	0,001	2,90E-70	5,61E-70	71002792	XP_756077.1	Pfs and NB-ARC domain protein
-4,64	0,02	0,001	2,26E-72	4,45E-72	70995466	XP_752488.1	HMG-CoA reductase
-4,58	0,02	0,001	2,90E-70	5,62E-70	70982600	XP_746828.1	calcium transporting ATPase (Pmc1)
-4,39	0,02	0,001	2,26E-72	4,42E-72	70984507	XP_747760.1	NACHT domain protein
-3,89	0,61	0,04	0	0	71000361	XP_754875.1	conserved hypothetical protein
-3,66	0,62	0,05	0	0	70982965	XP_747010.1	conserved hypothetical protein
-2,90	0,86	0,12	7,80E-274	2,44E-273	146323907	XP_751433.2	conserved hypothetical protein
-2,89	0,63	0,08	1,33E-272	4,16E-272	70981642	XP_746350.1	acid phosphatase
-2,89	0,92	0,12	7,04E-271	2,20E-270	70985741	XP_748376.1	conserved hypothetical protein
-2,89	0,89	0,12	7,04E-271	2,20E-270	70992213	XP_750955.1	conserved hypothetical protein
-2,67	0,35	0,06	2,11E-215	5,98E-215	70981596	XP_746327.1	DUF89 domain protein
-2,52	1,35	0,23	1,84E-200	5,05E-200	70989539	XP_749619.1	cell wall biogenesis protein
-2,50	1,85	0,33	0	0	70988552	XP_753998.1	DNA repair and transcription factor Ada
-2,42	0,34	0,06	2,05E-161	5,20E-161	70985992	XP_748499.1	alcohol dehydrogenase
-2,42	0,29	0,05	9,82E-161	2,49E-160	70989936	XP_749817.1	extracellular alpha-1,3-glucanase/mutanase
-2,42	0,22	0,04	9,82E-161	2,49E-160	70996710	XP_753110.1	tyrosinase
-2,41	0,26	0,05	2,79E-160	7,06E-160	146323113	XP_748341.2	hexose transporter protein
-2,41	0,58	0,11	7,91E-160	2,00E-159	70981774	XP_746416.1	O-methyltransferase
-2,41	0,48	0,09	3,78E-159	9,55E-159	70983824	XP_747438.1	monooxygenase
-2,41	0,40	0,08	3,78E-159	9,54E-159	70981582	XP_746320.1	4-nitrophenylphosphatase
-2,40	0,16	0,03	4,08E-157	1,03E-156	70985679	XP_748345.1	beta-glucosidase
-2,25	3,12	0,66	2,64E-262	8,11E-262	71000860	XP_755111.1	hypothetical protein AFUA_2G07760
-2,24	0,65	0,14	6,63E-260	2,03E-259	71002870	XP_756116.1	RTA1 domain protein
-2,18	0,61	0,13	5,21E-253	1,58E-252	70989023	XP_749361.1	conserved hypothetical protein
-2,17	0,68	0,15	5,24E-250	1,58E-249	70998775	XP_754109.1	conserved hypothetical protein
-2,16	1,28	0,29	0	0	70986754	XP_748866.1	F-box domain protein
-2,10	0,24	0,06	1,78E-109	3,94E-109	70992977	XP_751337.1	conserved hypothetical protein
-2,10	0,37	0,09	4,68E-109	1,04E-108	70994214	XP_751954.1	short chain dehydrogenase
-2,10	0,34	0,08	4,68E-109	1,04E-108	70984248	XP_747641.1	hypothetical protein AFUA_6G04370
-2,09	0,33	0,08	1,23E-108	2,72E-108	70998885	XP_754164.1	extracellular endo-1,5-alpha-L-arabinase
-2,09	2,58	0,61	2,82E-213	7,94E-213	70996698	XP_753104.1	heat shock protein Awh11
-2,09	0,30	0,07	1,38E-107	3,04E-107	70983073	XP_747064.1	quinone oxidoreductase
-2,09	0,62	0,15	2,82E-213	7,94E-213	146324759	XP_747383.2	DUF1295 domain protein
-2,08	1,51	0,36	0	0	70985440	XP_748226.1	MFS monosaccharide transporter
-2,08	0,22	0,05	5,86E-107	1,29E-106	70983757	XP_747405.1	alpha-1,3-glucanase
-2,08	0,31	0,07	5,86E-107	1,29E-106	71002860	XP_756111.1	integral membrane protein
-2,08	0,22	0,05	1,54E-106	3,38E-106	70995710	XP_752610.1	salicylate hydroxylase
-2,08	0,22	0,05	1,54E-106	3,38E-106	70985819	XP_748415.1	hypothetical protein AFUA_3G00940
-2,06	0,75	0,18	1,28E-103	2,79E-103	70986248	XP_748618.1	hypothetical protein AFUA_3G02960
-2,06	0,10	0,02	1,28E-103	2,79E-103	70981624	XP_746341.1	von Willebrand domain protein
-2,02	0,84	0,21	2,70E-206	7,49E-206	71001960	XP_755661.1	hypothetical protein AFUA_2G13280
-2,00	0,37	0,09	6,15E-200	1,69E-199	70990506	XP_750102.1	DnaJ domain protein
-1,96	0,48	0,12	2,50E-269	7,79E-269	70986727	XP_748853.1	FAD/FMN-containing isoamyl alcohol oxidase MreA-like
-1,96	0,50	0,13	4,07E-268	1,26E-267	70997315	XP_753407.1	hypothetical protein AFUA_5G12450
-1,96	0,38	0,10	1,64E-267	5,07E-267	70993564	XP_751629.1	conserved hypothetical protein
-1,94	0,26	0,07	2,51E-97	5,39E-97	146323331	XP_754867.2	conserved hypothetical protein
-1,92	1,00	0,26	6,33E-95	1,35E-94	70991953	XP_750825.1	hypothetical protein AFUA_6G09356
-1,91	0,54	0,14	1,02E-167	2,61E-167	70996612	XP_753061.1	phosphatidylserine decarboxylase
-1,90	0,41	0,11	6,21E-166	1,59E-165	71002184	XP_755773.1	conserved hypothetical protein
-1,89	0,51	0,14	3,76E-164	9,59E-164	70990186	XP_749942.1	plasma membrane protein Pth11-like
-1,82	1,68	0,48	2,26E-220	6,48E-220	70995070	XP_752301.1	hypothetical protein AFUA_1G09340
-1,81	0,58	0,17	4,62E-218	1,32E-217	70981955	XP_746506.1	conserved hypothetical protein
-1,77	0,27	0,08	1,09E-212	3,07E-212	70997575	XP_753531.1	nucleosome remodeling complex ATPase subunit (Snf2h)
-1,70	1,37	0,42	0	0	70983975	XP_747513.1	arsenic resistance protein ArsH
-1,69	0,38	0,12	4,75E-62	8,92E-62	70993166	XP_751431.1	short chain dehydrogenase/reductase family protein
-1,69	0,24	0,07	4,75E-62	8,92E-62	70983297	XP_747176.1	short-chain dehydrogenase
-1,68	0,97	0,30	2,59E-61	4,84E-61	146322384	XP_750046.2	conserved hypothetical protein
-1,68	0,34	0,11	2,59E-61	4,85E-61	70986814	XP_748895.1	endoglucanase
-1,68	0,24	0,07	2,59E-61	4,85E-61	70982025	XP_746541.1	integral membrane protein
-1,68	0,57	0,18	2,59E-61	4,84E-61	71000351	XP_754870.1	conserved hypothetical protein
-1,68	0,53	0,17	1,25E-120	2,84E-120	70987026	XP_748997.1	integral membrane protein (Pth11)
-1,68	2,03	0,63	0	0	71002232	XP_755797.1	protein kinase domain-containing protein
-1,68	0,60	0,19	9,19E-61	1,72E-60	70981961	XP_746509.1	extracellular guanyl-specific ribonuclease RntA
-1,68	0,66	0,21	1,20E-178	3,16E-178	70989537	XP_749618.1	pyruvate dehydrogenase E1 component alpha subunit
-1,68	0,43	0,13	1,20E-178	3,16E-178	70996658	XP_753084.1	MFS transporter
-1,67	0,91	0,28	3,81E-237	1,12E-236	70996660	XP_753085.1	TfdA family taurine dioxygenase
-1,67	1,14	0,36	1,57E-119	3,56E-119	71002388	XP_755875.1	hypothetical protein AFUA_2G15400
-1,67	0,44	0,14	8,85E-237	2,61E-236	146324805	XP_747537.2	C6 transcription factor
-1,67	0,42	0,13	2,14E-60	3,99E-60	70985735	XP_748373.1	ATP/GTP-binding protein
-1,67	0,71	0,22	8,05E-177	2,11E-176	146323456	XP_754338.2	RTA1 domain protein
-1,67	1,07	0,34	0	0	70999634	XP_754534.1	DUF821 domain protein
-1,67	0,04	0,01	7,55E-60	1,41E-59	146323460	XP_754329.2	nonribosomal peptide synthase GliP-like
-1,67	0,02	0,01	7,55E-60	1,41E-59	70981748	XP_746403.1	conserved hypothetical protein
-1,67	0,26	0,08	7,55E-60	1,41E-59	70997836	XP_753650.1	RTA1 domain protein
-1,67	0,99	0,31	7,55E-60	1,41E-59	70993240	XP_751467.1	hypothetical protein AFUA_4G13640
-1,66	0,25	0,08	1,75E-59	3,25E-59	70996857	XP_753183.1	fucose-specific lectin FleA
-1,66	0,32	0,10	1,75E-59	3,25E-59	70983171	XP_747113.1	chitosanase
-1,66	0,17	0,05	1,75E-59	3,25E-59	70989904	XP_749801.1	biotin-dependent 2-oxo acid dehydrogenases acyltransferase, putative
-1,66	0,30	0,09	1,75E-59	3,25E-59	70995245	XP_752384.1	conserved hypothetical protein
-1,66	0,14	0,04	1,75E-59	3,25E-59	70986258	XP_748623.1	phosphate-repressible Na+/phosphate cotransporter Pho89, putative
-1,66	2,47	0,78	0	0	146323125	XP_001481582.1	conserved hypothetical protein
-1,65	0,18	0,06	1,42E-58	2,63E-58	70983916	XP_747484.1	conserved hypothetical protein
-1,65	2,98	0,95	0	0	70991539	XP_750618.1	conserved hypothetical protein
-1,65	0,32	0,10	3,28E-58	6,04E-58	70995237	XP_752381.1	conserved hypothetical protein
-1,65	0,17	0,05	3,28E-58	6,04E-58	70998340	XP_753892.1	hypothetical protein AFUA_5G07420
-1,63	2,20	0,71	2,11E-172	5,49E-172	70983992	XP_747521.1	conserved hypothetical protein
-1,63	0,19	0,06	1,10E-171	2,86E-171	70986207	XP_748598.1	ABC multidrug transporter
-1,63	0,56	0,18	1,10E-171	2,86E-171	70989165	XP_749432.1	conserved hypothetical protein
-1,62	1,09	0,35	1,23E-168	3,15E-168	70994340	XP_751986.1	conserved hypothetical protein
-1,61	0,78	0,26	6,32E-224	1,82E-223	70984625	XP_747819.1	hypothetical protein AFUA_6G02580
-1,60	4,30	1,42	0	0	70986574	XP_748778.1	hypothetical protein AFUA_7G08370
-1,60	1,47	0,49	6,59E-221	1,89E-220	70994018	XP_751856.1	conserved hypothetical protein
-1,60	0,42	0,14	1,98E-111	4,40E-111	70996670	XP_753090.1	extracellular polygalacturonase
-1,59	0,37	0,12	6,73E-111	1,50E-110	70993256	XP_751376.1	metallo-beta-lactamase domain protein
-1,56	0,34	0,11	2,57E-197	7,04E-197	70984886	XP_747949.1	protein kinase
-1,55	0,63	0,21	4,69E-195	1,28E-194	70983017	XP_747036.1	haloalkane dehalogenase family protein
-1,54	0,11	0,04	3,07E-54	5,58E-54	70983095	XP_747075.1	ferric-chelate reductase (Fre2)
-1,53	0,30	0,10	7,39E-53	1,34E-52	71001772	XP_755567.1	conserved hypothetical protein
-1,51	0,17	0,06	1,17E-51	2,12E-51	70983179	XP_747117.1	MFS glucose transporter
-1,51	0,21	0,07	1,17E-51	2,12E-51	70983071	XP_747063.1	oxidoreductase, zinc-binding dehydrogenase family
-1,51	1,10	0,39	1,17E-51	2,12E-51	70996452	XP_752981.1	hypothetical protein AFUA_1G16140
-1,50	0,69	0,24	1,52E-135	3,59E-135	70997986	XP_753725.1	polysaccharide deacetylase family protein
-1,47	2,61	0,94	0	0	146323327	XP_001481608.1	conserved hypothetical protein
-1,47	0,80	0,29	1,61E-211	4,52E-211	146324191	XP_753377.2	CCCH zinc finger DNA binding protein

-1,46	2,09	0,76	6,13E-131	1,44E-130	70998002	XP_753733.1	hypothetical protein AFUA_5G09040
-1,46	1,33	0,48	4,18E-131	9,78E-131	70990248	XP_749973.1	conserved hypothetical protein
-1,43	0,90	0,33	8,25E-125	1,90E-124	70995054	XP_752293.1	acetyltransferase, GNAT family family
-1,42	0,96	0,36	1,12E-200	3,08E-200	70997311	XP_753405.1	oxidoreductase, 2OG-Fe(II) oxygenase family
-1,42	0,21	0,08	9,90E-79	2,02E-78	70983269	XP_747162.1	C6 finger transcription factor
-1,42	0,32	0,12	9,90E-79	2,02E-78	146323809	XP_751818.2	conserved hypothetical protein
-1,41	0,46	0,17	8,09E-155	2,02E-154	146324556	XP_001481478.1	MFS monosaccharide transporter
-1,41	0,22	0,08	2,10E-78	4,27E-78	71001186	XP_755274.1	cyclohexanone monooxygenase
-1,41	0,26	0,10	1,37E-77	2,78E-77	70992857	XP_751277.1	acyltransferase
-1,41	0,94	0,36	1,37E-77	2,78E-77	146324265	XP_001481439.1	conserved hypothetical protein
-1,41	2,38	0,90	7,20E-153	1,79E-152	70997862	XP_753663.1	hypothetical protein AFUA_5G09770
-1,40	0,83	0,31	6,09E-77	1,23E-76	44889997	CAF32115.1	hypothetical protein AfA33H4.001c [Aspergillus fumigatus]
-1,40	0,47	0,18	6,31E-151	1,56E-150	71001940	XP_755651.1	xylosidase : arabinofuranosidase
-1,40	0,45	0,17	3,93E-76	7,94E-76	70986535	XP_748759.1	hypothetical protein AFUA_7G08560
-1,38	1,23	0,48	0	0	70992337	XP_751017.1	histidine acid phosphatase
-1,35	0,44	0,17	1,26E-73	2,52E-73	70998498	XP_753971.1	conserved hypothetical protein
-1,35	4,17	1,64	4,45E-172	1,16E-171	70985314	XP_748163.1	hypothetical protein AFUA_5G01790
-1,35	0,46	0,18	1,32E-171	3,42E-171	70983153	XP_747104.1	HET domain protein
-1,34	0,87	0,34	7,03E-170	1,82E-169	71002760	XP_756061.1	MFS transporter
-1,33	0,72	0,29	1,01E-240	3,00E-240	70982756	XP_746906.1	MFS multidrug transporter
-1,32	0,78	0,31	6,34E-99	1,37E-98	70986752	XP_748865.1	hypothetical protein AFUA_7G06450
-1,32	1,32	0,53	6,87E-194	1,86E-193	70987066	XP_749015.1	lipase
-1,30	0,41	0,17	1,10E-95	2,36E-95	70983723	XP_747388.1	aromatic amino acid and leucine permease
-1,29	2,83	1,16	0	0	146323131	XP_748386.2	aminopeptidase Y
-1,27	1,13	0,47	3,29E-156	8,25E-156	70985410	XP_748211.1	RTA1 domain protein
-1,27	0,35	0,15	4,41E-93	9,36E-93	70985562	XP_748287.1	C6 transcription factor
-1,27	1,23	0,51	6,24E-93	1,32E-92	70998837	XP_754140.1	3-dehydroquinate dehydratase, type II
-1,26	3,53	1,47	1,94E-181	5,16E-181	70993784	XP_751739.1	hypothetical protein AFUA_4G10890
-1,26	0,33	0,14	7,90E-91	1,66E-90	70982075	XP_746566.1	serine peptidase, family S28
-1,25	1,19	0,50	1,27E-115	2,85E-115	70983996	XP_747523.1	integral membrane protein
-1,25	0,46	0,19	8,76E-90	1,84E-89	70992389	XP_751043.1	endoglucanase
-1,24	0,34	0,15	9,64E-89	2,02E-88	70988978	XP_749339.1	MFS transporter
-1,24	4,84	2,05	0	0	146323821	XP_751796.2	conserved hypothetical protein
-1,23	0,63	0,27	3,09E-113	6,92E-113	70988721	XP_749217.1	pectate lyase
-1,23	1,01	0,43	4,50E-137	1,07E-136	71002380	XP_755871.1	conserved hypothetical protein
-1,22	3,78	1,62	1,78E-111	3,96E-111	70983217	XP_747136.1	hypothetical protein AFUA_8G00690
-1,22	3,58	1,54	1,12E-220	3,20E-220	70996827	XP_753168.1	hypothetical protein AFUA_5G14890
-1,21	2,01	0,87	0	0	70998795	XP_754119.1	flavin-binding monooxygenase
-1,21	3,85	1,66	1,17E-158	2,94E-158	71001198	XP_755280.1	potassium transporter
-1,21	3,46	1,50	4,44E-158	1,12E-157	146324247	XP_001481523.1	RING-finger domain protein
-1,19	0,74	0,33	5,79E-108	1,28E-107	146324209	XP_753272.2	conserved hypothetical protein
-1,18	0,31	0,13	2,59E-153	6,46E-153	146323982	XP_001481488.1	class V chitinase
-1,18	2,69	1,19	4,98E-152	1,24E-151	146322442	XP_001481704.1	hypothetical protein AFUA_1G04455
-1,17	0,46	0,21	3,89E-81	7,98E-81	146324081	XP_753945.2	hypothetical protein AFUA_5G06880
-1,16	1,58	0,70	1,03E-149	2,55E-149	70986442	XP_748715.1	endoglucanase
-1,14	18,78	8,50	0	0	70982522	XP_746789.1	opsin
-1,14	1,48	0,67	1,20E-307	3,97E-307	146324506	XP_751145.2	beta-alanine synthase
-1,14	5,14	2,33	1,38E-164	3,53E-164	70991697	XP_750697.1	hypothetical protein AFUA_6G08060
-1,13	2,80	1,28	1,74E-249	5,23E-249	146323448	XP_001481624.1	conserved hypothetical protein
-1,12	2,76	1,27	0	0	70991130	XP_750414.1	actin filament organization protein App1-like
-1,12	33,00	15,19	0	0	71000689	XP_755026.1	GATA transcription factor LreA
-1,12	1,09	0,50	3,73E-264	1,15E-263	70991096	XP_750397.1	conserved hypothetical protein
-1,11	1,06	0,49	7,58E-118	1,71E-117	70990374	XP_750036.1	C2H2 transcription factor
-1,10	0,15	0,07	9,69E-23	1,55E-22	70987087	XP_749025.1	aldo-keto reductase
-1,10	0,13	0,06	9,69E-23	1,55E-22	70992001	XP_750849.1	zinc metallopeptidase
-1,10	0,17	0,08	9,69E-23	1,55E-22	70986662	XP_748821.1	branched-chain amino acid aminotransferase
-1,10	2,01	0,93	3,41E-220	9,74E-220	71000874	XP_755118.1	conserved hypothetical protein
-1,10	0,08	0,04	9,69E-23	1,55E-22	70992487	XP_751092.1	C6 transcription factor
-1,10	0,19	0,09	9,69E-23	1,55E-22	146323909	XP_001481574.1	spherulin 4-like cell surface protein
-1,10	2,43	1,13	1,07E-280	3,40E-280	146324415	XP_001481459.1	pyroglutamyl peptidase type I
-1,10	3,21	1,50	1,41E-299	4,62E-299	70986721	XP_748850.1	isochorismatase family hydrolase
-1,10	0,86	0,40	1,81E-22	2,88E-22	70995468	XP_752489.1	hypothetical protein AFUA_1G11240
-1,10	0,56	0,26	1,81E-22	2,88E-22	70986193	XP_748591.1	conserved hypothetical protein
-1,10	0,11	0,05	1,81E-22	2,89E-22	146322922	XP_755474.2	MFS transporter
-1,10	0,10	0,05	1,81E-22	2,89E-22	70989952	XP_749825.1	DnaJ domain protein
-1,10	0,10	0,05	1,81E-22	2,89E-22	71002878	XP_756120.1	MFS transporter
-1,10	0,68	0,32	1,81E-22	2,89E-22	70986028	XP_748516.1	conserved hypothetical protein
-1,10	0,05	0,02	1,81E-22	2,88E-22	146323549	XP_746431.2	WD40 repeat protein
-1,10	0,20	0,09	2,66E-43	4,66E-43	70995478	XP_752494.1	C6 finger domain protein
-1,10	0,24	0,11	1,81E-22	2,88E-22	70999282	XP_754360.1	conserved hypothetical protein
-1,10	0,37	0,17	1,81E-22	2,89E-22	70995722	XP_752616.1	conserved hypothetical protein
-1,10	0,44	0,20	1,81E-22	2,89E-22	70999390	XP_754414.1	conserved hypothetical protein
-1,10	0,16	0,07	1,81E-22	2,89E-22	70990458	XP_750078.1	MFS alpha-glucoside transporter
-1,10	0,10	0,05	1,81E-22	2,89E-22	70992377	XP_751037.1	conserved hypothetical protein
-1,09	2,35	1,10	1,89E-276	5,95E-276	70996332	XP_752921.1	hypothetical protein AFUA_1G15540
-1,09	0,40	0,19	4,63E-22	7,33E-22	70983798	XP_747425.1	hypothetical protein AFUA_3G06240
-1,09	0,23	0,11	4,63E-22	7,33E-22	70999908	XP_754671.1	15-hydroxyprostaglandin dehydrogenase (NAD(+))
-1,09	0,16	0,07	4,63E-22	7,32E-22	70991537	XP_750617.1	4-hydroxybenzoate polyprenyl transferase
-1,09	0,23	0,11	4,63E-22	7,33E-22	70982332	XP_746694.1	ferulic acid esterase (FaeA)
-1,09	0,09	0,04	4,63E-22	7,33E-22	70981584	XP_746321.1	pyoverdine/dityrosine biosynthesis family protein
-1,09	0,16	0,07	1,26E-42	2,20E-42	70983295	XP_747175.1	conserved hypothetical protein
-1,09	0,33	0,16	4,63E-22	7,33E-22	70999027	XP_754235.1	conserved hypothetical protein
-1,09	0,10	0,05	4,63E-22	7,33E-22	70987010	XP_748990.1	MFS alpha-glucoside transporter
-1,09	0,54	0,25	4,63E-22	7,33E-22	70989463	XP_749581.1	hypothetical protein AFUA_2G04500
-1,09	0,19	0,09	4,63E-22	7,33E-22	70981766	XP_746412.1	short chain dehydrogenase
-1,09	0,45	0,21	1,26E-42	2,20E-42	70988647	XP_749182.1	short-chain dehydrogenase/reductase family protein
-1,09	6,67	3,14	0	0	146323191	XP_001481590.1	conserved hypothetical protein
-1,09	1,87	0,88	5,79E-164	1,48E-163	146324245	XP_753173.2	hypothetical protein AFUA_5G14840
-1,09	1,73	0,81	3,29E-103	7,19E-103	146324830	XP_749018.2	hypothetical protein AFUA_7G04920
-1,09	1,26	0,59	2,34E-42	4,08E-42	70986404	XP_748696.1	hypothetical protein AFUA_3G03760
-1,09	0,11	0,05	2,34E-42	4,08E-42	146323253	XP_001481599.1	C2H2 type zinc finger domain protein
-1,09	4,31	2,03	0	0	70998180	XP_753818.1	cell wall protein
-1,08	1,25	0,59	2,47E-251	7,46E-251	70990188	XP_749943.1	MFS multidrug transporter
-1,08	0,18	0,09	5,90E-42	1,03E-41	70985368	XP_748190.1	MFS transporter
-1,08	0,16	0,08	1,09E-41	1,90E-41	70993028	XP_751362.1	C6 transcription factor
-1,08	0,12	0,05	1,60E-21	2,52E-21	70982768	XP_746912.1	dimethylallyl tryptophan synthase GliD1
-1,08	0,09	0,04	1,60E-21	2,52E-21	70997806	XP_753635.1	3-hydroxyacyl-CoA dehydrogenase
-1,08	0,41	0,19	1,60E-21	2,52E-21	70982598	XP_746827.1	cytidine deaminase
-1,08	0,10	0,05	1,60E-21	2,52E-21	70985906	XP_748458.1	MFS transporter
-1,08	0,16	0,08	1,60E-21	2,52E-21	146323275	XP_754989.2	mating type protein MAT1-2-1
-1,08	0,12	0,06	1,60E-21	2,52E-21	70993060	XP_751378.1	toxin biosynthesis regulatory protein AflJ
-1,08	0,13	0,06	1,60E-21	2,52E-21	70982762	XP_746909.1	conserved hypothetical protein
-1,07	0,44	0,21	1,30E-80	2,07E-80	70998320	XP_753882.1	cytochrome P450 oxidoreductase
-1,07	0,32	0,15	5,06E-41	8,78E-41	146324253	XP_753152.2	cytochrome P450 oxygenase
-1,07	0,50	0,24	2,11E-60	3,93E-60	70985468	XP_748240.1	conserved hypothetical protein
-1,07	0,67	0,32	2,01E-140	4,82E-140	70987093	XP_749028.1	C6 transcription factor
-1,07	0,11	0,05	4,01E-21	6,32E-21	70982606	XP_746831.1	aldehyde dehydrogenase
-1,07	0,17	0,08	4,01E-21	6,31E-21	70998825	XP_754134.1	FacC-like extracellular signaling protein
-1,07	0,20	0,10	4,01E-21	6,32E-21	146324327	XP_747689.2	hypothetical protein AFUA_6G03880
-1,07	0,62	0,29	4,01E-21	6,32E-21	70990536	XP_750117.1	hypothetical protein AFUA_1G03670
-1,07	0,43	0,21	4,01E-21	6,31E-21	70981931	XP_746494.1	C2H2 finger domain protein
-1,07	0,14	0,07	9,33E-41	1,62E-40	146324904	XP_748757.2	dynamin GTPase
-1,07	0,19	0,09	4,01E-21	6,32E-21	70996076	XP_752793.1	conserved fungal protein

-1,07	0,12	0,06	4,01E-21	6,31E-21	70985925	XP_748467.1	salicylate hydroxylase
-1,07	0,44	0,21	4,01E-21	6,31E-21	146323353	XP_001481612.1	conserved hypothetical protein
-1,07	0,96	0,46	9,33E-41	1,62E-40	70994392	XP_751994.1	conserved hypothetical protein
-1,06	2,11	1,01	5,56E-275	1,74E-274	70982223	XP_746640.1	C4-dicarboxylate transporter/malic acid transport protein, putative
-1,06	1,45	0,69	1,77E-59	3,29E-59	70987148	XP_749054.1	hypothetical protein AFUA_7G04560
-1,06	1,60	0,77	5,90E-166	1,51E-165	70996124	XP_752817.1	hypothetical protein AFUA_1G14480
-1,06	0,65	0,31	4,54E-99	9,81E-99	70994328	XP_751984.1	mannose-6-phosphate isomerase, class I
-1,05	3,22	1,55	5,14E-79	1,05E-78	71000928	XP_755145.1	hypothetical protein AFUA_2G08100
-1,05	2,57	1,24	0	0	70998362	XP_753903.1	DUF500 domain protein
-1,05	5,78	2,79	0	0	70983552	XP_747303.1	conserved hypothetical protein
-1,05	0,40	0,20	1,02E-96	2,19E-96	146323956	XP_001481480.1	conserved hypothetical protein
-1,05	0,07	0,04	3,37E-20	5,26E-20	146322896	XP_001481664.1	conserved hypothetical protein
-1,05	0,30	0,14	3,37E-20	5,26E-20	70985989	XP_748498.1	hypothetical protein AFUA_3G01770
-1,05	0,14	0,07	3,37E-20	5,26E-20	70986649	XP_748815.1	oxidoreductase
-1,05	0,16	0,08	3,37E-20	5,26E-20	71002888	XP_756125.1	RTA1 domain protein
-1,05	0,09	0,04	3,37E-20	5,26E-20	146324888	XP_748842.2	flavin-containing monooxygenase
-1,05	0,50	0,24	3,37E-20	5,26E-20	71002548	XP_755955.1	hypothetical protein AFUA_2G16190
-1,05	0,30	0,15	3,37E-20	5,26E-20	146323539	XP_001481525.1	conserved hypothetical protein
-1,05	1,90	0,92	0	0	70995688	XP_752599.1	GABA permease
-1,05	0,14	0,07	3,37E-20	5,26E-20	70983037	XP_747046.1	conserved hypothetical protein
-1,05	1,96	0,95	2,27E-116	5,11E-116	70998773	XP_754108.1	hypothetical protein AFUA_3G15160
-1,04	0,57	0,28	5,20E-59	9,64E-59	70997774	XP_753621.1	conserved hypothetical protein
-1,04	9,75	4,73	0	0	70984164	XP_747601.1	conserved hypothetical protein
-1,04	0,52	0,25	9,46E-59	1,75E-58	70985538	XP_748275.1	peroxisomal dehydratase
-1,04	0,47	0,23	3,70E-76	7,48E-76	70981626	XP_746342.1	N,N-dimethylglycine oxidase
-1,04	1,18	0,58	1,99E-131	4,67E-131	70988761	XP_749235.1	glycerophosphoryl diester phosphodiesterase family protein
-1,03	0,38	0,18	1,21E-75	2,44E-75	70981522	XP_731543.1	hypothetical protein AFUA_6G00140
-1,03	2,52	1,23	0	0	70999161	XP_754302.1	1,3-beta-glucanosyltransferase
-1,03	0,31	0,15	6,14E-39	1,06E-38	146323241	XP_748671.2	4-hydroxyphenylpyruvate dioxygenase
-1,02	3,34	1,64	1,28E-147	3,16E-147	70984619	XP_747816.1	conserved hypothetical protein
-1,02	0,51	0,25	5,26E-93	1,11E-92	70993516	XP_751605.1	MFS transporter (Mch2)
-1,02	0,34	0,17	1,11E-38	1,90E-38	70989197	XP_749448.1	conserved hypothetical protein
-1,02	0,39	0,19	2,68E-38	4,59E-38	70983979	XP_747515.1	conserved hypothetical protein
-1,02	0,25	0,12	2,68E-38	4,59E-38	70989429	XP_749564.1	NADH-dependent flavin oxidoreductase
-1,02	1,41	0,70	1,32E-91	2,79E-91	70993830	XP_751762.1	conserved hypothetical protein
-1,02	1,02	0,50	1,09E-127	2,52E-127	70982845	XP_746950.1	glyceraldehyde-3-phosphate dehydrogenase
-1,02	0,31	0,16	4,82E-38	8,24E-38	70984028	XP_747539.1	conserved hypothetical protein
-1,02	0,30	0,15	4,82E-38	8,24E-38	146323527	XP_754114.2	integral membrane protein
-1,02	1,74	0,86	9,34E-200	2,56E-199	70985464	XP_748238.1	glyceraldehyde 3-phosphate dehydrogenase
-1,01	0,88	0,43	2,55E-146	6,25E-146	70996292	XP_752901.1	conserved hypothetical protein
-1,01	1,11	0,55	4,47E-74	8,97E-74	146324582	XP_746775.2	conserved hypothetical protein
-1,01	1,50	0,74	0	0	70998360	XP_753902.1	poly(ADP)-ribose polymerase PARP
-1,01	0,25	0,12	1,16E-37	1,98E-37	70983380	XP_747217.1	N-acetylglucosamine-6-phosphate deacetylase (NagA)
-1,01	0,21	0,10	1,16E-37	1,98E-37	70997165	XP_753337.1	MATE efflux family protein subfamily
-1,01	0,39	0,19	1,07E-73	2,15E-73	146324745	XP_001481406.1	MFS transporter
-1,01	0,83	0,41	2,43E-90	5,11E-90	70998488	XP_753966.1	integral membrane protein
-1,01	70,23	34,87	0	0	70994164	XP_751929.1	GPI anchored cell wall protein
-1,01	2,80	1,39	0	0	70997053	XP_753281.1	NlpC/P60-like cell-wall peptidase
-1,00	0,66	0,33	3,72E-37	6,33E-37	71002764	XP_756063.1	hypothetical protein AFUA_2G17290
-1,00	0,37	0,18	3,05E-188	8,18E-188	70993156	XP_751426.1	conserved hypothetical protein
-1,00	32,75	16,36	0	0	70997049	XP_753279.1	calcium binding protein Caleosin
-1,00	2,62	1,31	1,09E-169	2,82E-169	146324834	XP_001481427.1	glyoxalase family protein

List of all down-regulated genes with a differential gene expression upon methylprednisolone exposure of a $\log_2$ ratio ≥ 1 and a false discovery rate (FDR) of ≤ 0,001. Gene expression was expressed as reads per kilo base per million reads (RPKM) in non-exposed (control) and methylprednisolone exposed micro-colonies (MP). The geninfo identifier (GI) number, accession, annotation and the protein name of the genes are depicted.

Supplemental table 3 • Expression of genes related to cell wall morphogenesis

GI number	control (RPKM)	MP (RPKM)	log2 Ratio	Expression	p-value	FDR	Accession	Annotation	Protein name	Carbohydrate
70989361	0,05	0,36	2,74	Up	6,71E-13	9,87E-13	XP_749530.1	alpha-1,3-glucanase/mutanase		α-(1,3)-glucan
70986546	0,49	1,21	1,32	Up	4,17E-12	5,95E-12	XP_748764.1	extracellular alpha-1,3-glucanase/mutanase		α-(1,3)-glucan
146322396	0,11	0,18	0,81	Indifferent	1,47E-13	2,20E-13	XP_001481700.1	alpha-1,3-glucanase/mutanase		α-(1,3)-glucan
70989791	1,27	1,35	0,09	Indifferent	5,52E-02	5,96E-02	XP_749745.1	alpha-1,3-glucanase/mutanase		α-(1,3)-glucan
71001558	1,20	1,22	0,02	Indifferent	4,79E-02	5,18E-02	XP_755460.1	alpha-1,3-glucan synthase Ags2	Ags2	α-(1,3)-glucan
70984888	6,48	6,57	0,02	Indifferent	9,99E-02	1,07E-01	XP_747950.1	alpha-1,3-glucanase		α-(1,3)-glucan
70986578	29,63	26,99	-0,13	Indifferent	2,98E-215	8,43E-215	XP_748780.1	alpha-1,3-glucanase		α-(1,3)-glucan
70985813	80,62	70,32	-0,20	Indifferent	0	0	XP_748412.1	alpha-1,3-glucan synthase Ags1	Ags1	α-(1,3)-glucan
70996314	13,38	11,00	-0,28	Indifferent	0	0	XP_752912.1	alpha-1,3-glucan synthase Ags3	Ags3	α-(1,3)-glucan
70983822	3,72	2,48	-0,59	Indifferent	0	0	XP_747437.1	alpha-1,3-glucanase		α-(1,3)-glucan
70983757	0,22	0,05	-2,08	Down	5,86E-107	1,29E-106	XP_747405.1	alpha-1,3-glucanase		α-(1,3)-glucan
70989936	0,29	0,05	-2,42	Down	9,82E-161	2,49E-160	XP_749817.1	extracellular alpha-1,3-glucanase/mutanase		α-(1,3)-glucan
70988641	0,03	0,07	1,49	Up	6,71E-13	9,86E-13	XP_749179.1	exo-beta-1,3-glucanase	Exg9	β-(1,3)-glucan
70986272	0,09	0,18	1,02	Up	0	0	XP_748630.1	endo-1,3(4)-beta-glucanase	Eng7	β-(1,3)-glucan
70992949	0,03	0,06	0,98	Indifferent	6,71E-13	9,87E-13	XP_751323.1	beta-glucosidase	Exg17	β-glucan
70990858	1,33	2,05	0,63	Indifferent	2,44E-11	3,38E-11	XP_750278.1	endo-1,3(4)-beta-glucanase	Eng3	β-(1,3)-glucan
70990956	0,63	0,84	0,40	Indifferent	2,79E-11	3,81E-11	XP_750327.1	beta-glucosidase	Exg12	β-glucan
70982931	4,18	5,01	0,26	Indifferent	0	0	XP_746993.1	1,3-beta-glucanosyltransferase	Gel5	β-(1,3)-glucan
71002176	7,88	9,38	0,25	Indifferent	1,95E-10	2,55E-10	XP_755769.1	GPI anchored endo-1,3(4)-beta-glucanase	Eng2	β-(1,3)-glucan
70995512	195,26	225,84	0,21	Indifferent	0	0	XP_752511.1	1,3-beta-glucanosyltransferase Bgt1	Bgt1	β-(1,3)-glucan
71001176	25,81	29,84	0,21	Indifferent	0	0	XP_755269.1	endo-beta-1,6-glucanase	Exg4	β-(1,6)-glucan
70981987	1,96	2,20	0,16	Indifferent	0	0	XP_746522.1	exo-beta-1,3-glucanase	Exg10	β-(1,3)-glucan
70993296	1,25	1,37	0,13	Indifferent	2,56E-05	2,93E-05	XP_751495.1	endo-1,3(4)-beta-glucanase	Eng5	β-(1,3)-glucan
70992347	247,15	261,29	0,08	Indifferent	0	0	XP_751022.1	1,3-beta-glucanosyltransferase Gel2	Gel2	β-(1,3)-glucan
70983670	1309,74	1381,90	0,08	Indifferent	1,31E-09	1,65E-09	XP_747362.1	cell wall glucanase (Scw11)	Scw11	β-(1,3)-glucan
70996706	1,76	1,83	0,06	Indifferent	6,45E-04	7,23E-04	XP_753108.1	beta-glucosidase	Exg15	β-glucan
70990522	8,76	9,09	0,05	Indifferent	1,34E-06	1,56E-06	XP_750110.1	exo-beta-1,3-glucanase (Exg1)	Exg1	β-(1,3)-glucan
70991821	3,65	3,70	0,02	Indifferent	1,67E-01	1,77E-01	XP_750759.1	beta glucosidase	Exg20	β-glucan
70996120	2,87	2,87	0,00	Indifferent	9,86E-01	9,86E-01	XP_752815.1	exo-beta-1,3-glucanase Exg0	Exg0/Exg8	β-(1,3)-glucan
70989629	558,17	557,08	0,00	Indifferent	2,13E-02	2,32E-02	XP_749664.1	1,3-beta-glucanosyltransferase	Gel4	β-(1,3)-glucan
70986816	0,07	0,07	-0,02	Indifferent	8,46E-01	8,51E-01	XP_748896.1	beta-D-glucoside glucohydrolase	Exg13	β-glucan
70986957	693,90	684,45	-0,02	Indifferent	1,29E-54	2,34E-54	XP_748964.1	SUN domain protein (Uth1)	Uth/Sun1	β-(1,3)-glucan
70985687	1341,97	1318,90	-0,03	Indifferent	5,57E-177	1,46E-176	XP_748349.1	GPI-anchored cell wall beta-1,3-endoglucanase EglC	EglC/Bgt2	β-(1,3)-glucan
70990648	4,62	4,44	-0,06	Indifferent	4,49E-08	5,36E-08	XP_750173.1	endo-1,3-beta-glucanase Eng1	Eng1	β-(1,3)-glucan
70992457	0,06	0,06	-0,07	Indifferent	4,68E-01	4,82E-01	XP_751077.1	exo-beta-1,3-glucanase	Exg5	β-(1,3)-glucan
70982754	0,38	0,36	-0,09	Indifferent	2,17E-02	2,36E-02	XP_746905.1	beta-glucosidase	Exg14	β-glucan
70991927	16,88	15,73	-0,10	Indifferent	9,24E-68	1,76E-67	XP_750812.1	exo-beta-1,3-glucanase	Exg2	β-(1,3)-glucan
70988799	540,93	492,14	-0,14	Indifferent	0	0	XP_749253.1	1,3-beta-glucanosyltransferase Gel1	Gel1	β-(1,3)-glucan
70992709	0,88	0,80	-0,15	Indifferent	2,97E-08	3,55E-08	XP_751203.1	exo-beta-1,3-glucanase	Exg6	β-(1,3)-glucan
71000329	1,96	1,75	-0,16	Indifferent	4,43E-20	6,91E-20	XP_754859.1	exo-beta-1,3-glucanase	Exg7	β-(1,3)-glucan
70986924	1,47	1,31	-0,17	Indifferent	4,28E-10	5,51E-10	XP_748948.1	glucanase	Exg3	β-(1,3)-glucan
70996999	2,72	2,42	-0,17	Indifferent	3,18E-17	4,87E-17	XP_753254.1	endo-1,3(4)-beta-glucanase	Eng8	β-(1,3)-glucan
70992541	1,46	1,26	-0,22	Indifferent	4,74E-18	7,31E-18	XP_751119.1	1,3-beta-glucanosyltransferase	Gel7	β-(1,3)-glucan
70992539	220,07	185,25	-0,25	Indifferent	0	0	XP_751118.1	1,3-beta-glucan synthase catalytic subunit FksP	Fksp	β-(1,3)-glucan
71001868	1,60	1,32	-0,27	Indifferent	2,82E-29	4,65E-29	XP_755615.1	1,3-beta-glucanosyltransferase Gel3	Gel3	β-(1,3)-glucan
70992535	2,35	1,93	-0,28	Indifferent	1,39E-31	2,31E-31	XP_751116.1	cell wall glucanase (Scw4)	Scw4	β-(1,3)-glucan
70998054	14,64	11,94	-0,29	Indifferent	0	0	XP_753759.1	cell wall glucanase	Bgt3	β-(1,3)-glucan
70984414	10,25	8,29	-0,31	Indifferent	0	0	XP_747720.1	beta-glucosidase	Exg21	β-glucan
70985216	3,51	2,80	-0,33	Indifferent	5,20E-64	9,80E-64	XP_748114.1	endo-1,3(4)-beta-glucanase	Eng4	β-(1,3)-glucan
70998386	5,94	4,70	-0,34	Indifferent	1,63E-238	4,82E-238	XP_753915.1	beta-glucosidase	Exg18	β-glucan
70999161	2,52	1,23	-1,03	Down	0	0	XP_754302.1	1,3-beta-glucanosyltransferase	Gel6	β-(1,3)-glucan
146323325	2,56	3,49	0,45	Indifferent	2,71E-11	3,70E-11	XP_754895.2	class III chitinase		chitin
146324032	0,13	0,17	0,43	Indifferent	6,43E-12	9,08E-12	XP_747948.2	class V chitinase Chi100		chitin
70983560	1,67	2,03	0,28	Indifferent	0	0	XP_747307.1	beta-N-acetylhexosaminidase NagA	NagA	chitin
70995750	1,38	1,62	0,23	Indifferent	0	0	XP_752630.1	chitin synthase D	ChsD	chitin
70983215	4,94	5,77	0,22	Indifferent	0	0	XP_747135.1	class III chitinase ChiA2	ChiA2	chitin
146324024	24,19	25,75	0,09	Indifferent	0	0	XP_747959.2	class III chitinase	ChiC	chitin
70984924	872,01	889,26	0,03	Indifferent	1,83E-08	2,19E-08	XP_747968.1	class III chitinase ChiA1	ChiA1	chitin
70992799	0,12	0,11	-0,07	Indifferent	2,06E-01	2,17E-01	XP_751248.1	class V chitinase		chitin
146323424	61,96	57,93	-0,10	Indifferent	2,34E-121	5,33E-121	XP_754491.2	class V chitinase		chitin
146322380	14,42	13,45	-0,10	Indifferent	1,84E-26	3,00E-26	XP_750029.2	class V chitinase		chitin
146323329	2,48	2,24	-0,15	Indifferent	1,15E-11	1,61E-11	XP_001481609.1	class V chitinase		chitin
146324443	75,64	66,10	-0,19	Indifferent	0	0	XP_750818.2	class V chitinase		chitin
71001992	71,70	62,16	-0,21	Indifferent	0	0	XP_756077.1	chitin synthase ChsE	ChsE	chitin
70985514	7,65	6,63	-0,21	Indifferent	1,48E-125	3,42E-125	XP_748263.1	chitin synthase C	ChsC	chitin
71001990	78,66	66,50	-0,24	Indifferent	0	0	XP_755676.1	chitin synthase		chitin
70988943	16,26	13,61	-0,26	Indifferent	0	0	XP_749322.1	chitin synthase A	ChsA	chitin
70982151	18,15	14,68	-0,31	Indifferent	0	0	XP_746604.1	chitin synthase B	ChsB	chitin
70983674	54,78	43,27	-0,34	Indifferent	0	0	XP_747364.1	chitin synthase F	ChsF	chitin
70998925	61,40	47,70	-0,36	Indifferent	0	0	XP_754184.1	chitin synthase ChsG	ChsG	chitin
70993970	3,21	2,10	-0,61	Indifferent	5,98E-118	1,35E-117	XP_751832.1	polysaccharide deacetylase family protein		chitin
70984970	1,07	0,68	-0,65	Indifferent	5,61E-65	1,06E-64	XP_747991.1	class III chitinase		chitin
70992165	1,09	0,61	-0,83	Indifferent	3,95E-131	9,25E-131	XP_750931.1	chitin deacetylase		chitin
146323982	0,31	0,13	-1,18	Down	2,59E-153	6,46E-153	XP_001481488.1	class V chitinase		chitin
70987022	0,06	0,00	-5,84	Down	2,90E-70	5,61E-70	XP_748995.1	class III chitinase		chitin
146322309	0,06	0,00	-5,94	Down	2,26E-72	4,43E-72	XP_749783.2	class V chitinase		chitin
70988857	40,40	40,82	0,01	Indifferent	5,36E-03	5,91E-03	XP_749281.1	alpha-1,6 mannosyltransferase subunit (Mnn9)	Mnn9	galactomannan
70999266	353,82	350,52	-0,01	Indifferent	3,88E-18	5,98E-18	XP_754352.1	conserved hypothetical protein	GlfA	galactofuranose
70990078	31,87	31,36	-0,02	Indifferent	7,45E-04	8,34E-04	XP_749888.1	alpha-1,6-mannosyltransferase subunit (Hoc1)	Hoc1	galactomannan
70998096	12,53	11,81	-0,09	Indifferent	1,89E-19	2,93E-19	XP_753779.1	alpha-1,6-mannosyltransferase subunit (Och1)	Och1	galactomannan
70992859	1,61	1,35	-0,26	Indifferent	3,37E-20	5,26E-20	XP_751278.1	glycosyl transferase	Och2	galactomannan
71000257	92,02	70,24	-0,39	Indifferent	0	0	XP_754823.1	UDP-glucose 4-epimerase	Uge3	galactosaminogalactan
146323048	23,56	17,17	-0,46	Indifferent	0	0	XP_001481682.1	mannan polymerase II complex ANP1 subunit Anp1	Anp1	galactomannan
70982949	1,20	0,66	-0,86	Indifferent	8,41E-125	1,93E-124	XP_747002.1	glycosyl transferase	Och3	galactomannan
70984495	1,78	2,47	0,47	Indifferent	3,26E-11	4,44E-11	XP_747754.1	cell wall glucanase/allergen F16-like	Crh1	cross-linking
3510010	954,78	888,98	-0,10	Indifferent	0	0	XP_752985.1	extracellular cell wall glucanase Crf1/allergen Asp F9	Crh5	cross-linking
146323378	103,91	95,41	-0,12	Indifferent	1,92E-260	5,87E-260	XP_754695.2	cell wall glucanase	Crh3	cross-linking
70994734	971,90	888,09	-0,13	Indifferent	0	0	XP_752144.1	GPI-anchored cell wall organization protein Ecm33	Ecm33	cross-linking
70989195	340,57	268,44	-0,34	Indifferent	0	0	XP_749447.1	cell wall glucanase (Utr2)	Utr2/Crh2	cross-linking
70991783	30,79	23,18	-0,41	Indifferent	0	0	XP_750740.1	cell wall glucanase	Crh4	cross-linking

List of all genes related to cell wall morphogenesis that were expressed in any condition. Gene expression was expressed as reads per kilo base per million reads (RPKM) in non-exposed (control) and methylprednisolone exposed micro-colonies (MP). Gene expression was considered differential if the expression between the groups has a $\log_2$ difference of ≤ -1 or ≥ 1 and a false discovery rate (FDR) of ≤ 0,001. The geninfo identifier (GI) number, accession, annotation, protein name of the genes are depicted. The last column describes the carbohydrate that is modified by the gene-encoded protein.

Supplemental table 4 • Over-represented Gene Ontology (GO) terms in methylprednisolone treated samples

Over-represented biological processes amongst genes having increased expression

Accession	GO term	List hits	List total	Population hits	Population total	p-value
GO:0071702	organic substance transport	12	148	118	4309	4,01E-02

Over-represented cellular componenst amongst genes having decreased expression

Accession	GO term	List hits	List total	Population hits	Population total	p-value
GO:0031224	intrinsic to membrane	33	67	618	2482	1,20E-04
GO:0044425	membrane part	33	67	678	2482	9,60E-04
GO:0016020	membrane	35	67	761	2482	1,70E-03

Over-represented molecular functions amongst genes having increased expression

Accession	GO term	List hits	List total	Population hits	Population total	p-value
GO:0016491	oxidoreductase activity	34	179	500	5087	7,53E-03
GO:0005506	iron ion binding	14	179	228	5087	1,00E-02

Over-represented molecular functions amongst genes having decreased expression

Accession	GO term	List hits	List total	Population hits	Population total	p-value
GO:0016798	hydrolase activity, acting on glycosyl bonds	23	215	218	5087	3,16E-03
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	17	215	143	5087	7,91E-03

Gene ontology (GO) enrichment analysis was performed. Over-represented GO terms of biological processes, cellular components and molecular functions with enriched differentially expressed genes of methylprednisolone-exposed micro-colonies compared to their occurrence in the *A. fumigatus* Af293 genome were shown. GO terms with a calculated p-value ≤ 0,05 with Bonferroni Correction were considered to be significantly enriched. The population total illustrates the amount of genes that are mapped to any term in the biological process, cellular component or molecular function ontology, which are present in the *A. fumigatus* Af293 genome. The population hits illustrates the amount of genes that are mapped to the particular GO term, which are present in the *A. fumigatus* Af293 genome. The list total depicts the amount of genes that are mapped to any term in the biological process, cellular component or molecular function ontology, which are differentially expressed upon methylprednisolone treatment. The list hits illustrates the amount of genes that are mapped to the particular GO term, which are differentially expressed upon methylprednisolone treatment.

Supplemental table 5 • Genes with up-regulated gene expression levels after methylprednisolone treatment assigned with GO term intrinsic to membrane

GI number	control (RPKM)	MP (RPKM)	log, Ratio	Expression	p-value	FDR	Accession	Annotation
70986068	1,00	0,74	9,53	Up	0	0	XP_748534.1	ion channel
70985837	1,00	0,28	8,12	Up	0	0	XP_748424.1	RTA1 domain protein
70981959	1,00	0,25	7,98	Up	0	0	XP_746508.1	amino acid permease
146322311	1,00	0,16	7,35	Up	0	0	XP_749787.2	metalloreductase
70982905	1,00	0,12	6,89	Up	0	0	XP_746980.1	neutral amino acid permease
70999073	1,00	0,11	6,83	Up	0	0	XP_754258.1	Ctr copper transporter family protein
70986234	1,00	0,11	6,73	Up	0	0	XP_748611.1	MFS sugar transporter
71002336	1,00	0,10	6,67	Up	0	0	XP_755849.1	choline transport protein
70992427	1,00	0,09	6,57	Up	0	0	XP_751062.1	RTA1 domain protein
70996897	1,00	0,09	6,55	Up	0	0	XP_753203.1	MFS monosaccharide transporter
70981875	1,00	0,09	6,51	Up	0	0	XP_746466.1	haemolysin-III family protein
70999075	1,00	0,06	6,00	Up	0	0	XP_754259.1	integral membrane protein
146323583	1,00	0,05	5,71	Up	0	0	XP_746335.2	GABA permease
70983201	1,00	0,05	5,58	Up	0	0	XP_747128.1	sugar transporter family protein
70981590	1,00	0,05	5,55	Up	0	0	XP_746324.1	MFS monosaccharide transporter
70985715	1,00	0,05	5,52	Up	0	0	XP_748363.1	high-affinity glucose transporter
70983121	0,05	0,34	2,76	Up	6,71E-13	9,82E-13	XP_747088.1	MFS sugar transporter
70996716	0,05	0,32	2,57	Up	6,71E-13	9,86E-13	XP_753113.1	high affinity nitrate transporter NrtB
70992447	0,05	0,19	1,91	Up	6,71E-13	9,85E-13	XP_751072.1	MFS alpha-glucoside transporter
146322751	0,20	0,64	1,71	Up	2,08E-12	3,00E-12	XP_749273.2	GABA permease
70992469	0,15	0,42	1,49	Up	2,98E-13	4,41E-13	XP_751083.1	MFS sugar permease
70985484	0,04	0,12	1,48	Up	2,12E-13	3,15E-13	XP_748248.1	amino acid permease
70981658	0,90	2,43	1,43	Up	2,63E-11	3,60E-11	XP_746358.1	MFS quinate transporter
70986000	0,58	1,30	1,17	Up	3,43E-12	4,92E-12	XP_748503.1	amino acid permease
70984769	0,05	0,10	1,05	Up	6,71E-13	9,83E-13	XP_747891.1	MFS lactose permease
70985937	0,29	0,59	1,02	Up	3,53E-13	5,22E-13	XP_748473.1	MFS multidrug transporter

Supplemental table 6 • Differential expression of hydrophobin genes

GI number	control (RPKM)	MP (RPKM)	log, Ratio	Expression	p-value	FDR	Accession	Annotation	Protein name
70983957	0,001	1,97	10,94	Up	0	0	XP_747504.1	hydrophobin	RodC
70996676	0,001	0,35	8,47	Up	0	0	XP_753093.1	conidial hydrophobin RodB	RodB
70983729	0,73	2,63	1,85	Up	1,30E-12	1,88E-12	XP_747391.1	hydrophobin	RodE
70997898	5,48	13,14	1,26	Up	4,58E-12	6,51E-12	XP_753681.1	conidial hydrophobin Hyp1/RodA	RodA
70985374	3,03	5,59	0,89	Indifferent	1,95E-11	2,70E-11	XP_748193.1	hypothetical protein AFUA_5G01490	RodD
146324012	2,77	3,37	0,28	Indifferent	2,94E-11	4,02E-11	XP_748015.2	conserved hypothetical protein	RodF
146323018	-	-	-	Not expressed	-	-	XP_755798.2	hydrophobin	

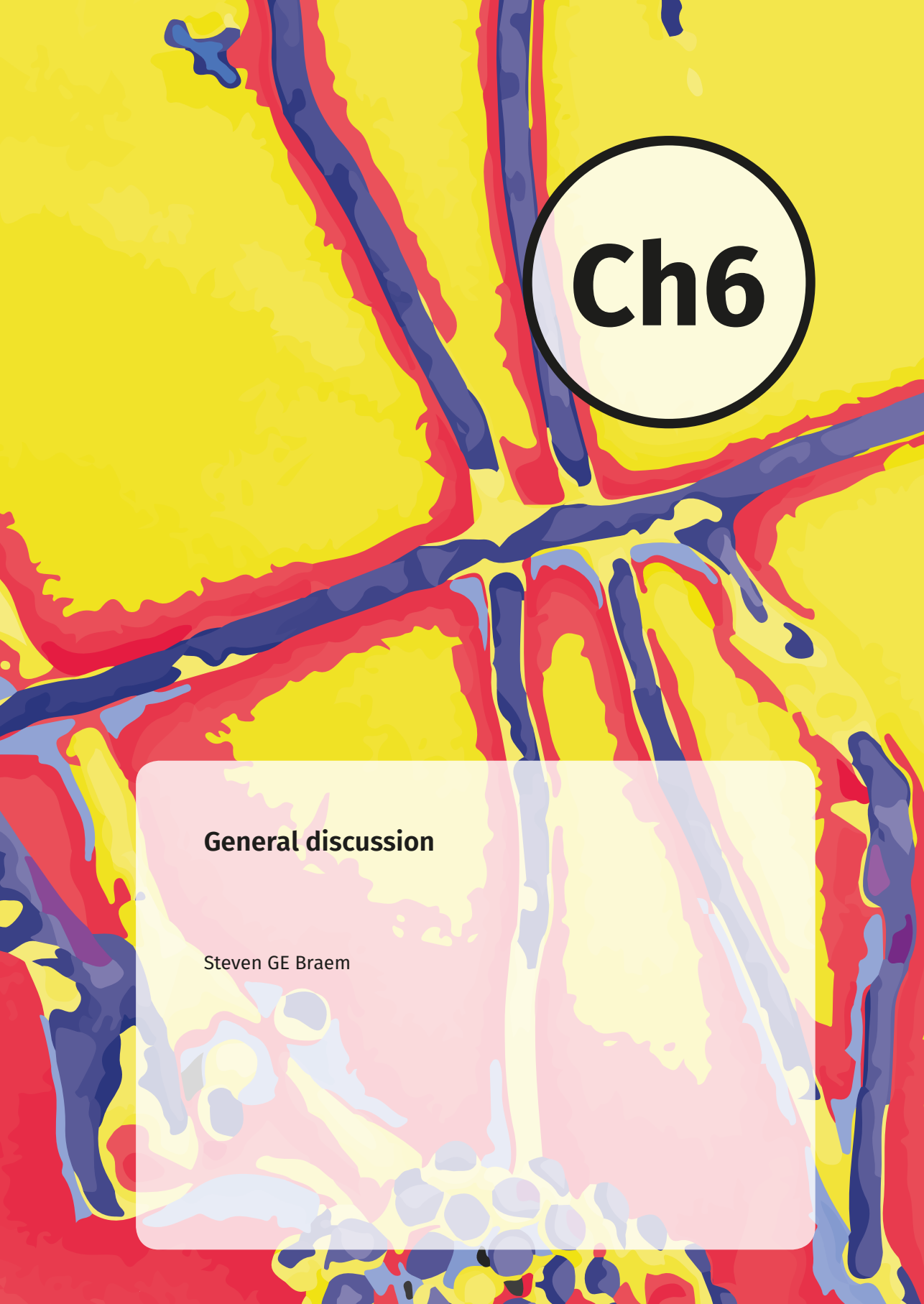
Supplemental table 7 • Differential expression of melanin biosynthesis genes

GI number	control (RPKM)	MP (RPKM)	log, Ratio	Expression	p-value	FDR	Accession	Annotation	Protein name
71002824	0,001	0,30	8,20	Up	0	0	XP_756093.1	conidial pigment biosynthesis scytalone dehydratase Arp1	Arp1
71002820	0,29	0,75	1,35	Up	4,60E-14	6,89E-14	XP_756091.1	conidial pigment biosynthesis 1,3,6,8-tetrahydroxynaphthalene reductase Arp2	Arp2
146323096	0,14	0,18	0,38	Indifferent	7,75E-08	9,20E-08	XP_756088.2	conidial pigment biosynthesis oxidase Arb2	Arb2
71002828	2,19	2,54	0,21	Indifferent	2,05E-10	2,69E-10	XP_756095.1	conidial pigment polyketide synthase PksP/Alb1	Alb1
146323098	0,04	0,04	0,05	Indifferent	6,83E-01	6,94E-01	XP_756089.2	conidial pigment biosynthesis oxidase Abr1/brown 1	Abr2
71002818	0,19	0,001	-7,60	Down	3,71E-214	1,05E-213	XP_756090.1	conidial pigment biosynthesis protein Ayg1	Ayg1

Supplemental table 8 • Differential expression of gliotoxin biosynthesis genes

GI number	control (RPKM)	MP (RPKM)	log, Ratio	Expression	p-value	FDR	Accession	Annotation	Protein name
70992025	0,001	0,10	6,60	Up	0	0	XP_750861.1	methyltransferase GliN	GliN
70992013	0,01	0,07	2,49	Up	6,71E-13	9,84E-13	XP_750855.1	nonribosomal peptide synthase GliP	GliP
146324463	0,15	0,42	1,49	Up	6,71E-13	9,82E-13	XP_750864.2	conserved hypothetical protein	GliH
71001528	5,53	8,01	0,53	Indifferent	0	0	XP_755445.1	conserved hypothetical protein	GtmA
70992007	1,24	1,69	0,45	Indifferent	9,74E-12	1,37E-11	XP_750852.1	C6 finger domain protein GliZ	GliZ
70992027	7,21	6,54	-0,14	Indifferent	6,56E-34	1,10E-33	XP_750862.1	cytochrome P450 oxidoreductase GliF	GliF
70992029	59,31	51,78	-0,20	Indifferent	0	0	XP_750863.1	thioredoxin reductase GliT	GliT
70992023	0,05	0,001	-5,63	Down	2,83E-73	5,66E-73	XP_750860.1	MFS gliotoxin efflux transporter GliA	GliA
146324455	0,05	0,00	-5,65	Down	2,90E-70	5,59E-70	XP_750856.2	cytochrome P450 oxidoreductase GliC	GliC
146324459	-	-	-	Not expressed	-	-	XP_750858.2	glutathione S-transferase GliG	GliG
70992009	-	-	-	Not expressed	-	-	XP_750853.1	aminotransferase GliI	GliI
146324453	-	-	-	Not expressed	-	-	XP_750854.2	membrane dipeptidase GliJ	GliJ
146324461	-	-	-	Not expressed	-	-	XP_750859.2	gliotoxin biosynthesis protein GliK	GliK
146324457	-	-	-	Not expressed	-	-	XP_750857.2	O-methyltransferase GliM	GliM

Gene expression was expressed as reads per kilo base per million reads (RPKM) in non-exposed (control) and methylprednisolone exposed micro-colonies (MP). Gene expression was considered differential if the expression between the groups has a $\log_2$ difference of ≤ -1 or ≥ 1 and a false discovery rate (FDR) of ≤ 0,001. If the transcript was not present in both situations, the gene was considered not to be expressed. The geninfo identifier (GI) number, accession, annotation, protein name of the genes are depicted.



General discussion

Steven GE Braem

DISCUSSION

Opsonins

Opsonins are proteins that label surfaces of invading pathogens to improve the recognition, phagocytosis and consequent removal by host cells. Opsonins are found throughout the human body in blood, tissue and other body fluids. High concentrations of opsonins are found in serum, e.g. antibodies, complement proteins, and pentraxins. Other opsonins, e.g. surfactant proteins, are present on the surface area of the lung. The effect of opsonization, labeling surfaces with opsonins to enhance phagocytosis, has already been described in 1903[1]. In general, the opsonization of pathogens leads to a more efficient uptake of the pathogen by professional phagocytes. In this thesis we investigated two processes leading to the opsonization of *A. fumigatus*.

Antibodies

Antibodies can opsonize pathogens leading to a direct or indirect recognition by neutrophils. Immunoglobulin M (IgM) is the first antibody that is secreted upon initial infection and presentation of an antigen. Although it cannot be recognized by receptors on immune cells directly, IgM is a potent activator of the complement system resulting in C3b opsonization of the invading pathogen. The role of IgM in the immune response against *A. fumigatus* is not thoroughly studied. Although we tested sequential serum samples of hematological patients before initial *A. fumigatus* infection, we were unable to measure any IgM in these sera.

IgG serves as an opsonin that can be recognized directly by Fcγ receptors (FcγRs) or indirectly via complement-mediated opsonization. Generally, the recognition of antibody-antigen complexes by FcγRs induces the phagocytosis of pathogens by neutrophils. Opsonization of *A. fumigatus* with only antibodies does not result in neutrophil phagocytosis. In contrast, other fungal and bacterial pathogens, like *Candida albicans*, *Staphylococcus aureus* and *Streptococcus pneumoniae,* are efficiently phagocytosed by neutrophils when opsonized with antibodies alone[2-4]. In these cases, neutrophil phagocytosis was mediated by FcγRs. Antibody opsonization in the presence of complement proteins, like in serum, also leads to the activation of the classical complement pathway on microbial surfaces. The classical pathway induces the complement activation on *A. fumigatus* swollen conidia and germ tubes resulting in the opsonization with C3b. The complement-mediated opsonization of *A. fumigatus* leads to phagocytosis and subsequent killing by neutrophils.

The presence of opsonic antibodies recognizing surface epitopes on microbial surfaces is required to activate the classical complement pathway. Opsonic antibodies are present in all healthy individuals and hematological patients. Since the antibody levels varied considerably between individuals, we could investigate the role of antibodies in the host defense against *A. fumigatus* in more detail. We correlated the levels of opsonic antibodies to the efficiency of complement opsonization and neutrophil phagocytosis. Additionally, *A. fumigatus* killing by neutrophils was also related to high levels of opsonic antibodies. Interestingly, low levels of opsonic antibodies prior treatment increased the change of the development of early-onset invasive aspergillosis in hematological patients.

In general, patients with hypogammaglobulinemia are more susceptible to

infections with encapsulated bacteria and not specifically to fungal infections. However, patients with hypogammaglobulinemia (<400 mg/dl IgG) after lung transplantation developed invasive aspergillosis more often than lung transplant patients with normal antibody levels[5]. This suggests that antibodies have a protective role in the host defense against *A. fumigatus*.

The existence of a protective effect of antibodies in particular patient populations indicates the potential benefit of antibody-based therapy in *A. fumigatus* infections. Passive immunization with opsonic antibodies against *A. fumigatus* or active immunization by vaccination could reduce the risk to develop invasive aspergillosis. In mice, passive immunization by the administration of monoclonal antibodies against a cell wall glycoprotein or a sialylated oligosaccharide enhances protection in a systemic *A. fumigatus* infection model[6,7]. Vaccination with a β-glucan conjugate vaccine or fixed group B *Streptococcus* type Ib also enhances survival in systemic infection models in mice[6,8]. Although immunization would be possible, the search for good epitopes will be challenging. In addition, the antibody subtype and its affinity determine the anti-fungal response. Since FcγR-mediated phagocytosis of *A. fumigatus* is neglectable, the antibodies induced by immunization should bind the fungal surface, being optimal complement activators.

It should be stressed that opsonic antibodies alone are not sufficient to combat *A. fumigatus*. Neutrophils play a key role in the clearance of *A. fumigatus*[9]. Neutropenia and neutrophil dysfunction are main risk factors in the development of invasive aspergillosis. Moreover, neutropenic mice are unable to clear *A. fumigatus* infections. Mice in which neutrophils are depleted three hours after intratracheal infection will succumb, while mice depleted from neutrophils six hours after infection will survive[9]. Antibodies cannot overcome this immunosuppressed status during neutropenia. However, antibodies aid in the host defense when neutrophil counts reestablish[6].

Complement

Complement-mediated opsonization of pathogens is important for efficient phagocytosis by neutrophils. Activation of the complement system by any of the three initiation pathways leads to the opsonization of C3b. Neutrophils recognize C3b via complement receptors, resulting in efficient phagocytosis of the pathogen. We showed that complement is mainly initiated via the classical pathway on swollen conidia and germ tubes of *A. fumigatus*. Complement activation leads to an instant labeling with C3b molecules on the fungal surface. The C3b opsonization is essential for neutrophil phagocytosis and killing of *A. fumigatus*. Since antibody opsonization has no direct role in the phagocytosis by neutrophils, it should be underlined that the effect of opsonic antibodies in *A. fumigatus* host defense is mainly dependent on the activation of the classical complement pathway.

Although complement deficiencies in humans do not directly correlate with increased susceptibility to *A. fumigatus* infection, the complement system does play an important role in *A. fumigatus* immunity. The best proof for this comes from studies with genetically engineered mice. MBL-deficient mice are as susceptible as wild type mice[10]. However, C1q-deficient mice are more susceptible to *A. fumigatus* infections, again indicating the importance of antibody-dependent opsonization[11]. Another link between antibodies and the complement system is the fact that passive transfer of

opsonic antibodies is protective in wild type mice, but not in C3-deficient mice[6]. This let us speculate that the protective role of opsonic antibodies is linked via the antibody-dependent classical complement pathway activation. In addition, C5-deficient mice are more susceptible in an intratracheal infection model[12]. This is explained by the fact that these mice are unable to generate the chemoattractant C5a upon complement activation resulting in reduced neutrophil recruitment towards the site of infection.

In conclusion, the host defense against *A. fumigatus* is particularly mediated by neutrophils. However, the aid of opsonic antibodies and the activation of the complement system are essential to opsonize *A. fumigatus* with C3b, to prime and attract neutrophils inducing rapid and efficient phagocytosis and subsequent killing of *A. fumigatus*.

Pentraxin 3

Although not studied in this thesis, the long pentraxin 3 (PTX3) is an important opsonin in the *A. fumigatus* defense. PTX3 is produced and secreted by phagocytes upon activation by conidia[11,13]. PTX3 binds directly to *A. fumigatus* conidia via the cell wall polysaccharide galactomannan, and induces phagocytosis and killing by macrophages and neutrophils[11,13,14]. The opsonic property of PTX3 is dependent on FcγRs and the activation of the complement system[13]. PTX3 can influence the complement-mediated opsonization in several ways. Binding of C1q to PTX3 opsonized-surfaces induces the classical complement pathway activation. PTX3 can also bind C1q in fluid phase, preventing the binding of C1q to antibody-antigen complexes and thus the activation of the classical complement pathway[15]. Another way how PTX3 acts on the complement system is by synergizing the complement activation induced by ficolin-2[16]. Moreover, the attraction of factor H (FH) to PTX3 opsonized-surfaces can modulate the alternative pathway activity[17].

The importance of PTX3 in the immunity against *A. fumigatus* is stressed in mice and humans lacking or having reduced amounts of PTX3. PTX3-deficient mice are more susceptible to *A. fumigatus* infection than wild type mice caused by reduced phagocytosis and killing by phagocytes[11,14]. In human, it has been shown that patients receiving a bone marrow transplant from a donor, with a homozygous SNP in *PTX3*, are at higher risk to develop invasive aspergillosis. This is due to a lower concentration of PTX3 in neutrophils, resulting in a reduced phagocytosis and killing of *A. fumigatus* conidia[18].

Surfactant protein A

Surfactant protein A (SP-A) is a collectin that is mainly produced in the lung and binds to numerous fungal, bacterial and viral pathogens. Binding of SP-A to invading pathogens can lead to agglutination, which enhances the phagocytosis efficacy. SP-A also induces phagocytosis independent of agglutination via opsonization of the pathogen, activation of the immune cell or the upregulation of surface receptors involved in the direct recognition of the pathogen. Binding of SP-A to *A. fumigatus* conidia has been reported to induce agglutination, phagocytosis and killing by neutrophils and macrophages[19]. However, we could not confirm agglutination and neutrophil phagocytosis upon SP-A opsonization (data not shown).

An immediate lung defense upon encountering *A. fumigatus* would be

beneficial for the host. However, SP-A-deficient mice are more resistant to an intranasal challenge with *A. fumigatus* than wild type mice[20]. In contrast, SP-A-deficient mice are more susceptible to an arsenal of pathogens e.g. viruses (respiratory syncytial virus, influenza A virus, adenovirus), bacteria (group B *Streptococcus*, *Pseudomonas aeruginosa*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *S. aureus*), and other fungi (*Pneumocystis jirovecii*)[21-29].

This observation suggests that *A. fumigatus* utilizes SP-A for successful colonization and infection. SP-A binding to conidia could enhance adherence and uptake by lung epithelial cells. Since lung epithelial cells are non-professional phagocytes, conidia can survive and hide from the effective immune response by neutrophils. SP-A could also influence the activation of the classical complement pathway. SP-A binds to C1q and prevents C1 complex formation and C1 binding to immune complexes[30]. This could lead to a reduction in complement-mediated opsonization on *A. fumigatus* conidia.

We identified a cell wall protein XP_748409 that binds SP-A. XP_748409 is secreted and anchored to the membrane via a glycosylphosphatidylinositol (GPI) anchor. Since XP_748409 is only 182 amino acids long, it is unlikely that the N-terminus of the protein can reach the extracellular milieu when anchored to the membrane and is thus unable to bind SP-A. However, its GPI anchor can be cleaved by phospholipase C or proteases releasing XP_748409. Probably, XP_748409 will be partly retained in the cell wall due to its heavily glycosylated C-terminal serine-rich domain. This movement of XP_748409 from the cell membrane to the cell wall could enable the exposure of the N-terminal part to the extracellular milieu and potentiates the binding of SP-A to XP_748409. Undoubtedly, this protein location disables the opportunity of XP_748409 to activate a downstream signal cascade after engagement.

Since SP-A can recognize sugar moieties on the fungal surface directly, the expression of a SP-A binding protein would not be necessary. However, it could be that the orientation of SP-A is different when SP-A is bound to the cell wall protein XP_748409 or when SP-A is bound to the cell wall polysaccharides. SP-A binds *A. fumigatus* conidia and XP_748409 in a Ca^{2+}-dependent manner, which indicates that binding of SP-A is mediated via its lectin domains. Also, the phagocytosis of SP-A opsonized conidia by neutrophils and macrophages suggests a lectin dependent binding of SP-A[19]. Although it is questionable whether agglutination of *A. fumigatus* conidia occurs, agglutination can only be induced via the binding of the SP-A lectin domains to various conidia. However, the identification of a cell wall protein attracting SP-A is indicative for another role and orientation of SP-A. Although XP_748409 binds SP-A dependent on Ca^{2+}, the orientation of SP-A could be different than its native configuration on the cell wall. Moreover, the reduced susceptibility of SP-A-deficient mice compared to wild type mice to *A. fumigatus* infection indicates another role for SP-A in the *A. fumigatus* host defense than increased phagocytosis and killing. Of interest, the cellular response differs strongly depending on the orientation of SP-A bound to its receptor. Receptor binding to the SP-A collagen-like domain will induce an inflammatory response, while binding to the SP-A lectin domain will induce an anti-inflammatory response[31].

Various factors influence the immune response induced by SP-A. The presence and type of pathogen, the type and activation status of the immune cell, and the time of exposure influence the function and importance of SP-A in host defense, and result in

the description of reports with contradicting observations. Therefore, the exact role of SP-A as opsonin in the immunity against *A. fumigatus* remains currently unclear.

CONCLUSION

Several opsonins are involved in the immunity against *A. fumigatus*. Upon infection, *A. fumigatus* is labeled by opsonins to enhance recognition resulting in fast and efficient clearance. Although all opsonins have their own function and strategy, the interplay between the individual opsonins determines in which manner the immune system is activated, and how *A. fumigatus* is cleared from the human host.

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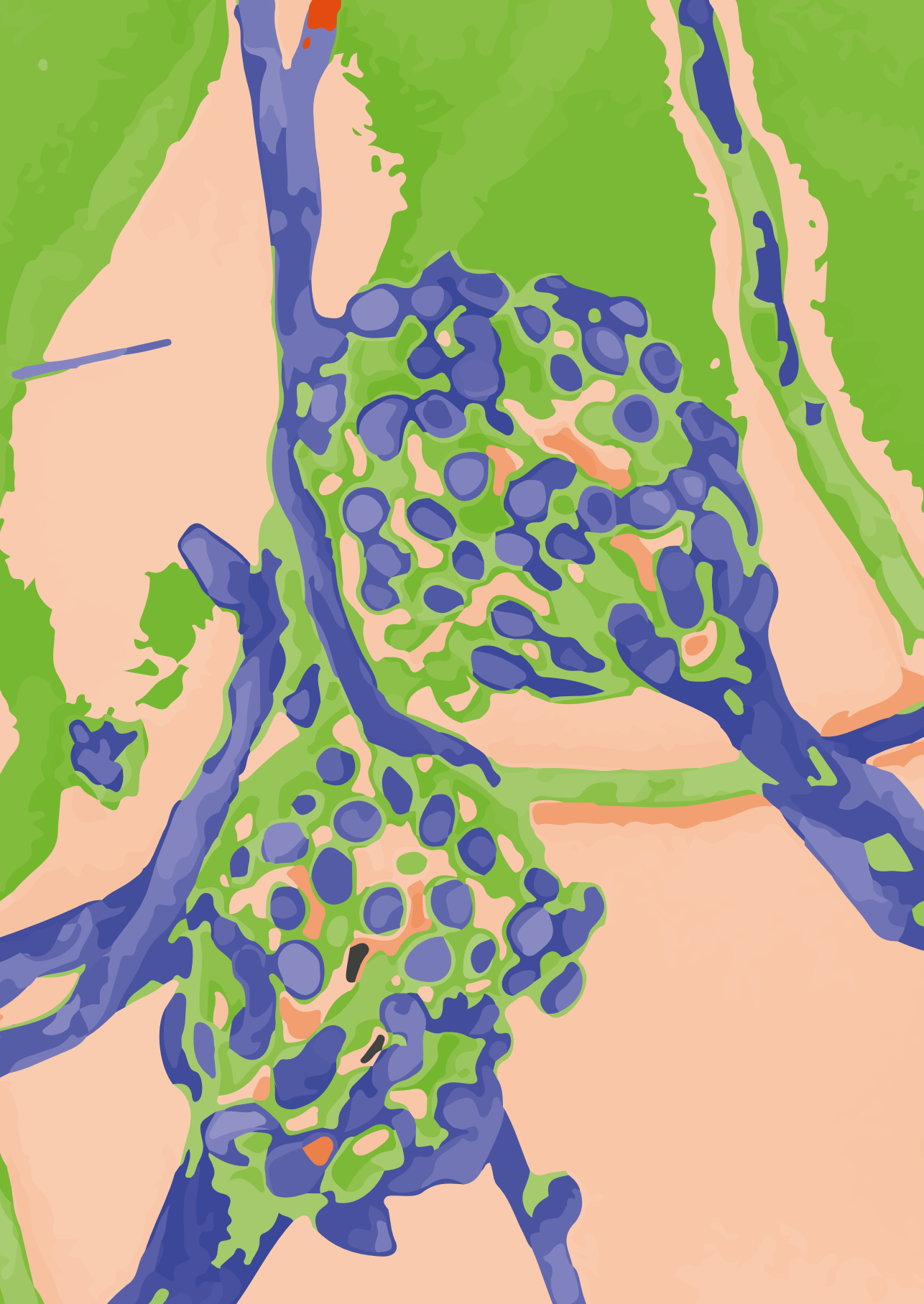

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NEDERLANDSE SAMENVATTING

Aspergilli zijn schimmels die overal in het milieu voorkomen en bestaan uit een groep van meer dan 200 soorten. Enkele *Aspergillus* soorten kunnen ziekten veroorzaken bij de mens. Een van deze soorten is *Aspergillus fumigatus*, welke verantwoordelijk is voor meer dan 90% van de ziekten die veroorzaakt worden door *Aspergillus* soorten. Infecties met *A. fumigatus* kunnen leiden tot verschillende longaandoeningen, zoals allergie (allergische bronchopulmonaire aspergillose) en invasieve groei in longweefsel (invasieve aspergillose). Invasieve aspergillose komt voornamelijk voor bij patiënten met een ernstig verzwakt immuunsysteem. Vooral leukemie patiënten en transplantatie patiënten hebben een verhoogde kans op het ontwikkelen van invasieve aspergillose, omdat hun imuunsysteem ernstig verzwakt is ten gevolge van de behandeling van hun ziekte.

Aspergillus fumigatus verspreidt zich door de lucht via kleine hydrofobe sporen (0.002 - 0.003 mm). Deze sporen zijn continu aanwezig in de lucht in een relatief hoge concentratie, variërend van 10 tot 400 sporen per m^3. Hierdoor ademt de mens iedere dag honderden sporen in. De meeste sporen zullen worden opgeruimd door de trilharen in de neus en bronchiën. Sporen die dieper in de long terechtkomen zullen worden opgeruimd door het immuunsysteem. Patiënten met een verzwakt immuunsysteem kunnen deze sporen minder goed opruimen. Hierdoor krijgen de sporen de kans om uit te groeien. De sporen zullen eerst opzwellen en vervolgens ontkiemen. Deze ontkiemde sporen groeien verder uit en uiteindelijke ontstaat er een netwerk van schimmeldraden.

Het aangeboren immuunsysteem is belangrijk in de afweer tegen *A. fumigatus.* Dit aangeboren immuunsysteem werkt razendsnel en bevat verschillende eiwitten en immuuncellen, die in samenwerking de infectie bestrijden. Op het moment dat sporen diep in de long terecht komen, worden ze gemarkeerd met opsonine eiwitten zoals surfactant eiwitten, antilichamen en complement eiwitten. Deze markering leidt tot een betere herkenning, opname en vernietiging van de schimmel door phagocyten. Neutrofielen zijn phagocyten en zijn de belangrijkste immuuncellen in de afweer tegen *A. fumigatus*. Daardoor hebben patiënten met weinig of slecht functionerende neutrofielen een verhoogde kans op de ontwikkeling van invasieve aspergillose.

Neutrofielen functioneren beter als de ziekteverwekker gemarkeerd is met opsoninen. Belangrijke opsoninen in het bloed zijn antilichamen en complement eiwitten. Complement wordt geactiveerd via de klassieke, lectine en alternatieve weg. De klassieke weg wordt geïnitieerd door de binding van antilichamen aan bepaalde eiwitten en suikers op het oppervlak van de ziekteverwekker. De lectine weg wordt geïnitieerd door directe herkenning van suikers op het oppervlak van de ziekteverwekker. De alternatieve weg zorgt voornamelijk voor een toename van activatie van complement via de klassieke en lectine weg. De activatie van complement zorgt voor de opsonisatie met C3b. C3b is een belangrijke opsonine voor de efficiënte opnamen van ziekteverwekkers door neutrofielen. Complement activatie resulteert ook in de generatie van C3a en C5a, wat ervoor zorgt dat neutrofielen worden aangetrokken en migreren naar de plek van de infectie.

In **hoofdstuk 2** hebben we het belang van complement in de afweer tegen *A. fumigatus* onderzocht. We hebben de activatie van complement onderzocht in aanwezigheid

van verschillende typen *A. fumigatus* sporen (in de lucht zwevende sporen, gezwollen sporen en ontkiemde sporen). Alle typen sporen activeren complement, wat leidt tot opname en vernietiging door neutrofielen. Opsonisatie van ziekteverwekkers met alleen antilichamen kan leiden tot opname door neutrofielen. Echter, *A. fumigatus* wordt niet opgenomen door neutrofielen wanneer de schimmel alleen met antilichamen is geopsoniseerd. Daarnaast is gebleken dat de klassieke weg het meest belangrijk is voor de initiatie van complement op gezwollen en ontkiemde sporen.

Aangezien de klassieke weg geïnitieerd wordt door antilichamen hebben we het belang van antilichamen vervolgens onderzocht in gezonde individuen. Dit wordt beschreven in **hoofdstuk 3**. Gezonde individuen hebben antilichamen tegen de buitenkant van gezwollen sporen. Echter, de hoeveelheid bindende antilichamen is verschillend per individu. Aanwezigheid van veel bindende antilichamen zorgt voor een goede complement activatie, opname en vernietiging van gezwollen sporen. Deze processen zijn minder efficiënt wanneer weinig bindende antilichamen aanwezig zijn. Tevens hebben we het belang van antilichamen onderzocht in patiënten met leukemie. De hoeveelheid bindende antilichamen werd gemeten in het bloed, voordat de patiënt een behandeling kreeg tegen de leukemie. Patiënten met een lage hoeveelheid aan bindende antilichamen bleken een 7 keer groter risico te hebben op invasieve aspergillose in de eerste 40 dagen na behandeling, dan patiënten met een hogere hoeveelheid bindende antilichamen. Kortom, antilichamen spelen een belangrijke rol in de activatie van complement, resulterend in een efficiënte opname en vernietiging van *A. fumigatus*.

In **hoofdstuk 4** beschrijven we de ontdekking van een eiwit van *A. fumigatus* wat surfactant eiwit SP-A kan binden. SP-A is ook een opsonine en komt voornamelijk voor in de long. SP-A bindt aan sporen van *A. fumigatus*. Met behulp van een eiwitten bibliotheek is een eiwit geïdentificeerd van *A. fumigatus* dat SP-A bind. Dit eiwit kan alleen aan SP-A binden in de aanwezigheid van calcium. In de aanwezigheid van het eiwit wordt de binding van SP-A aan sporen geremd. Meer onderzoek is nodig om te achterhalen wat de precieze functie is van dit eiwit in de afweer tegen *A. fumigatus*.

In **hoofdstuk 5** beschrijven we het effect van glucocorticoïden op de groei en genexpressie van *A. fumigatus*. Het gebruik van glucocorticoïden resulteert in de onderdrukking van de werking van het immuunsysteem. Glucocorticoïden worden onder andere gebruikt om afstoting van orgaan transplantatie tegen te gaan en om het menselijke lichaam te beschermen tegen overmatige immuunreacties. In deze studie bekijken we de effecten van drie verschillende glucocorticoïden (dexamethason, hydrocortison en methylprednisolon) op de groei van verschillende *Aspergillus* soorten. De groei van *A. fumigatus* wordt bevorderd door blootstelling aan hydrocortison en methylprednisolon. Echter, beide glucocorticoïden remmen de groei van twee minder ziekmakende *Aspergillus* soorten, *A. niger* en *A. tubingensis*. Om te bepalen hoe *A. fumigatus* reageert op de blootstelling aan methylprednisolon (groeibevorderend glucocorticoïd), is gekeken naar de expressie van *A. fumigatus* genen. De blootstelling van methylprednisolon resulteerde in veranderde expressie van genen, gerelateerd aan de opbouw van de celwand, de aanmaak van melanine en de productie van gliotoxine. Vooral de expressie van hydrofobine genen was toegenomen. De veranderingen in

genexpressie van *A. fumigatus* door blootstelling aan methylprednisolon zou kunnen leiden tot een agressiever infectie.

Het immuunsysteem heeft een heleboel opsoninen die betrokken zijn bij de bestrijding van infecties met *A. fumigatus*. Een aantal opsoninen zijn beschreven in dit proefschrift. De bevindingen in dit proefschrift geven meer inzicht in het infectieproces van *A. fumigatus*, de afweer tegen *A. fumigatus* en dragen bij aan de ontwikkeling van nieuwe therapieën.

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of wat kopwerk te mogen verrichten op een dijk tegen de wind in.

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LIST OF PUBLICATIONS

Braem SG, Rooijakkers SH, van Kessel KP, de Cock H, Wösten HA, van Strijp JA, Haas PJ. *Effective Neutrophil Phagocytosis of Aspergillus fumigatus Is Mediated by Classical Pathway Complement Activation.* J Innate Immun. 2015; 7:364-374

van Gent M, **Braem SG**, de Jong A, Delagic N, Peeters JG, Boer IG, Moynagh PN, Kremmer E, Wiertz EJ, Ovaa H, Griffin BD, Ressing ME. *Epstein-Barr virus large tegument protein BPLF1 contributes to innate immune evasion through interference with toll-like receptor signaling.* PLoS Pathog. 2014; 10(2):e1003960

CURRICULUM VITAE

Steven Braem was born at November 11th 1985 in Oostburg, Zeeland, the Netherlands. In 2004 he completed his secondary school at the Zwincollege in Oostburg, with a focus on biology, chemistry, physics and mathematics. In the same year he started his bachelor's study Biomedical Sciences at the Utrecht University. In 2007 he had a fulltime position as vice-chairman of the study society of Biomedical Sciences for one year. In 2008 he obtained his Bachelor of Science degree and started his master's study Infection and Immunity in the Graduate School of Life Sciences at Utrecht University. During his study he had the opportunity to perform an internship at the department of Immunology at the Wilhelmina Children's Hospital in Utrecht in the immune regulation group of prof. dr. Linde Meyaard. A second internship was performed at the department of Medical Microbiology at the University Medical Center Utrecht in the experimental virology group of prof. dr. Emmanuel Wiertz. In 2010 he obtained his Master of Science degree and started working as a research technician in the group of prof. dr. Emmanuel Wiertz for four months. In 2011 he obtained a PhD position at the department of Medical Microbiology at the University Medical Center Utrecht under the supervision of dr. Pieter-Jan Haas and prof. dr. Jos van Strijp. During his PhD training he studied the interaction between the pathogenic fungus *Aspergillus fumigatus* and the host defense. The findings of his PhD research have been described in this thesis.