Interaction between the opportunistic pathogen *Aspergillus fumigatus* and its host

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Interaction between the opportunistic pathogen *Aspergillus fumigatus* and its host

Interactie tussen de opportunistische pathogeen *Aspergillus fumigatus* en zijn gastheer

(met een samenvatting in het Nederlands)

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te Dronten

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What were once only hopes for the future have now come to pass.

Queen Elizabeth II

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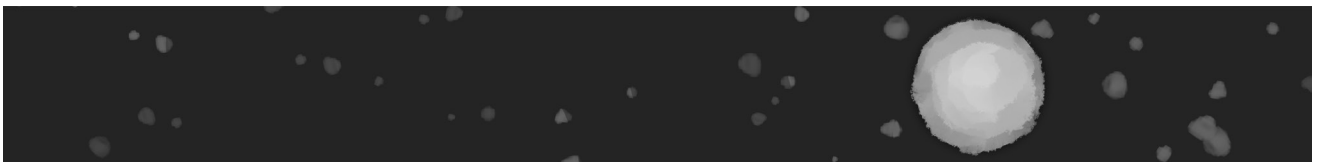

1

General Introduction

Esther M. Keizer

Aspergillus species are omnipresent fungi. They can grow in a wide variety of environmental conditions. For instance, they can grow at a relatively low and high humidity and at a wide pH and temperature range (Krijgsheld et al., 2013). To date, 446 species belong to the genus *Aspergillus* that is divided in the subgenera *Aspergillus, Circumdati, Cremei, Fumigati, Nidulantes* and *Polypaecilum*. For instance, *Aspergillus fumigatus* and *Aspergillus niger* belong to *Fumigati* and *Circumdati*, respectively (Houbraken et al., 2020).

Aspergilli are saprotrophic fungi that grow on organic debris (Latgé, 1999). Germination of conidia initiates the colonization of the substrate resulting in an interconnected hyphal network called mycelium (Adams et al., 1998). Hyphae in the mycelium specialize by forming secretion hyphae, resistor hyphae and aerial hyphae (Champe & Simon, 1992; Tegelaar et al., 2020). Both thin and wide aerial hyphae are distinguished. The latter will further develop into conidiophores that produce conidia, the end product of asexual reproduction. *A. fumigatus,* but not *A. niger,* also has a sexual reproduction stage, which is exclusively heterothallic (O'Gorman et al., 2009). The conditions for sexual reproduction of *A. fumigatus* had been described to be very restrictive in nature and under laboratory conditions (Kwon-Chung & Sugui, 2009). Yet, recent results show that sexual reproduction in *A. fumigatus* occurs more frequently than previously assumed after assessing the number of sexual fruiting bodies, called cleistothecia, that were formed after mating of several isolates (Swilaiman et al., 2020). For other aspergilli, such as *Aspergillus nidulans*, the conditions are less restrictive and sexual reproduction occurs more frequent (Seo et al., 2004). Sexual spores are formed in fruiting bodies and are therefore believed not to be efficiently dispersed. In contrast, conidia can be dispersed effectively by for instance wind, insects and water droplets (Teertstra et al., 2017). On average, we inhale several hundred *A. fumigatus* conidia a day (Mullins et al., 1984). Consequently, life-threatening infections can develop in patients with a compromised immune system (Brakhage, 2005). *A. fumigatus*, and to a lesser extent *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans*, can cause invasive and non-invasive pulmonary aspergillosis (Stevens et al., 2000). On a global level more than 10 million patients are diagnosed with an invasive and a non-invasive aspergillosis infection each year (Bongomin et al., 2017).

Infections caused by *A. fumigatus*

Conidia of *A. fumigatus* can cause lung infections upon inhalation (Figure 1). The fact that there are up to a 100 conidia of this fungus per m^3 air (Wéry, 2014) and that *Aspergillus* DNA is detected in 37 % of lung biopsies of healthy individuals shows that we are continuously exposed to this fungus (Denning et al., 2011). The conidia do not cause infections in healthy individuals and are removed by mucociliary clearance and mucosal defence mechanisms (Kerr et al., 2016). Infections caused by *A. fumigatus* in non-healthy individuals are divided in invasive and non-invasive lung infections (Figure 1). Invasive infections are the most severe *A. fumigatus* infections that occur in immunocompromised patients. Invasive pulmonary aspergillosis (IPA) is characterized by hyphae invading the lung tissue causing damage and inflammation. Eventually, the fungus can also disseminate into other organs, mostly the brain, via vascular invasion (Zmeili & Soubani, 2007). IPA is the most common invasive fungal infection in hematopoietic stem cell (HSCT) and solid organ recipients and can also be found in low-risk groups such as patients with autoimmune or inflammatory disease combined with immune metabolic

abnormalities (Herbrecht et al., 2012). In non-neutropenic patients the non-angio invasive form of IPA is mostly observed (Stergiopoulou et al., 2007), while the bronchial aspergillosis (IBA) form of IPA, characterized by necrotising infection of the lung, is observed in patients with a solid organ transplant (Latgé & Chamilos, 2019).

Non-invasive lung infections caused by *A. fumigatus* can occur in patients with asthma or cystic fibrosis. Allergic bronchopulmonary aspergillosis (ABPA) occurs due to hypersensitivity towards *A. fumigatus* allergens (Agarwal, 2009). Next to ABPA, patients can have increased sensitization towards *Aspergillus* or other fungal allergens, which is called severe asthma with fungal sensitisation (SAFS) (Agarwal et al., 2013). *A. fumigatus* can also cause *Aspergillus* bronchitis, which is a chronic superficial infection of the lower airways (Chrdle et al., 2012). A more severe non-invasive infection is an aspergilloma (fungal ball). In this case, the fungus colonizes and proliferates in pre-existing lung cavities. Aspergillomas most commonly occur in patients who have recovered from pulmonary tuberculosis but are also found in patients with sarcoidosis and other lung cavity diseases (Denning et al., 2016). In these patient groups, chronic pulmonary aspergillosis can also be found. This infection is typified by chronic inflammation and fibrosis (Denning et al., 2016; Latgé & Chamilos, 2019).

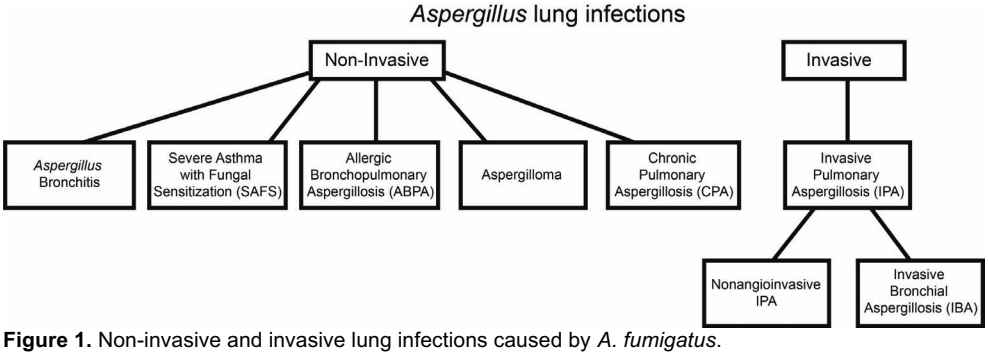

Figure 1. Non-invasive and invasive lung infections caused by *A. fumigatus*.

Amphotericin B (AMB), azoles and echinocandins are used to treat *Aspergillus* infections. AMB binds irreversibly to ergosterol, thereby affecting the barrier function of the plasma membrane resulting in fungal death (Gray et al., 2012). Azoles also target ergosterol but in this case the synthesis of this sterol is affected. Azoles block the demethylation of lanosterol, resulting in a depletion of ergosterol and accumulation of the toxic sterol 14α-methyl-3,6-diol (Watson et al., 1989). The inhibition of lanosterol methylation leads to stress as is shown by upregulation of the cell wall integrity pathway, resulting in chitin and glucan patches at the cell wall. Invagination of the plasma membrane is observed at these patches, resulting in fungal killing (Geißel et al., 2018). The enzyme for demethylation of lanosterol is encoded by the *CYP51A* and *CYP51B* genes. Mutations in *CYP51A* are frequently reported in azole resistant strains of *A. fumigatus* (Wagener & Loiko, 2017). Echinocandins represent the third class of anti-fungals used to treat *A. fumigatus* infections. These compounds inhibit β(1,3)-glucan synthase, which is a key enzyme for fungal cell wall synthesis (Dudakova et al., 2017).

Conidia and hyphal cell wall

The cell wall protects hyphae and conidia from environmental stress (Bernard & Latgé, 2001). The main structural components of the cell wall of *A. fumigatus* hyphae are α(1,3)-glucan, β(1,3)-glucan, mannan, galactomannan and chitin, where glucans represent 50 - 60 % of the cell wall, mannan and galactomannan between 20 - 30 %, while the remaining 10 - 20 % of the cell wall consists of chitin (Bowman & Free, 2006; Latgé et al., 2017) (Figure 2). Chitin and α(1,3)-glucan form a rigid scaffold for the fungal cell wall (Kang et al., 2018). Chitin is linked via a β(1,4)-linkage to the β(1,3)-glucan matrix on top of the chitin and α(1,3)-glucan scaffold (Fontaine et al., 2000; Kang et al., 2018). The outer layer of the cell wall consists of a dynamic shell of mannose and α(1,3)-glucan and glycoproteins such as galactomannan and galactosaminogalactan (GAG), the latter is only found in the vegetative mycelium (Fontaine et al., 2011; Kang et al., 2018).

Several enzymes are needed to produce the cell wall polysaccharides. The *A. fumigatus* genome contains 8 chitin synthase (CHS) genes that encode enzymes that polymerize GlcNAc into chitin (Mellado et al., 1995). Only deletion of the *chsA* and *chsE* genes reduces chitin levels in the cell wall (Aufauvre-Brown et al., 1997; Rogg et al., 2011). Yet, single deletion of each of the CHS genes causes a growth defect (Muszkieta et al., 2014). A plasma membrane bound glucan synthase complex produces β(1,3)-glucan in the cytoplasm using UDP-glucose as a substrate. The β(1,3)-glucan is deposited at the outer surface of the plasma membrane through a pore in the membrane. The catalytic subunit of this complex is encoded by *FSK1* and is regulated via Rho GTPases (Beauvais et al., 1993; Beauvais et al., 2001). The long chains of β(1,3)-glucan are made by glucanosyltransferases, which elongate the β(1,3)-glucan chains by splitting the β(1,3)-glucan and then transferring the exposed reducing end to a non-reducing end of another β(1,3)-glucan (Mouyna et al., 2000). Deletion of the genes involved in β(1,3)-glucan synthesis decreases the amount of β(1,3)-glucan in the hyphal cell wall. This decrease is compensated by an increase of chitin and galactosaminogalactan, but also in a decrease in galactomannan (Dichtl et al., 2015). The α(1,3)-glucan is synthesized by the α(1,3)-glucan synthases (AGS1, AGS2 and AGS3) (Henry et al., 2012). Deletion of the encoding genes completely abolishes α(1,3)-glucan synthesis, coinciding with an increased amount of β(1,3)-glucan and chitin (Henry et al., 2012). The *Uge5* gene is needed for the synthesis of galactomannan and the galactofuranose attached to the galactomannan (Lee et al., 2014), while mannan is synthesized via mannosyltransferases (Henry et al., 2016).

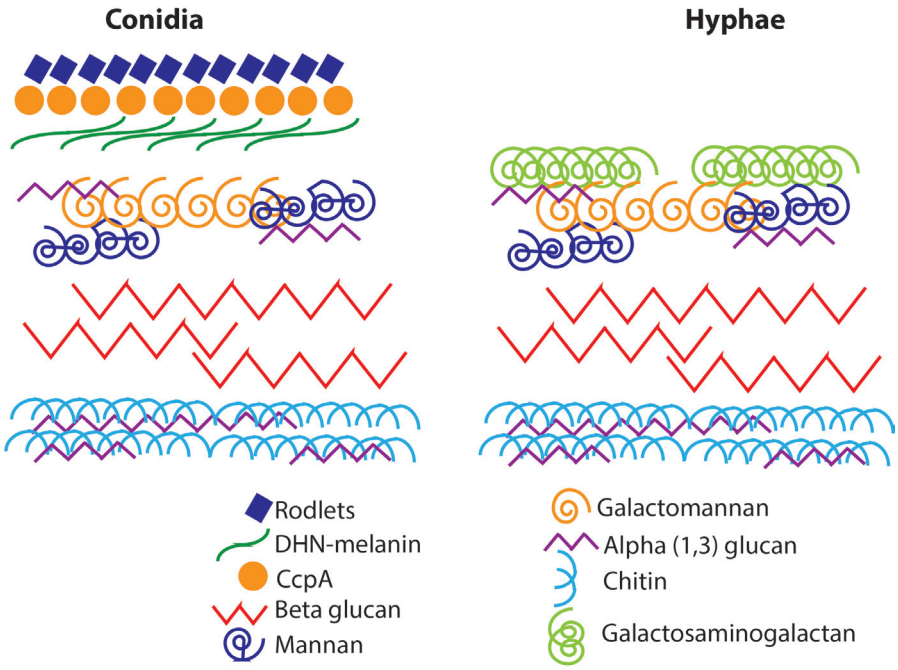

Figure 2. Schematic overview of the cell wall of hyphae (A) and conidia (B) of *A. fumigatus*. Adapted from (Garcia-Rubio et al., 2020; Kang et al., 2018).

Conidial surface

The surface layer of *A. fumigatus* conidia consists of a network of amphipathic proteins called hydrophobins. These proteins make the conidia hydrophobic by forming the rodlet layer. There are seven hydrophobin genes in *A. fumigatus* (*rodA - rodG*). Only deletion of the gene encoding the hydrophobin RodA makes the conidia more hydrophilic and abolishes rodlet formation, whereas deletion of the other hydrophobin genes do not alter the conidia (Girardin et al., 1999; Paris et al., 2003; Thau et al., 1994; Valsecchi et al., 2017). Stealth protein cell wall protein A (CcpA) is also located near the conidial surface. CcpA is just as abundant as RodA and like this hydrophobin, it prevents recognition of the conidia by the innate immune system (Voltersen et al., 2018). The layer of hydrophobins and CcpA covers a green pigmented layer consisting of 1,8-dihydroxynaphthalene (DHN)-melanin. The first and essential gene in the biosynthesis pathway of DHN-melanin is the polyketide synthase *pksP* (Jahn et al., 1997; Langfelder et al., 1998; Tsai et al., 1998). Genes *ayg1* (hydrolase), *arp2* (reductase), *arp1* (dehydratase), *abr1* (oxidase) and *abr2* (laccase) are also needed for the synthesis of DHN-melanin (Bayry et al., 2014) (Figure 3), but deletion of these genes does not abolish pigment formation completely (Heinekamp et al., 2012). Next to the lack of pigment formation, deletion of the *pksP* gene also leads to hydrophilic conidia due to an unorganized rodlet layer and the presence of glycoproteins on top of the hydrophobin layer (Bayry et al., 2014). Even with these three layers at the conidial surface (CcpA, hydrophobins and melanin), still some of the underlying cell wall structure can be exposed, such as small β-glucan patches (Steele et al., 2005).

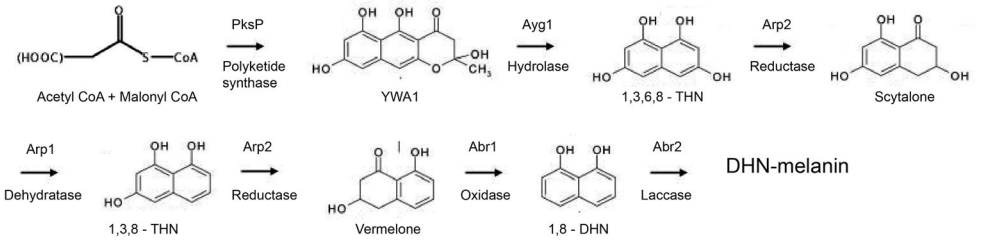

Figure 3. Synthesis pathway of DHN-melanin. Adapted from (Bayry et al., 2014; Eisenman & Casadevall, 2012; Heinekamp et al., 2012).

Immune response of the host

Innate immune response in the lung

Due to their small size (<5 µm) *A. fumigatus* conidia can reach deeper parts of the lung and, as a consequence, the lung alveolar epithelium (McCormick et al., 2010). In healthy individuals, the conidia are cleared by mucociliary clearance and mucosal defence mechanisms of epithelial and immune cells (Figure 4). One of the defence mechanisms is the secretion of antimicrobial peptides and proteins, such as the surfactant proteins A and D (SP-A and SP-D), which reduce fungal growth and increase clearance of the fungus by neutrophils (Madan et al., 1997; Ordonez et al., 2019). The alveolar epithelium consists of the type I and the type II lung epithelial cells. The type I cells cover 95 % of the epithelial surface and are involved in gas exchange. They are in close contact with the type II cells that secrete surfactant. Different parts of the cell wall of the hyphae interact with the epithelial cells. For example, chitin and galactosaminogalactan bind to the fibrinogen C domain-containing protein (FIBCD1) (Beaussart et al., 2015; Jepsen et al., 2018). Conidia can also be taken up by the epithelial cells and surveiling alveolar macrophages. Uptake has been studied *in vitro* in epithelial cells, for example type II A549 lung epithelial cells or airway epithelial cells. It depends on actin dynamics (Escobar et al., 2016; Wasylnka & Moore, 2002), Dectin-1 (Han et al., 2011) and the Arp 2/3 complex working together with WIPF2 (Culibrk et al., 2019). Upon internalization, conidia end up in phagolysosomes, which acidify thus killing the conidia. However, conidia can survive by blocking the acidification of the phagolysosomes with DHN-melanin (Amin et al., 2014). Germinating conidia trigger the release of interleukin-8 (IL-8 or CXCL8) by epithelial cells (Balloy et al., 2008).

Alveolar macrophages are professional phagocytes, which take up conidia via the DC-SIGN and Dectin-1 receptor (Faro-Trindade et al., 2012; Serrano-Gómez et al., 2004). Activation of the Dectin-1/Syk signalling pathway by β-glucan regulates the maturation of the phagosome (Mansour et al., 2013). This leads to the acidification of the phagosome and production of NAPDH-oxidase dependent reactive oxygen species (ROS), which enable killing of the conidia. The absence of NADPH-oxidases leads to a reduced killing of phagocytosed conidia (Philippe et al., 2003). LC3-associated phagocytosis (LAP), which promotes fungal killing, is also activated by the Dectin-1/Syk signalling pathway (Akoumianaki et al., 2016). DHN-melanin inhibits the activation of LAP, which is also shown in the increase of LC3 recruitment to macrophages containing swollen conidia compared to macrophages containing dormant conidia (Sprenkeler et al., 2016). Next to this, there are other signalling pathways in macrophages, for example, the MelLec receptor, which

recognizes DHN-melanin, that regulate inflammatory responses (Stappers et al., 2018).

Neutrophils are also important in the defence against fungal infection. They are activated by the release of chemokines such as CXCL1, CXCL2 and CXCL5 (Jhingran et al., 2015). Conidia are killed by the release of ROS by neutrophils, which trigger the apoptosis program in conidia (Shlezinger et al., 2017). Secreted proteases and nutritional immunity induced by the neutrophils also facilitate killing of the conidia (Leal et al., 2013; Tkalcevic et al., 2000). Nutritional immunity is able to inhibit germination via lactoferrin mediated iron depletion (Zarember et al., 2007). Patients with chronic granulomatous disease (CGD) lack ROS production in neutrophils, due to a NADPH oxidase defect, and are characterized by recurrent fungal infections (Gazendam et al., 2016; Goldblatt & Thrasher, 2000). Germination of conidia is also inhibited by the secretion of antifungal extracellular vesicles, which are enriched in antimicrobial peptides and limit growth and induce cell damage by delivering antifungal proteins (Shopova et al., 2020). Neutrophil extracellular traps (NETs) consisting of antimicrobial peptides and chromatin fibres are made by neutrophils when they encounter conidia or hyphae from *A. fumigatus*. NET formation is maximal when neutrophils are exposed to hyphae, but reduced with resting and swollen conidia, which is explained by the inhibitory role of RodA on NET formation (Bruns et al., 2010). Even though NET formation is mainly observed when neutrophils encounter hyphae, it is not involved in the killing of hyphae. Hyphae are killed by neutrophils via the activation of NADPH via Syk-PI3K-PKC which leads to the production of ROS and release of meyeoloperoxidase (MPO) (Gazendam et al., 2016).

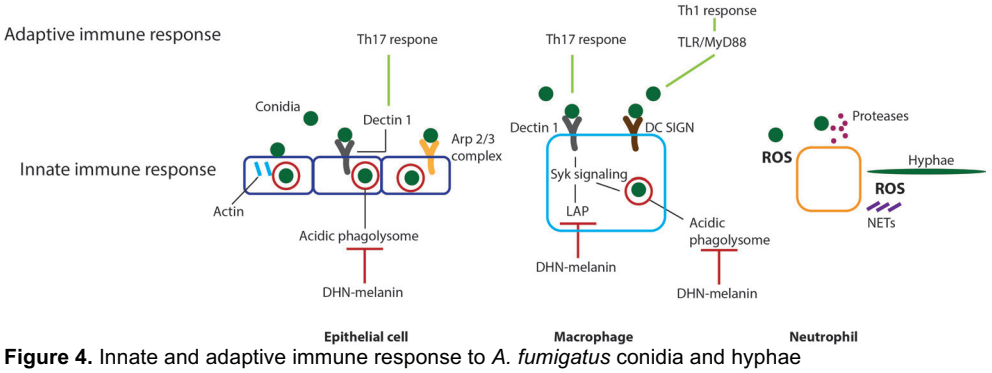

Figure 4. Innate and adaptive immune response to *A. fumigatus* conidia and hyphae

Other innate immune cells that clear a fungal infection are monocytes. They are important for the uptake and killing of conidia. Depletion of monocytes increases the development of aspergillosis. Cytokines are released upon activation of monocytes by fungal recognition, which in their turn activates neutrophils (Espinosa et al., 2014). Eosinophils are also involved in fungal killing, next to this they assist in fungal clearance by recruiting monocytes and macrophages and regulating inflammatory cytokine responses upon a fungal infection (Guerra et al., 2017). Also, humoral immunity plays a role in the defence against *A. fumigatus* infections, especially the pentraxin 3 (PTRX3) effector, which is released by epithelial cells and phagocytes. It promotes the phagocytosis of conidia by opsonisation. Deletion of this

effector leads to an increase in susceptibility towards invasive aspergillosis (Garlanda et al., 2002).

The complement system is important in the recognition and elimination of fungi, the initiation of an inflammatory response and the regulation of the adaptive immune response (Parente et al., 2020). An inactive complement system leads to an increased susceptibility to invasive aspergillosis infections but decreases the allergic reaction in ABPA patients (Garlanda et al., 2002; Hogaboam et al., 2004). The complement system consists of three pathways; the classical, lectin and alternative pathway (Merle et al., 2015). Dormant conidia are recognized by the alternative pathway via mannose binding lectins to the C3 component of the complement system (Dumestre-Pérard et al., 2008). This is also observed by a patchy C3 distribution on the conidia (Braem et al., 2015). Inhibition of the classical pathway shows that the lectin pathway is also involved in the recognition of dormant conidia (Braem et al., 2015). Upon germination, exposure of β-glucan on conidia increases, thus activating the lectin pathway via binding of ficolin-1 to this polysaccharide (Jensen et al., 2017). Hyphae also activate the classical and lectin pathway. In this case, ficolin-1 binds to chitin and mannose binding lectins interact with galactomannan (Jensen et al., 2017; Kaur et al., 2007). PTRX3, which is described above, is also able to activate the classical and lectin pathway of the complement system (Parente et al., 2020).

Adaptive immune response

The adaptive immune response against *A. fumigatus* is less studied than the innate immune response (Figure 4). CD4 and CD8 positive T-cells mediate the protective immunity against *Aspergillus* infections (Carvalho et al., 2012). The immunoglobin E and G (IgE and IgG, respectively) produced by these T-cells can also be used as a diagnostic marker for SAFS and ABPA (Farrant et al., 2016; Skov et al., 1999). Generation of Th1 anti-*Aspergillus* immunity is via TLR/MyD88, whereas Th17 immunity is generated via Dectin-1 signalling. An optimal Th17 response is obtained via IL-2 production of dendritic cells (Zelante et al., 2015). In patients with ABPA an increase in Th17 cells is observed, which contributes to the disease manifestation seen in these patients (Bacher et al., 2019). Regulatory T cells (Tregs) are important for immune homeostasis. Next to this a disbalance between the Treg and the Th2 responses is observed, leading to the allergic reaction (Bacher et al., 2016).

Fungal immune evasion

A. fumigatus has developed several defence mechanisms to escape recognition by the immune system and inhibition of growth and killing by the host. The rodlet, melanin and CcpA layer surrounding the conidia play an important role. It shields the β(1,3)-glucan and galactomannan in the cell wall, making the conidia immunological inert (Aimanianda et al., 2009; Luther et al., 2007; Voltersen et al., 2018). CcpA was the most recent discovered component that shields conidia from immune recognition. Deletion of its encoding gene induces a higher IL-8 release by neutrophils and increases the production of reactive oxygen intermediates (ROI) compared to wild-type conidia (Voltersen et al., 2018). Upon germination, the conidia start to swell and the rodlet, melanin and CcpA layer is fragmented and broken down. This leads to immune recognition of the swollen conidia and more phagocytes are attracted to the conidia (Hohl et al., 2005; Luther et al., 2007; Voltersen et al., 2018).

Growth of the fungus can be inhibited by limitation of nutrients such as iron. Iron is an essential element for growth (Kaplan & Kaplan, 2009). *A. fumigatus* uses siderophores to scavenge and store iron. Extracellular iron is scavenged by secreted siderophores (fusarinine and tracetylfusarinine C), while intracellular iron is scavenged by specific hyphal (ferricrocin) and conidial (hydroxy ferricrocin) siderophores (Haas, 2014). Deletion of *sidA* abolishes the production of siderophores and decreases virulence of *A. fumigatus* conidia in a mouse model (Schrettl et al., 2004).

Next to shielding the conidial surface, DHN-melanin plays different roles in the evasion of the immune system. DHN-melanin blocks the acidification of the lysosomes upon internalization in epithelial, macrophages and neutrophils cells (Amin et al., 2014; Escobar et al., 2016). Removal of DHN-melanin increases the phagocytosis rate of the conidia by the macrophages but reduces the uptake by type II A549 lung epithelial cells (Amin et al., 2014; Thywißen et al., 2011). Production of ROS by phagocytes is also increased when DHN-melanin is absent (Jahn et al., 1997). The PI3-Akt signalling pathway is activated by DHN-melanin, which leads to an inhibition of apoptosis in macrophages. Further inhibition of apoptosis by melanin is induced via prevention of caspase activation and cytochrome c release (Volling et al., 2011).

Activation of the complement system can be evaded via the formation of a fibrous capsule by recruited immune cells around the infection site to avoid dissemination of the fungus, but also prevents access of complement components (Parente et al., 2020) and by masking the conidia surface by rodlets, CcpA and DHN-melanin (Chai et al., 2010). But also, by the recruitment or production of inhibitors of the complement system such as Factor H or Aspf2 by *A. fumigatus* (Behnsen et al., 2008; Dasari et al., 2018; Washburn et al., 1986). Proteolytic enzymes such as Alp1 and Mep1 of *A. fumigatus*, which target and degrade complement proteins, can also be used to evade activation of the complement system (Behnsen et al., 2010; Shende et al., 2018).

Secondary metabolites

Secondary metabolites are molecules with a low molecular mass and a great diversity in structure. These metabolites are not required for growth but can be used as a signal for communication or to defend the habitat of fungi by inhibition of growth of competitors (Yim et al., 2007). Secondary metabolites can be divided into four classes. The most predominant are the non-ribosomal peptides (NRPS) and the polyketides (PKS). Examples of NRPS are antibiotics such as penicillium and cephalosporin or the fungal toxin gliotoxin (Brakhage, 1998; Scharf et al., 2012). Lovastatin, produced by *A. terreus*, lowers cholesterol in humans and is a well-known example of a PK (Hoffmeister & Keller, 2007). Next to these two groups, terpenes (or terpenoids) and fatty acids are distinguished, which are derivatives of isoprene and acetate, respectively (Bömke & Tudzynski, 2009; Brodhun & Feussner, 2011).

The multidomain synthesizing systems of NRPS and PKS use amino acids and malonyl CoA, respectively, as building blocks. The core structure of these enzymes consists of three domains. The NRPS consist of an adenylation domain - needed for amino acid activation, a peptidyl carrier - which acts as a cofactor for the activation amino acids, and a condensation domain for peptide bond formation. The PKS consist of an acetyltransferase - for selection and transfer, an acyl-carrier

protein - for extension, and a ketoacyl synthase domain for condensation (Brakhage & Schroeckh, 2011). The NRPS and PKS systems are activated by a 4'phosphopantetheinyl transferase (PPTase). Deletion of its encoding gene *pptA* abolishes the production of secondary metabolites via the PKS and NRPS pathway in *A. fumigatus, A. nidulans* and *A. niger* (Brakhage, 2013; Jørgensen et al., 2011; Keszenman-Pereyra et al., 2003; Márquez-Fernández et al., 2007).

Genes required for synthesis of secondary metabolites are located in gene clusters (Smith et al., 1990). On average *Aspergillus* species contain 50 secondary metabolite gene clusters (von Döhren, 2009). Genes in these clusters can be regulated via global regulators, which in general do not belong to the gene cluster and also regulate other genes, or pathway specific regulators that belong to the cluster they regulate (Brakhage, 2013). Pathway specific regulators for NRPS are very diverse, whereas PKS specific regulators mostly belong to the Zn cluster family of transcription factors (Shelest, 2008). Examples for pathway specific regulators are *gliZ*, which regulates gliotoxin production (Schrettl et al., 2010) or *apdR*, the regulator of aspyridone in *A. nidulans* (Bergmann et al., 2007).

Examples of global regulators of secondary metabolites are the transcription factor *pacC*, which is important for fungal pH response and regulates amongst other penicillium and sterigmatocystin synthesis (Tilburn et al., 1995). The CBC complex is another example of a global regulator and is involved in the regulation of penicillium production and senses the redox status and iron availability in cells (Thön et al., 2010). *AreA* is involved in the regulation of nitrogen and the regulation of the secondary metabolites gibberellin and fumonisin (Kim & Woloshuk, 2008; Tudzynski et al., 1999). The putative methyltransferase *laeA*, is a global regulator of secondary metabolism and regulates up to 40 % of the secondary metabolite genes in *A. fumigatus* (Perrin et al., 2007). It has been postulated to regulate gene expression via chromatin remodelling using histone methylation (Bok & Keller, 2004). Next to this, *laeA* is part of the velvet complex, which is involved in the production of secondary metabolites in response to light (Bayram et al., 2008).

Scope of this thesis

Aspergillus species are very abundant on the globe, but even though this genus has a wide variety of species, most infections are caused by *A. fumigatus*. Knowledge of the virulence mechanisms of *A. fumigatus* is increasing, for example how the immune response is evaded and how the conidia interact with host cells. However, knowledge at the molecular level is still limited. Furthermore, research has mainly focussed on a limited set of *A. fumigatus* isolates whereas (genetic) heterogeneity might result in differences in virulence. The aim of this Thesis was to obtain more detailed information on molecular interactions between fungal components of *A. fumigatus* and the host to unravel what their role is in infection and immune evasion. In addition, I addressed strain heterogeneity in context of virulence in different infection models.

The EphA2 receptor is expressed on epithelial cells and recognizes β-glucan. **Chapter 2** describes the role of this receptor in the internalization of conidia of *A. fumigatus*. Receptor activity was blocked either by antibody inhibition or by the kinase inhibitor dasatinib, which led to a 50 % reduction in internalization of conidia. The Dectin-1 receptor also recognizes β-glucan and was also described to play a role in the internalization of conidia. Dual inhibition of both receptors decreased the

internalization of conidia further, but did not abolish it, showing that there are also other receptors or pathways involved in the internalization of conidia.

The role of DHN-melanin in the protection of conidia against killing by hydrogen peroxide is described in **Chapter 3**. This protective role was proposed more than 20 years ago and represents a paradigm in *A. fumigatus* virulence. It was postulated to be required for surviving killing by immune cells that produce peroxide. In contrast to what has been described so far, it is now shown that DHN-melanin does not protect against hydrogen peroxide. In fact, the strain originally used in these studies contained an additional mutation in one of the catalase genes, which is proposed to be responsible for the increase in hydrogen peroxide sensitivity in particular of hyphae.

Chapter 4 focusses on the potential role of secreted molecules of *Aspergillus* species in immune evasion due to their ability to interact with cell surface receptors on human immune cells. This chapter shows that different *Aspergillus* species secrete molecules that interact with such receptors. The *A. niger LaeA* mutant secretes small molecules (≤ 3 kDa) which compete for binding with a subset of antibodies that interact with surface-expressed receptors of human immune cells. These results indicate that CD88, CD181 and CD182 are potential targets of these small molecules. Deletion of *laeA* stopped medium acidification. Molecules competing for binding with the CD181 receptor were still produced in other non-acidifying *A. niger* strains. The size of the molecules, as well as the heat stability and the lack of a proteinase K response, suggests that these molecules are secondary metabolites.

The virulence of five isolates of *A. fumigatus* derived from invasive or non-invasive infections was studied in type II A549 lung epithelial cells, *Protostelium aurantium* amoebae, *Galleria melonella* larvae and zebrafish embryos (**Chapter 5)**. It is shown that the virulence of the strains differs between the four different infection models. This difference is not related with the origin of the isolates from invasive or non-invasive infections but is related to genetic difference between strains. One isolate, Af293, was much less virulent compared to the other four strains. Genetic analysis indicated that Af293 contains more SNPs in virulence related genes. The reduced ability to germinate upon infection, together with other genetic differences, might be related to the reduced virulence of this isolate.

Results are summarized and discussed in **Chapter 6**.

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EphA2-dependent internalization of *Aspergillus fumigatus* conidia in A549 lung cells is modulated by DHN-melanin

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Abstract

Dectin-1 and EphA2 receptors recognize β-glucan present in the fungal cell wall. Inhibition of Dectin-1 with the monoclonal 2a11 antibody was shown to reduce internalization of conidia of the human pathogen *Aspergillus fumigatus* into epithelial cells. In this study we investigated the role of the EphA2 receptor present on A549 epithelial type II lung cells in the interaction with *A. fumigatus* conidia. We assessed whether EphA2 is involved in association and internalization of conidia by receptor inhibition by an antibody or by using the kinase inhibitor dasatinib. Internalization of conidia was reduced by 50 % when this receptor was blocked with either the EphA2-specific monoclonal antibody or dasatinib. A similar response was found when Dectin-1 was inhibited with the 2a11 monoclonal antibody. Inhibition of both receptors reduced the internalization to 40 %. EphA2 inhibition was also assessed in a hydrophobin deletion strain (Δ*rodA*) that exposes more β-glucan and a DHN-melanin deletion strain (Δ*pksP*) that exposes more glucosamine and glycoproteins. The Δ*rodA* strain behaved similar as the wild-type strain with or without EphA2 inhibition. In contrast, the Δ*pksP* mutant showed an increase in association to the A549 cells and a decrease in internalization. Internalization was not further decreased by EphA2 inhibition. Taken together, the EphA2-dependent internalization of *Aspergillus fumigatus* conidia in A549 lung cells is modulated by DHN-melanin.

Introduction

Aspergillus fumigatus is a saprotrophic fungus, which is able to colonize a large variety of dead organic material and living organisms (Krijgsheld et al., 2013). Colonies of this fungus produce asexual spores that are dispersed via the air. On average, we inhale several hundred of these conidia per day (Mullins et al., 1984). Due to their small diameter of 2 - 3 µm (Brakhage & Langfelder, 2002), they can reach the deeper parts of the respiratory tract (Moore et al., 2011). There, conidia can attach to lung epithelial cells, after which they can be internalized (Wasylnka & Moore, 2002). These characteristics contribute to the virulence of *A. fumigatus*, making it an opportunistic pathogen that can cause severe invasive infections especially in immunocompromised patients (Kosmidis & Denning, 2015).

The interaction of conidia and lung epithelial cells differs between fungal species and even strains. For example, no difference was observed between adherence of conidia of *Aspergillus niger* and *A. fumigatus* to A549 type II lung epithelial cells. In contrast, *A. fumigatus* conidia were internalized more efficiently, while germination within a 12 h period was also much less compared to *A. niger* (Escobar et al., 2016). Transcriptome analysis revealed that the immune response of the lung cells differs upon interaction with these two aspergilli. In contrast to *A. niger, A. fumigatus* downregulates a set of genes involved in the immune response. On the other hand, both aspergilli upregulate IL-8, where the IL-8 upregulation is dependent on the multiplicity of infection (MOI) of the conidia. A higher MOI results in increased IL-8 expression for both *A. fumigatus* and *A. niger* (Escobar et al., 2018).

One of the receptors of epithelial cells that is involved in the internalization of conidia and initiation of the immune response is Dectin-1. This is a c-type lectin receptor that recognizes the cell wall component β(1-3)-glucan (Brown & Gordon, 2001). It is essential for the initiation of the immune response by producing inflammatory molecules (Brown et al., 2002). Together with toll-like receptor 2 (TLR2), Dectin-1 is involved in the production of reactive oxygen species (ROS) and mediation of the inflammatory response of macrophages (Gantner et al., 2003). Inhibition of this receptor reduces internalization of *A. fumigatus* conidia in lung epithelial cells (Han et al., 2011). In the case of *Candida albicans,* it has been shown that the ephrin type-A receptor (EphA2) is activated by β-glucan. This activation initiates the immune response and together with the epidermal growth factor receptor (EGFR) EphA2 is involved in the endocytosis of *C. albicans* by the host cell (Swidergall et al., 2018). In addition, EphA2 is required for the antifungal activity of neutrophils and the control of fungal proliferation during *C. albicans* infection (Swidergall et al., 2019). During a *Cryptoccocus neoformans* infection, which can cause cryptococcal meningitis, the EphA2 receptor enables transport of the fungus across the blood brain barrier (Aaron et al., 2018).

Dormant conidia of *A. fumigatus* are covered with a hydrophobic rodlet layer consisting of the rodlet proteins RodA and RodB, and a green pigment layer which consists of 1,8-dihydroxynaphthalene (DHN)-melanin (Latgé & Beauvais, 2014). When germination is induced, conidia start to swell and the rodlet and melanin layers fragment. As a result, conidia increasingly expose hydrophilic polysaccharide and glycoprotein patches, becoming completely hydrophilic at the end of the breakdown process (Dague et al., 2008). The rodlet and melanin layers cover immunogenic cell wall components like chitin, β-glucan and glycoproteins, masking the dormant

conidia for immune recognition (Latgé & Beauvais, 2014). Removal of the rodlet (Δ*rodA*) and/or the DHN-melanin layer (Δ*pksP*) leads to a rearrangement of the conidial cell wall. Conidia of the Δ*rodA* strain are more hydrophilic and expose more β-glucan (Carrion et al., 2013), while conidia of the Δ*pksP* strain expose more ConA reactive glycoproteins, glucosamine-containing components and chitin (Bayry et al., 2014; Valsecchi et al., 2019). Conidia of the double deletion strain (Δ*rodA*Δ*pksP*) expose more chitin but not more β-glucan (Bayry et al., 2014; Valsecchi et al., 2019). Inactivation of the melanin synthesis genes *ayg1* or *arp2* also results in reorganization of the conidial surface, similar to that of Δ*pksP*, which is explained by the absence of the early melanin intermediate scytalone. These cell wall rearrangements result in an increased immune response (Bayry et al., 2014; Bruns et al., 2010; Tsai et al., 1998).

In this study, we show that inhibition of EphA2 results in reduced internalization of dormant, swollen, or heat-killed conidia of *A. fumigatus*, but does not affect association of these conidia to the A549 lung epithelial cells. In contrast with the Δ*rodA* strain, A549 cell association was strongly increased for the Δ*pksP* strain, whereas internalization decreased. No further decrease was observed upon EphA2 inhibition in the case of the Δ*pksP* strain. Dual inhibition of the EphA2 and Dectin-1 receptor showed a stronger reduction of internalization of Af293 and CEA10 strain when compared to the single inhibitors but internalization was not completely blocked. Taken together, DHN-melanin modulates the conidial internalization into A549 cells to an EphA2-dependent mechanism.

Material and methods

Strains and growth conditions

Strains used in this study (Table 1) were grown for 3 days on potato dextrose agar (PDA, Difco) at 37 °C. Conidia were harvested with 0.85 % (w/v) NaCl and filtered through 3 layers of miracloth (Merck Millipore) to remove remnants of mycelium and hyphae. Conidia were counted using a Bürker Türk counting chamber.

Table 1. Strains used in this study

Strain	Description	Reference
A. fumigatus		
Af293.1	pRG3AMA1-RFP	(Leal et al., 2010)
CEA10		(Girardin et al., 1993)
CEA10Δ*KU80*	pyrG^AF::Δ*ku80* in CEA10	(da Silva Ferreira et al., 2006)
CEA10Δ*pksP*	Δ*pksP*::hph in CEA10Δ*KU80*	This Chapter
CEA10*pksP*C	*pksP*$^{+}$ derivative of CEA10Δ*pksP*	This Chapter
CEA10Δ*rodA*	Δ*rodA*::hph in CEA10Δ*KU80*	This Chapter
A. niger		
AV112d.7	*PglaA*:dTomato	(Vinck et al., 2011)

Transformation of *A. fumigatus*

Knock-out vectors for *rodA* and *pksP* were created by amplifying their left flanks using primer pairs *rodA*LF*BamHI*FW / *rodA*LF*XbaI*Rev and *pksP*LF*BamHI*FW / *pksP*LF*XbaI*Rev, respectively (Table 2). Similarly, the right flanks were amplified using primer pairs *rodA*RF*XbaI*FW / *rodA*RF*PstI*Rev and *pksP*RF*XbaI*FW / *pksP*RF*SacI*Rev. Both flanks were ligated in *BamHI* / *PstI* and *BamHI* / *SacI* linearized pUC20 for r*odA* and *pksP*, respectively, using T4 polymerase according

to the manufacturer's protocol (Thermofisher Scientific). In the next step, a 3031 bp *XbaI* fragment containing the hygromycin resistant cassette (Punt et al., 1987) was ligated in between the left and the right flanks in the pUC20 derivatives after digestion with *XbaI*.

A. fumigatus CEA10Δ*ku80* conidia were transformed using the PEG-mediated transformation protocol (Meyer et al., 2010). In short, conidia were grown overnight in complete medium consisting of minimal medium (MM; 6 g L^{-1} $NaNO_3$, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 mL L^{-1} Vishniac, pH 6.0) supplemented with 2 g L^{-1} tryptone, 1 g L^{-1} casamino acids, 1 g L^{-1} yeast extract, and 0.5 g L^{-1} yeast ribonucleic acids. Mycelium was isolated by filtering over a double layer of miracloth and incubated at 37 °C and 80 rpm for a maximum of 60 minutes in lysing enzymes from *Trichoderma harzianum* (Sigma Aldrich) in osmotic medium (OM, 0.01 M Pb buffer pH 5.8, 2.45 M $MgSO_4 \cdot 7H_2O$). Protoplasts were filtered through a double layer of Miracloth, collected by centrifugation for 10 min at 1120 g, and incubated for 5 min with PEG-6000 (Acros Organics) and a *SmaI* or *EcorV* linearized plasmid for the *pksP* and r*odA* deletion plasmids, respectively. The protoplasts were regenerated on MMS-agar (MM + 0.95 M sucrose, 25 mg mL^{-1} caffeine (Sigma Aldrich), 100 µg mL^{-1} hygromycin (Sigma Aldrich), and 1.2 % bacteriological agar (Scharlau)). After 4 - 5 days, transformants were transferred to fresh MM plates with 50 µg mL^{-1} hygromycin. Genomic DNA was isolated from mycelium using phenol / chloroform, which was used to confirm deletion of the genes. To this end, primer pairs *upstreamrodA*LFFW / *h*yg*rodA*Rev and *upstreampksP*LFFW / *h*yg*pksP*Rev (Table 2) were used for PCR for the *rodA* and *pksP* deletion strains, respectively.

Table 2. Primers used in this study. Restriction sites within primers are underlined.

Name	Sequence
rodA LF *BamHI* FW	5' TAGGATCCCACGAGCCTGGCTAAAG 3'
rodA LF *XbaI* Rev	5' GATCTAGAACAGCAGCGCTCAAAG 3'
rodA RF *XbaI* FW	5' CCTCTAGACTACTCGTCGCTTCTG 3'
rodA RF *PstI* Rev	5' ATCTGCAGTTGTCGGCCTGGTTTG 3'
pksP LF *BamHI* FW	5' TAGGATCCGCACGGCCAACGTAG 3'
pksP LF *XbaI* Rev	5' AGTCTAGATGGCGAGTGGTTTGC 3'
pksP RF *XbaI* FW	5' ATTCTAGACCACGGCCATGAGTTCC 3'
pksP RF *SacI* Rev	5' CAGAGCTCAGCGCAGGATGACAGAC 3'
upstream *rodA* LF FW	5' GTACGCATCTACGTGCTCCA 3'
hyg *rodA* Rev	5' GTCCAAGCAGCAAAGAGTG 3'
upstream *pksP* LF FW	5' GGAGATAGGTGCAGGTGTTC 3'
hyg *pksP* Rev	5' GCCGTGGTTGGCTTGTATG 3'
pksP FW	5' TGGCATTTGGGATAAGCACG 3'
pksP Rev	5' GCAGGGCATGGCATTCTTAA 3'
pksP check FW	5' CGACTCGATTGCATTGCTCA 3'
pksP check Rev	5' CTGCTGTGCCAATTCATCGA 3'
plasmid FW	5' TTATCTTTGCGAACCCAGGG 3'
plasmid Rev	5' CAACCCTAGTACGCCCTTCA 3'

For complementation of strain CEA10Δ*pksP*, the *pksP* gene was amplified using primers *pksP*FW and *pksP*Rev (Table 2) and directly used for transformation. To distinguish transformants with a wild-type phenotype from wild-type contamination, plasmid pGDPGFP containing the reporter gene *GFP* (Lagopodi et al., 2002) was co-transformed enabling selection of fluorescent strains. Integration of the gene was checked with primers *pksP*checkFW and *pksP*checkRev, while presence of

pGDPGFP was checked using primers plasmidFW and plasmidRev (Table 2, Supplementary Figure 1).

Cell culture and fungal infections

The human lung carcinoma epithelial cell line A549 (ATCC, CCL-185) was maintained by serial passage in Dulbecco's modified Eagle medium (DMEM) (Ref. code: 11995-065, Gibco) with 10 % fetal bovine serum (FBS) (Gibco). Fungal infection experiments were done as described (Escobar et al., 2016). Briefly, cells were seeded at a concentration of 2 10^5 cells mL^{-1} and cultured at 37 °C and 5 % CO_2 until a confluent monolayer was formed consisting of 2 10^6 cells mL^{-1}. Cells in 12 or 48 wells plates (Corning®, Costar®) were challenged with 2 10^5, 2 10^6 or 2 10^7 conidia mL^{-1} in DMEM + 10 % FBS resulting in a MOI of 0.1, 1 or 10, respectively. Cells were cultured in 48 wells plates containing 8 mm glass coverslips (ThermoFisher Scientific) for internalization and association experiments (see below).

Internalization and association of conidia

Internalization and association experiments were performed as described (Escobar et al., 2016). In short, A549 cells were grown on 8 mm glass coverslips (ThermoFisher Scientific) until a confluent layer had been formed. A549 cells were incubated with dormant conidia, dormant heat-killed conidia or swollen conidia. Spores were killed at 90 °C for 20 min (Supplementary Figure 2), while swollen conidia were obtained by a 2 h incubation at 37 °C in DMEM + 10 % FBS. Dormant, swollen, or heat killed conidia that did not express a plasmid containing a fluorescent gene, such as *RFP* in Af293.1, were stained with 16 - 20 µg mL^{-1} *Aspergillus* FITC labeled antibody (ThermoFisher Scientific) for 60 min at room temperature before addition to the A549 cells. Unbound antibody was not removed by washing, to avoid loss of conidia during pelleting. Conidia were added to A549 cells at a MOI of 1 and incubated for 2 h. Unbound conidia were removed by washing three times with DMEM + 10 % FBS after 2 h of incubation, after which incubation continued for 2 more hours. Conidia adhering to the A549 cells were visualized with 1 % calcofluor white (CFW; Sigma Aldrich) in DMEM + 10 % FBS. To this end, the dye was added for 10 min at 37 °C followed by one washing step with DMEM + 10 % FBS. Cells and conidia were fixed with 4 % paraformaldehyde (PFA) (VWR international) for 5 min at 4 °C and 20 min at room temperature. Background fluorescence of PFA was quenched with 20 mM NH_4Cl (Acros Organics) for 20 min at room temperature. A549 cells were visualized with 1 µg mL^{-1} Hoechst (BD Biosciences). Coverslips were mounted onto glass slides using FluorSave^{tm} (Merck Millipore) and dried overnight followed by confocal analysis. To determine the number of conidia associated to the A549 epithelial cells (number of conidia per A549 cell) 10 fields at the coverslip were randomly chosen for imaging. Internalization was determined by analysing z-stacks made at 10 randomly chosen sites at the coverslip. Conidia that were red or green were counted as internalized. Conidia that were also stained with CFW were counted as adhering non-internalized spores, as CFW cannot penetrate into the A549 cells. Internalization values were expressed as the percentage of total conidia that associated with the cells. Experiments were performed in biological triplicate. At least 100 conidia were counted per strain in each experiment.

EphA2 and Dectin-1 receptor inhibition

EphA2 receptor activity was blocked by adding 2.5 µM dasatinib (Cell Signaling Technology) (Swidergall et al., 2018) or a 50 times dilution of the EphA2 antibody (#6997, Cell Signaling) 1 h prior to infection to the A549 cells. The Dectin-1 receptor was blocked by adding 20 µg mL^{-1} 2a11 antibody (ab82888, Abcam) for 1 h and washed 3 times with DMEM + 10 % FBS before addition of the conidia (Brown et al., 2002).

Surface exposure of β(1-3)-glucan

Conidia incubated for 2 h in DMEM + 10 % FBS at 37 °C, until they were swollen before fixation with 4 % PFA. Swelling of conidia was confirmed by staining with a β(1,3)-glucan antibody (2G8, ab233743 Abcam). PFA background fluorescence was quenched with 20 mM NH_4Cl. Conidia were blocked with 0.3 % bovine serum albumin (BSA) (Sigma) for 60 min. The 2G8 antibody was diluted 100-fold and incubated with conidia for 120 min. After washing once with PBS the conidia were incubated with a 1000-fold dilution of the secondary antibody (Goat Anti-Mouse, Alexa Fluor® 488, ab150113 Abcam) for 45 min. Conidia were mounted on a glass slide with FluorSave and dried overnight.

Confocal microscopy

Confocal images were acquired with a Zeiss LSM 700 microscope using the Plan-Apochromat 63 x 1.40 oil DIC (WD=0.19) objective. Images were taken using the 405, 488 and 555 nm laser lines. Fluorescence emission of CFW and Hoechst was detected using the 400-490 nm spectral band. Red fluorescence emission of mRFP and dTomato was detected with the 560-700 nm spectral band and FITC fluorescence was detected with the 490-555 nm spectral band. Images were analysed and processed with the Fiji image processing package of ImageJ (www.fiji.sc).

Quantification of IL-8 secretion

Confluent layers of A549 cells in 12 wells plates (Corning®, Costar®) were challenged with conidia for 2 h, after which unbound conidia were removed by washing 3 times with pre-warmed DMEM. After washing, exposure was continued for 2 or 10 h. The culture medium was added to 96 wells IL-8 ELISA plates (ThermoFisher Scientific) according to the manufacturer's instructions. Experiments were performed in biological triplicate.

A549 cell damage

Confluent layers of A549 cells in 24 wells plates (Corning®, Costar®) were challenged with conidia for 2 h, after which unbound conidia were removed by washing three times with pre-warmed DMEM. Exposure of the A549 lung cells to the conidia was continued for another 2 or 10 h. Cell damage after both periods of challenging (4 and 12 hours in total) was measured by lactate dehydrogenase (LDH) released into the medium. The medium was added to a transparent 96 wells plate (Corning®, Costar®) and LDH activity was measured using an LDH activity kit (Sigma Aldrich) according to manufacturer's instructions. A549 cells not challenged with conidia served as control. Experiments were performed in biological triplicate and technical duplicate.

Statistical analysis

Differences in association, IL-8 release and LDH activity were analysed using a one-way ANOVA with a p-value ≤ 0.05. A Tukey test with a Bonferroni correction for multiple testing was used as a post-hoc test. For the analysis of the internalization of conidia values were scored as in or out and treated as binary data. Differences were analysed using a Pearson chi-square test with p-values ≤ 0.05 considered significant. Separate t-tests with a Bonferroni correction for multiple testing were used as a post-hoc test.

Results

The role of EphA2 in conidial internalization and association

The EphA2 receptor of A549 epithelial lung cells was inhibited with a specific EphA2 antibody to assess the role of this receptor in association and internalization of conidia. To this end, the clinical *A. fumigatus* Af293 and CEA10 wild-type strains were used. Conidia were incubated with a confluent layer of A549 cells at a multiplicity of infection (MOI) of 1. The CEA10 and Af293 conidia associated to the A549 cells with efficiencies of 0.25 and 0.08 spores per cell within 4 h of incubation, respectively (Figure 1A). Inhibition of the EphA2 receptor did not result in a significant reduction of conidial association of both strains. Inhibition of the EphA2 receptor did reduce the internalization of conidia in A549 cells from 81 % to 56 % for Af293 and 75 % to 42 % for CEA10 (Figure 1B). Thus, the efficiency of internalization of the two *A. fumigatus* strains into A549 epithelial lung cells differs and this process proceeds, at least in part, via the EphA2 receptor. The EphA2 receptor could also be inhibited with the broad range kinase inhibitor dasatinib. This inhibition resulted in a reduction of internalization of conidia from 93 % to 51 % and 73 % to 47 % for Af293 and CEA10, respectively (Supplementary Figure 3B). Association and internalization were also assessed for the non-pathogenic *A. niger* N402 strain. Inhibition of EphA2 by dasatinib did not affect association to A549 cells but a similar decrease of internalization of conidia was observed as with the *A. fumigatus* strains (i.e. from 72 % to 31 %) (Supplementary Figure 4).

Next, we investigated if association and internalization of heat-killed and swollen conidia were affected by EphA2. The EphA2 receptor is activated upon recognition of β-glucan that is present in the fungal cell wall (Swidergall et al., 2018). We hypothesized that internalization of swollen conidia is more effective since β-glucan is more exposed when compared to dormant spores due to the absence of the rodlet and melanin layers. It was observed that a 2 h incubation in culture medium resulted in increased exposure of β-glucan on the conidial surface of both strains (Supplementary Figure 5). Dormant conidia of CEA10 did expose more β-glucan as compared to Af293. In contrast, heat-killed conidia of both strains did not expose increased amounts of β-glucan at their surface when compared to the dormant spores. In fact, heat killing resulted in reduced detection of β-glucan in CEA10 (Supplementary Figure 5). Remarkably, internalization of heat-killed or swollen conidia of Af293 did not change significantly (Figure 1B) and thus does not correspond with β-glucan exposure at the surface of the swollen conidia (Supplementary Figure 5). Internalization of swollen and heat-killed conidia of CEA10 increased with 8 % and 4 %, respectively (Figure 1B). Inhibition of the EphA2 receptor with a specific EphA2 antibody resulted in a reduction of internalization of swollen and heat-killed conidia from 83 % to 49 % and from 83 % to 45 %,

respectively, for Af293 (Figure 1B) and a reduction from 83 % to 46 % and 79 % to 44 %, respectively, for CEA10 (Figure 1B). Internalization of swollen and heat-killed conidia upon inhibition of the EphA2 receptor by dasatinib resulted in a similar decrease in internalization (Supplementary Figure 3B). These results show that the increased exposure of β-glucan in swollen conidia does not increase the efficiency of internalization of conidia as compared to dormant conidia and that conidia do not need to be alive for efficient internalization.

Association of heat-killed conidia of Af293 was increased from 0.08 to 0.24 conidia per cell when compared to dormant conidia (Figure 1A), while association of swollen conidia of Af293 was similar to dormant conidia. This was not observed with conidia of CEA10. The association of heat-killed conidia increased from 0.25 to 0.4, while the association of swollen conidia decreased from 0.25 to 0.18 (Figure 1A). These results suggest that the swollen conidia of Af293 and CEA10 WT interact differently with the A549 epithelial lung cells. Association of heat-killed conidia of *A. niger* was reduced irrespective of the absence or presence of EphA2 (Supplementary Figure 4A), suggesting that conidia of *A. niger* N402 and *A. fumigatus* use different mechanisms for association to the A549 lung epithelial cells.

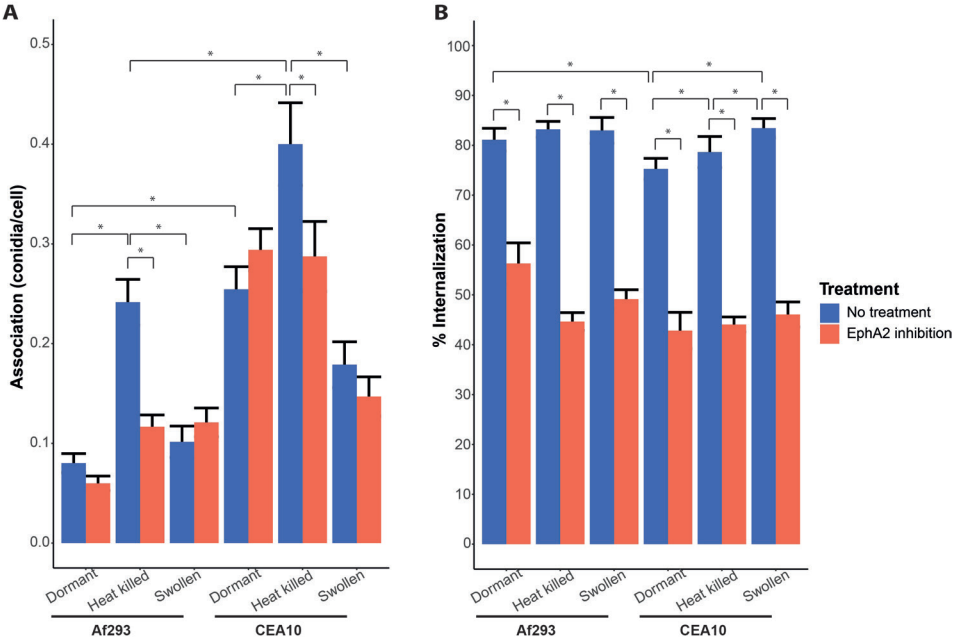

Figure 1. Internalization and association of *A. fumigatus* conidia of strains Af293 (expressing the *RFP* gene) and CEA10 WT (stained with the *Aspergillus*-FITC antibody) after 4 h of infection, with (red bar) or without (blue bar) inhibition of the EphA2 receptor by the EphA2 antibody. A) Association of dormant, heat killed or swollen Af293 and CEA10 conidia. B) Internalization of dormant, heat killed or swollen Af293 and CEA10 conidia. Bars represent the average of three separate experiments consisting of 10 pictures per condition/strain with the standard error.

Effect of DHN-melanin and RodA deletion on conidial internalization and association

Inactivation of the rodlet gene *rodA* results in increased exposure of β-glucan (Carrion et al., 2013) (Supplementary Figure 5), whereas deletion of the *pksP* gene results in increased glycoprotein and chitin exposure (Bayry et al., 2014; Valsecchi et al., 2019). The association of the conidia to the A549 lung cells was not significantly affected by the absence of the RodA protein (Figure 2A). Interestingly, a five-fold increase in association was observed in the case of Δ*pksP* conidia, whereas the *pksP* complementation strain had a similar association as the CEA10 wild-type strain (Figure 2A). Association of conidia of the Δ*rodA* and Δ*pksP* strains as well as the *pksP* complementation strain did not change when the EphA2 receptor was inhibited by the EphA2 antibody but did increase when the EphA2 receptor was inhibited by dasatinib (Figure 2A, Supplementary Figure 6A), contrasting their cognate wild-type strain. Deletion of *rodA* in CEA10 did not alter internalization incidence of the conidia after 4 h of incubation when compared to the wild-type (Figure 2B). However, the Δ*pksP* strain showed decreased internalization, whereas the complementation strain showed a similar internalization as the wild-type strain (Figure 2B). Similar to the wild-type, internalization of conidia of the Δ*rodA* and *pksP* complementation strains were reduced when the EphA2 receptor was inhibited by the EphA2 antibody (i.e. from 72 % to 50 % and 68 % to 34 %, respectively). In contrast, no inhibition was observed in the case of the Δ*pksP* strain (39 % and 36 % with and without inhibition, respectively). Inhibition of the EphA2 receptor with dasatinib did not reduce the internalization of the Δ*pksP* strain (26 % and 22 % with and without inhibition, respectively) but a decrease from 84 % to 42 % and 79 % to 29 % for the *pksP* complementation strain and the Δ*rodA* strain was observed, respectively (Supplementary Figure 6B). These results show that EphA2-dependent internalization of *A. fumigatus* conidia requires the presence of DHN-melanin.

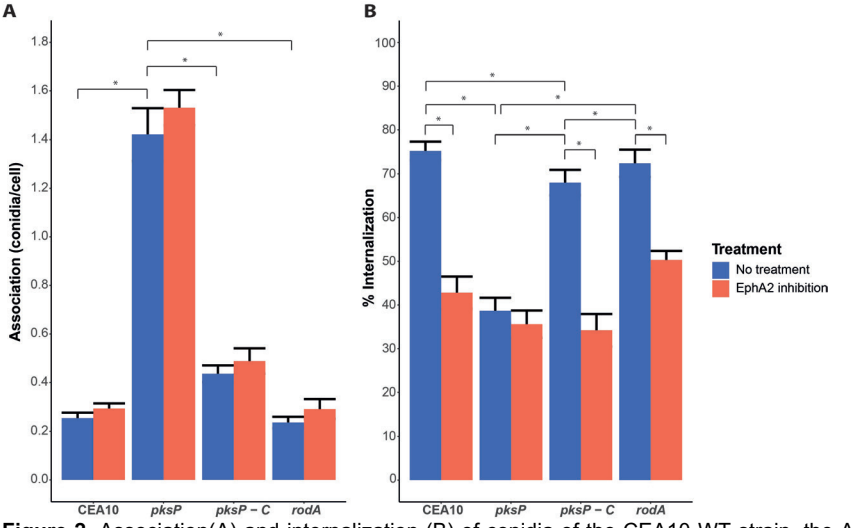

Figure 2. Association(A) and internalization (B) of conidia of the CEA10 WT strain, the Δ*pksP* (lacking DHN-melanin) and Δ*rodA* (lacking the hydrophobin RodA) deletion strains, and the complemented Δ*pksP* strain after 4 h of infection with (red bar) or without (blue bar) inhibition of the EphA2 receptor by an antibody. All conidia are stained with the *Aspergillus*-FITC antibody. Bars represent the average of three separate experiments consisting of 10 pictures per condition/strain with the standard error.

Dual inhibition of both EphA2 and Dectin-1

Dectin-1 is a β-glucan receptor like EphA2 and also plays a role in the internalization of *A. fumigatus* conidia into lung epithelial cells (Han et al., 2011). Inhibition of the Dectin-1 receptor with the 2a11 antibody resulted in a similar reduction of internalized conidia of Af293 and CEA10 WT as compared to EphA2 inhibition (Figure 3A). Dectin-1 inhibition reduces internalization from 79 % to 56 % for Af293 and from 78 % to 46 % for CEA10 WT. Interestingly, inhibition of both Dectin-1 and EphA2 decreased internalization of conidia further to 41 % and 39 % for Af293 and CEA10, respectively (Figure 3A). Since the inhibition of the EphA2 receptor did not result in a decrease in internalization of the Δ*pksP* strain, we investigated if the internalization of conidia lacking DHN-melanin was also independent of Dectin-1. Indeed, inhibition of the Dectin-1 receptor with 2a11 antibody did result in a further decrease in internalization of conidia lacking DHN-melanin (Figure 3B). This decrease was less pronounced as observed with the wild-type strain, suggesting that the Dectin-1 receptor is less important in the internalization of conidia lacking DHN-melanin.

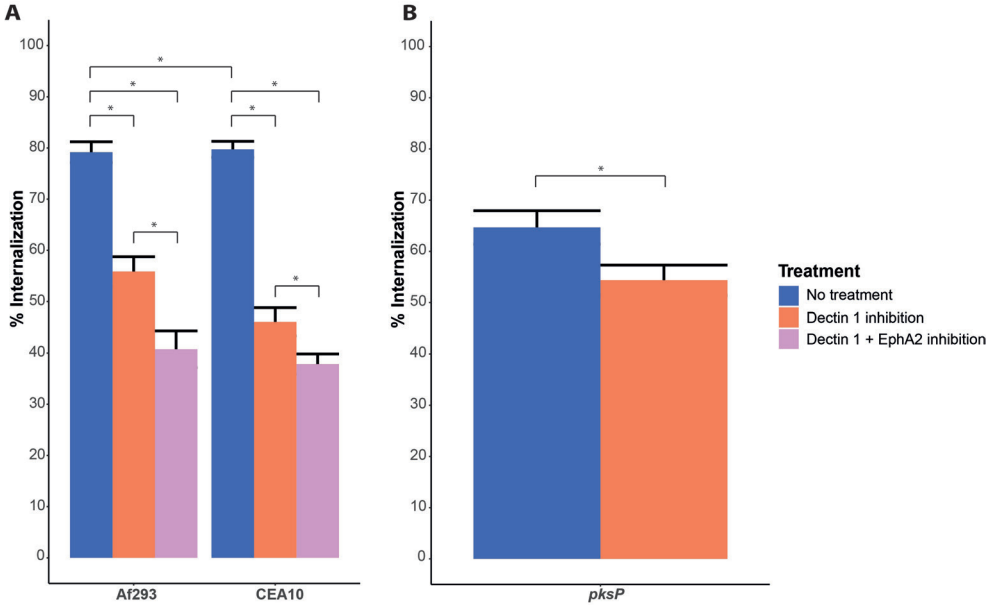

Figure 3. Internalization of conidia after EphA2 inhibition by dasatinib or Dectin-1 inhibition by the 2a11 antibody. A) Internalization of *A. fumigatus* dormant conidia of strains Af293 (expressing *RFP*) and CEA10 WT (stained with *Aspergillus*-FITC antibody), after 4 h of infection without (blue bar) or Dectin-1 receptor inhibition with 2a11 antibody (orange bar) or inhibition of both EphA2 (with an antibody) and Dectin-1 receptor (with 2a11 antibody) (pink bar). B) Internalization of *A. fumigatus* CEA10 Δ*pksP* dormant conidia (stained with *Aspergillus*-FITC antibody), after 4 h of infection without (light bar) or with Dectin-1 receptor inhibition with 2a11 antibody (light grey bar). Bars represent the average of three separate experiments consisting of 10 pictures per condition/strain with the standard error.

Discussion

This study shows that the β-glucan recognizing EphA2 receptor plays a role in the early interaction between *A. fumigatus* conidia and A549 epithelial cells next to Dectin-1 (Figure 4). Inhibition of the EphA2 receptor with the small molecule dasatinib leads to a 50 % reduction in internalization. Since dasatinib does not only inhibit the activation of the EphA2 receptor but acts as a broad range tyrosine kinase inhibitor, it also inhibis other kinases such as BRC-Abl, Scr and Syk family kinases (Baell & Holloway, 2010; Schade et al., 2008; Song et al., 2010). Therefor, we also used an EphA2-specific antibody. Antibody-mediated inhibition gave similar results when compared to dasatinib. These results show that the EphA2 receptor is indeed involved in the internalization of *A. fumigatus* conidia, which is in line with the role of the EphA2 receptor in *C. albicans* (Swidergall et al., 2018).

The Syk family kinase is both inhibited via dasatinib and Dectin-1 inhibition. Interestingly, Syk signalling is also important for the maturation of phagolysosomes and fungal killing in monocytes (Kyrmizi et al., 2013), but a role in the internalization of conidia into epithelial cells has not been reported. It might be that part of the internalization of the conidia via the Dectin-1 receptor and the receptors inhibited by dasatinib is regulated via Syk signalling.

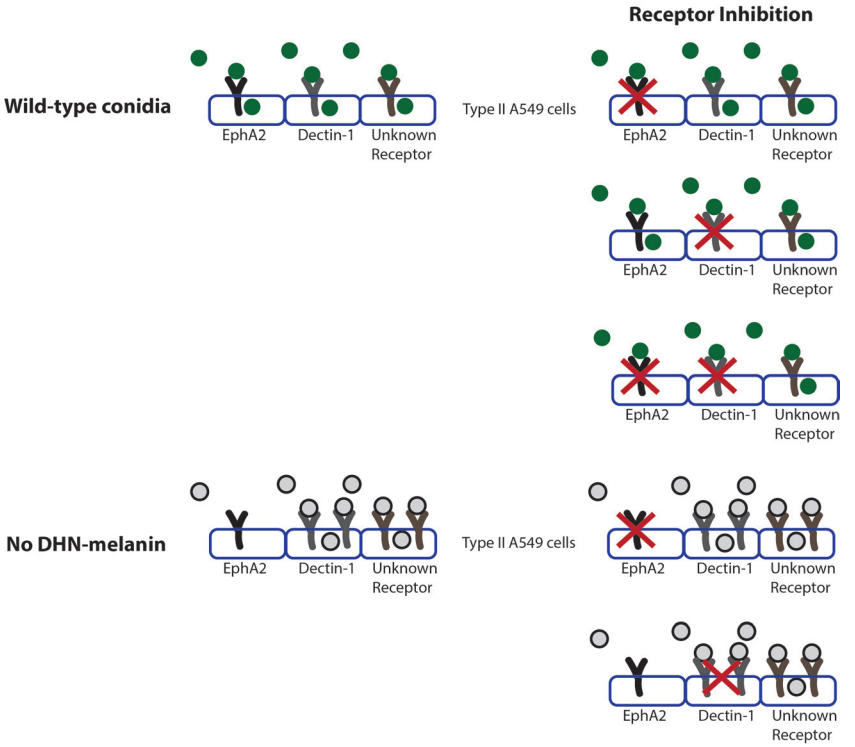

Figure 4. Schematic overview of the role of receptors in internalization and association of wild-type conidia (in dark green) or conidia lacking DHN-melanin (grey with black line). Conidia will associate to various indicated receptors present on the type II A549 epithelial lung cells, and the EphA2, Dectin-1 and at least one other receptor will be activated, which results in the internalization of conidia. In conidia lacking DHN-melanin, association of conidia is increased, and internalization of conidia is facilitated in an EphA2 independent and a Dectin-1 dependent manner.

Differences in the association and internalization of conidia of the Af293 and CEA10 wild-type strains were observed in the A549 cell culture system. The conidia of Af293 have a lower association to the A549 cells and are more efficiently internalized when compared to CEA10 conidia (Figure 1). These differences are not due to the fact Af293 was expressing a red fluorescent plasmid and the CEA10 strain was labelled with an anti-*Aspergillus*-FITC antibody. Labelling of the Af293.1 strain with this antibody did not alter internalization and association of conidia (results not shown). Differences between these two strains have also been reported in other infection systems. For instance, they differ in gene expression during an infection of airway epithelial cells (Watkins et al., 2018). Moreover, Af293 has a lower fitness at low-oxygen concentrations, which leads to a decrease in virulence in a mouse invasive pulmonary aspergillosis (IPA) model (Kowalski et al., 2016). The Af293 strain is also more virulent in a zebrafish model when compared to the CEA10 strain, which was explained by faster germination of CEA10 conidia and, consequently, a more rapid clearance by the host immune system (Rosowski et al., 2018).

The reduction of internalization of Af293 and CEA10 wild-type conidia after EphA2 receptor inhibition indicates an important role for β-glucan in the internalization of *A. fumigatus* conidia (Figure 1 and 4). This internalization depends on the presence of DHN-melanin since internalization of a Δ*pksP* mutant was not affected by inhibition of EphA2 (Figure 2B and 4). It could be that conidia lacking DHN-melanin use alternative receptors, which remain to be identified. In this context it is remarkable that inhibition of the Dectin-1 receptor results in a modest decrease in internalization of conidia lacking DHN-melanin. This suggests that Dectin-1 is mainly used as receptor for internalization if DHN-melanin is present (Figure 3B and 4). Notably, association of conidia lacking DHN-melanin with A549 cells was actually higher as compared to its wild type and the complemented strain. This finding may be explained by changes in the cell wall architecture that impact interactions with cell surface receptors. How the presence of DHN-melanin affects the EphA2-dependent uptake remains to be clarified but it may be that a co-receptor for DHN-melanin interacts with EphA2. Absence of DHN-melanin could result in a shift to other receptors for binding and uptake. We observed that only 40 % of the Δ*pksP* conidia were internalized indicating that a large amount of conidia remain cell-surface associated. Some alternative conidial surface components of *A. fumigatus* have been implicated in binding and internalization such as sialic acid (Warwas et al., 2007) and the CalA protein (Liu et al., 2017). Whether these surface components are involved in increased binding and / or internalization of conidia lacking DHN-melanin needs to be determined.

Dormant conidia expose small amounts of β-glucan at their surface. This amount is apparently sufficient for interactions with EphA2 because we did not find a relation between increased exposure of β-glucan and association and internalization. Swollen conidia of Af293 expose more β-glucan yet internalize with similar efficiency as compared to dormant conidia. Dormant conidia of strains lacking RodA also expose more β-glucan (Valsecchi et al., 2019) (Supplementary Figure 5), yet association and internalization was similar as compared to WT conidia. Association of conidia lacking DHN-melanin is much higher as compared to WT and its complemented strain. Conidia of a Δ*pksP* strain expose much more chitin and glycoproteins at their surface, but not more β-glucan (Valsecchi et al., 2019) (Supplementary Figure 5).

Association of heat-killed Af293 and CEA10 conidia was reduced by inhibition of the EphA2 receptor, but was higher when compared to dormant conidia. This difference in association might be caused by altered conidial surface structures as result of the heat inactivation. However, no difference in internalization was observed between dormant and heat-killed spores, which is in line with the result of Wasylnka & Moore, (2002). It should be noted that the internalization efficiencies of conidia into the epithelial cells differ between studies. This difference can be due to the use of different MOI, different *A. fumigatus* strains and / or time of exposure to epithelial cells. In our experiment conidia which did not associate to the A549 epithelial cells were removed after 2 h, meaning that only the associated conidia were used to determine the internalization. When the number of internalized conidia is determined based on the MOI at the start of the infection (Supplementary table 1), it becomes clear that the internalization of the conidia is still higher than in other studies (Wasylnka & Moore, 2002).

In a macrophage model, it was observed that swollen conidia were more efficiently internalized when compared to dormant conidia, which was explained by the increased exposure of β-glucan (Luther et al., 2007). The reported 50 % increase in internalization was not observed in our experiments (Figure 1B). This could be due to the difference in infection model and / or in swelling time and therefore the amount of exposed β-glucan. The swelling time for the macrophage model was 6 h, while we used 2 h in our experiments. Nevertheless, the 2 h swollen conidia in our experiments did show increased exposure of β(1-3)-glucan (Supplementary Figure 5).

Since both EphA2 and the Dectin-1 receptor recognize β-glucan, we hypothesized that dual inhibition of both receptors completely abolishes internalization of conidia. This is not the case. It might be that other cellular receptors and processes sustain internalization under these conditions as is depicted in a schematic overview (Figure 4). Furthermore, it is known that the actin dynamics in host cells are important for the internalization of conidia (Culibrk et al., 2019; Escobar et al., 2016). Phospholipase D (PLD) activity is associated with actin dynamics and inhibition of PLD leads to a 50 % reduction in internalization of conidia. Inhibition of the Dectin-1 receptor, leading to a reduction in internalized conidia, also leads to a reduction in PLD activity (Han et al., 2011). Further research in the involvement of actin and PLD activity in the internalization of conidia is required. *A. fumigatus* also has PLD isoforms. A PLD deletion strain of *A. fumigatus* has decreased intracellular and extracellular PLD activity and internalization of conidia is decreased, similar to PLD inhibition on A549 epithelial cells (Li et al., 2012). Aspf2 is another component of *A. fumigatus*, which could sustain internalization after EphA2 and Dectin-1 receptor inhibition. Aspf2 suppresses the host immune response by binding to plasminogen and to negative regulators of the complement system. The binding to plasminogen leads to increased damage of A549 cells after adhesion of the conidia. Deletion of Aspf2 leads to a decrease in A549 cell damage, but also to a decrease in internalization in macrophages (Dasari et al., 2018).

Interesting for future research is the MelLec receptor. This is, just as Dectin-1, a c-type lectin receptor, but instead of β-glucan it recognizes DHN-melanin (Stappers et al., 2018). The internalization of conidia lacking DHN-melanin is decreased and the observed internalization is partly via the Dectin-1 receptor but not the EphA2 receptor. More research is required to determine if the MelLec receptor is involved in the internalization of conidia and whether the decrease in

internalization of conidia lacking DHN-melanin is due to the MelLec receptor unable to recognize these conidia.

The role of the EphA2 receptor in the transport of *C. neoformans* over the blood brain barrier (Aaron et al., 2018) and the importance of EphA2 activation for the antifungal activity of neutrophils (Swidergall et al., 2019) highlights the important role of EhpA2 in fungal infections. It also questions the ability of this receptor to function as a druggable target for fungal infections. Inhibition of the EphA2 receptor may lead to a decrease in fungal uptake and crossing of the fungi over the blood brain barrier but could also inhibit a proper neutrophil response against the fungal infection, therefore making it more difficult to control and clear the infection. To explore the potential of the EhpA2 receptor as a druggable target for fungal infections research should be done in more expanded models: such as using immune cells to follow the processes that differ in these models (immune cell recruitment, fungal uptake, fungal proliferation and killing).

Previously, an EphA2-dependent upregulation of IL-8 was observed with *C. albicans* (Swidergall et al., 2018). We therefore investigated whether an EphA2-dependent immune response of A549 cells could also be observed with *A. fumigatus*. We investigated IL-8 production after a 12 h co-incubation by an IL-8 specific ELISA. We did not observe an increased IL-8 production, which could support the proposed downregulating effect of *A. fumigatus* on the immune response by (Escobar et al., 2018) (Supplementary Figure 7A). This contrasts with other studies that did show induction of IL-8 (Chen et al., 2015; Oosthuizen et al., 2011) and it might be explained by the fact that we remove non-associated conidia after 2 h of infection. Externally germinating conidia could be responsible for the induction of IL-8 in other studies. A decrease in IL-8 production upon EphA2 receptor inhibition as described by (Swidergall et al., 2018) was not observed in our system after a 12 h inhibition with an EphA2 specific antibody (Supplementary Figure 7A, 8A). Additionally, we investigated if A549 cells were damaged due to outgrowth of *A. fumigatus*. In line with the results of (Seidel et al., 2020) we did not see an increase in cell damage expressed by LDH activity (Supplementary Figure 7B, 8B), even though hyphae escaping A549 cells were observed (Escobar et al., 2018). This suggests non-lytic exit as described recently (Seidel et al., 2020). We cannot rule out the possibility that in our experimental setting the inhibition with the antibody did not last for the 12 h infection period. Alternatively, or in addition, other receptors might be involved to support the IL-8 production during inhibition of the EphA2 receptor.

Since the absence of DHN-melanin results in loss of EphA2-dependent uptake it is tempting to speculate that sequentially interactions take place during germination at the lung epithelial surface. Dormant conidia containing DHN-melanin might be processed preferentially via the EphA2 receptor and internalized. Upon initiation of germination and subsequent swelling, DHN-melanin is gradually lost allowing a shift to other receptors. This shift might affect internalization, since absence of DHN-melanin results in increased surface association. This suggests that fast swelling and germinating conidia can be more efficiently cleared by the immune system than conidia that germinate more slowly. As slow swelling and germinating conidia seem to be taken up more efficiently by epithelial cells, thereby hiding from the immune system.

Conclusion

It was shown previously that inhibition of Dectin-1 reduces internalization of conidia of *A. fumigatus* into epithelial cells. Here we showed that the EphA2 receptor is also involved in internalization of *A. fumigatus* conidia during infection. Next to EphA2 and Dectin-1 also other receptors play a role in internalization since the dual inhibition of these β-glucan recognizing receptors did not lead to a complete block in internalization of conidia. In addition, we showed that DHN-melanin modulates association and internalization into lung epithelial cells. Its removal leads to a 60 % reduction of internalization and becomes EphA2 independent while association to A549 cells is increased five-fold. Our results suggest a shift in receptor usage during swelling and germination at the lung cell surface affecting association and internalization.

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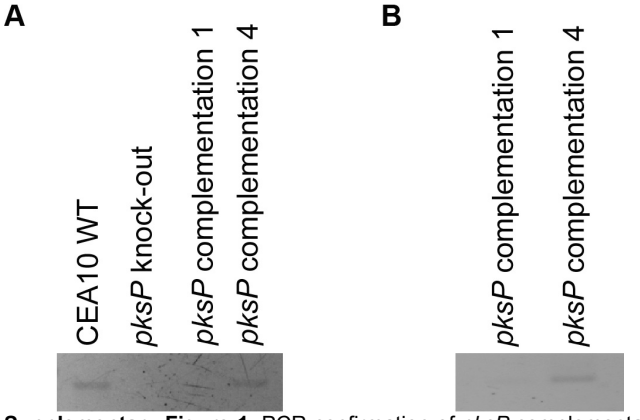
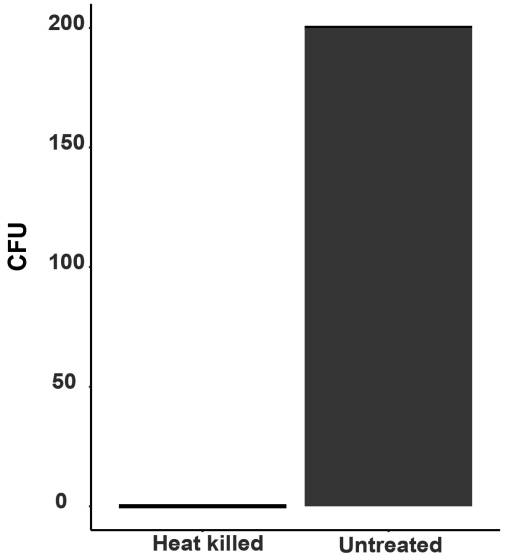
Supplementary data

Supplementary Table 1. Conidia removed by washing. Numbers are averages of 3 independent experiments, with 10 pictures in each experiment and at least 100 counted conidia. The association and internalization data is also used for the graph in Supplementary Figure 3.

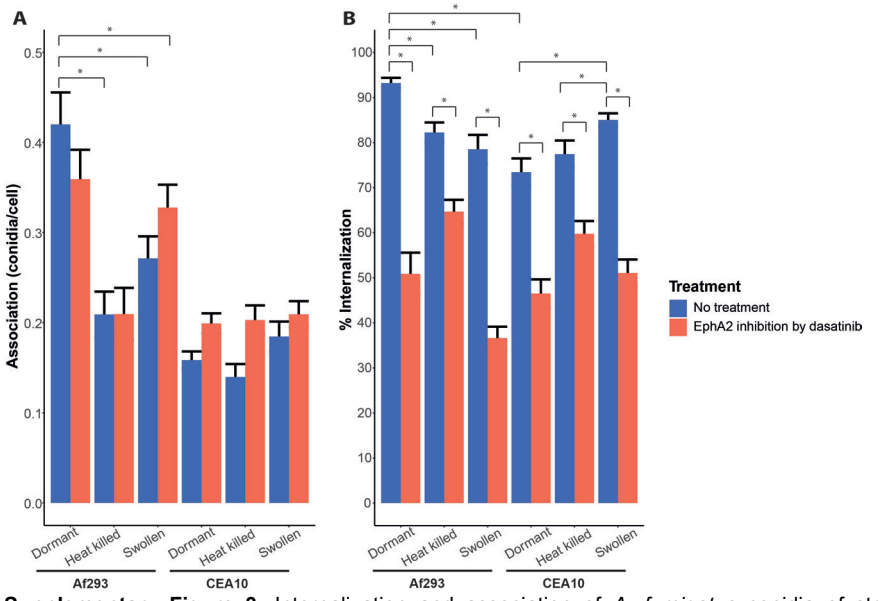
	Af293	**CEA10**
Start (conidia/cell)	1	1
Association after 4 hours (including washing) (conidia/cell)	0.08	0.25
Removed by washing (conidia/cell)	0.92	0.75
Internalized conidia (%) of the associated conidia	81 %	75 %
Internalized conidia (conidia/cell) of the associated conidia	0.0648	0.1875
Internalized conidia (%) of the start inoculum	6.48 %	18.75 %



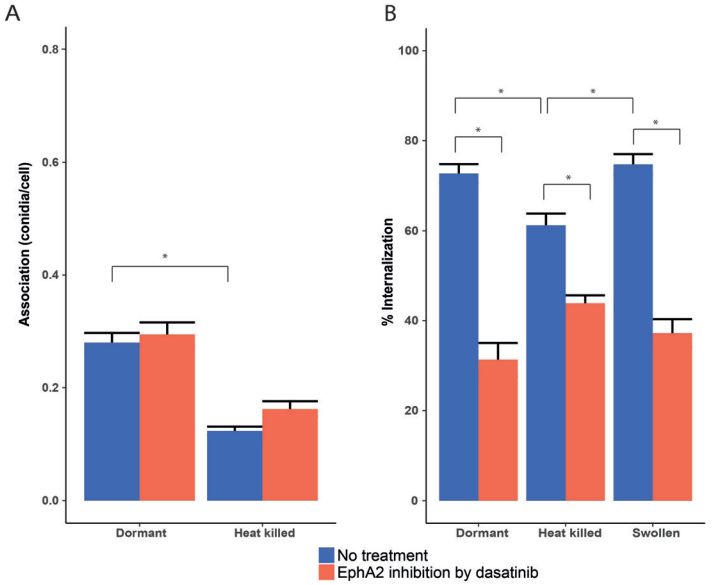
Supplementary Figure 1. PCR confirmation of *pksP* complementation. Complementation transformant 4 was both positive in PCR for the *pksP* gene (A) and the presence of the reporter plasmid (B) and was used for all experiments.



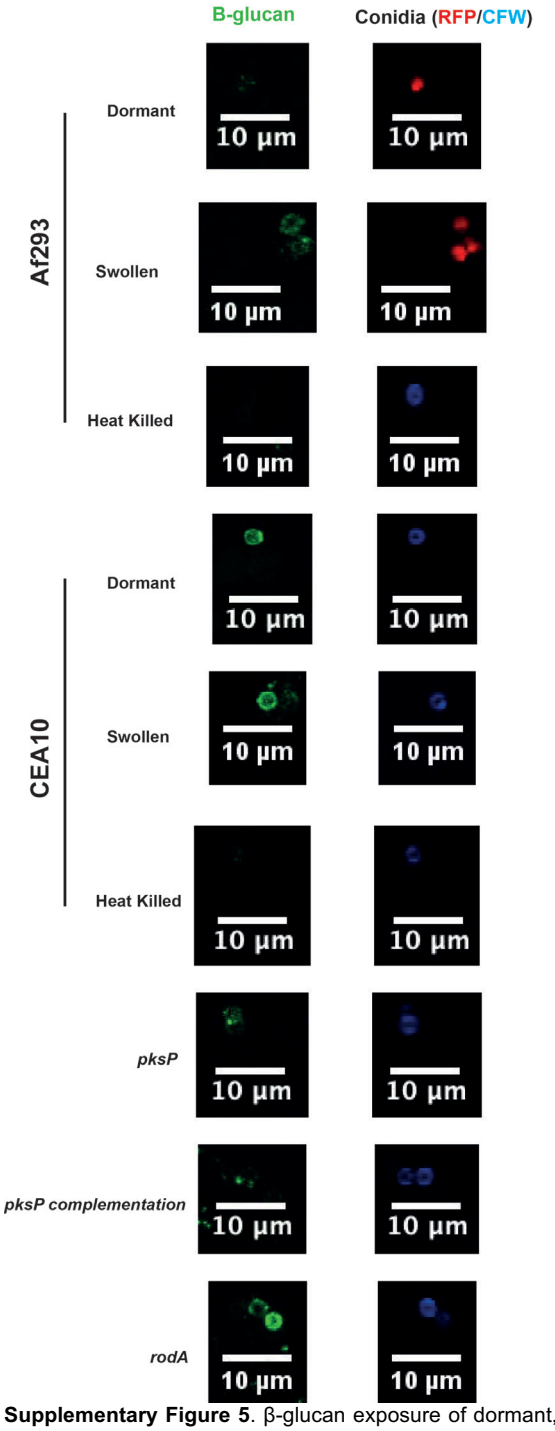
Supplementary Figure 2. CFU counts after heat treatment (90 °C, 20 min) of conidia. Bars represent the average of three separate experiments.



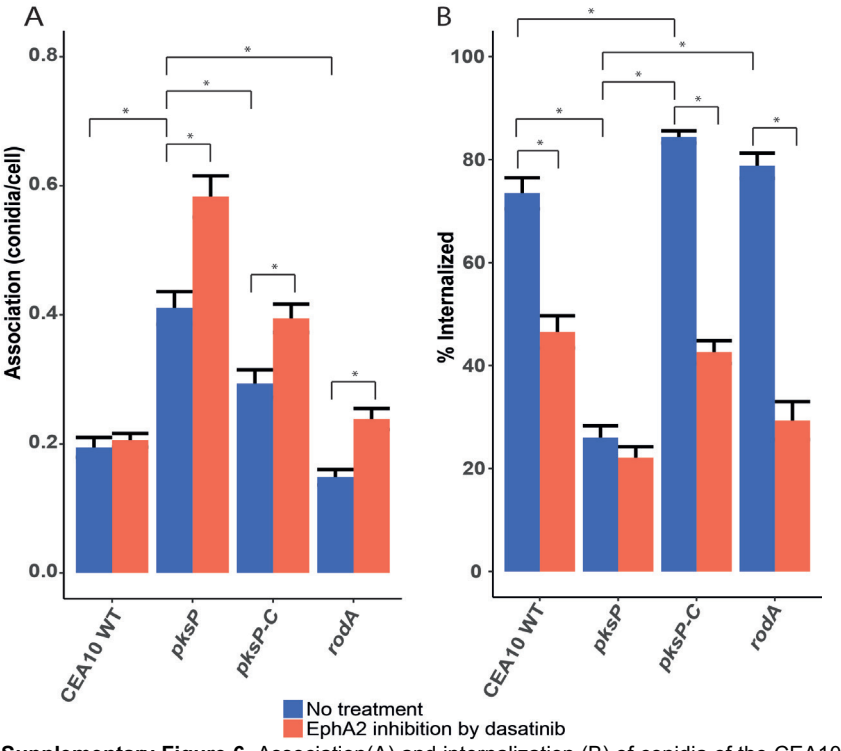
Supplementary Figure 3. Internalization and association of *A. fumigatus* conidia of strains Af293 (expressing the *RFP* gene) and CEA10 WT (stained with the *Aspergillus*-FITC antibody) after 4 h of infection, with (red bar) or without (blue bar) inhibition of the EphA2 receptor by dasatinib. A) Association of dormant, heat killed or swollen Af293 and CEA10 conidia. B) Internalization of dormant, heat killed or swollen Af293 and CEA10 conidia. Bars represent the average of three separate experiments consisting of 10 pictures per condition/strain with the standard error.



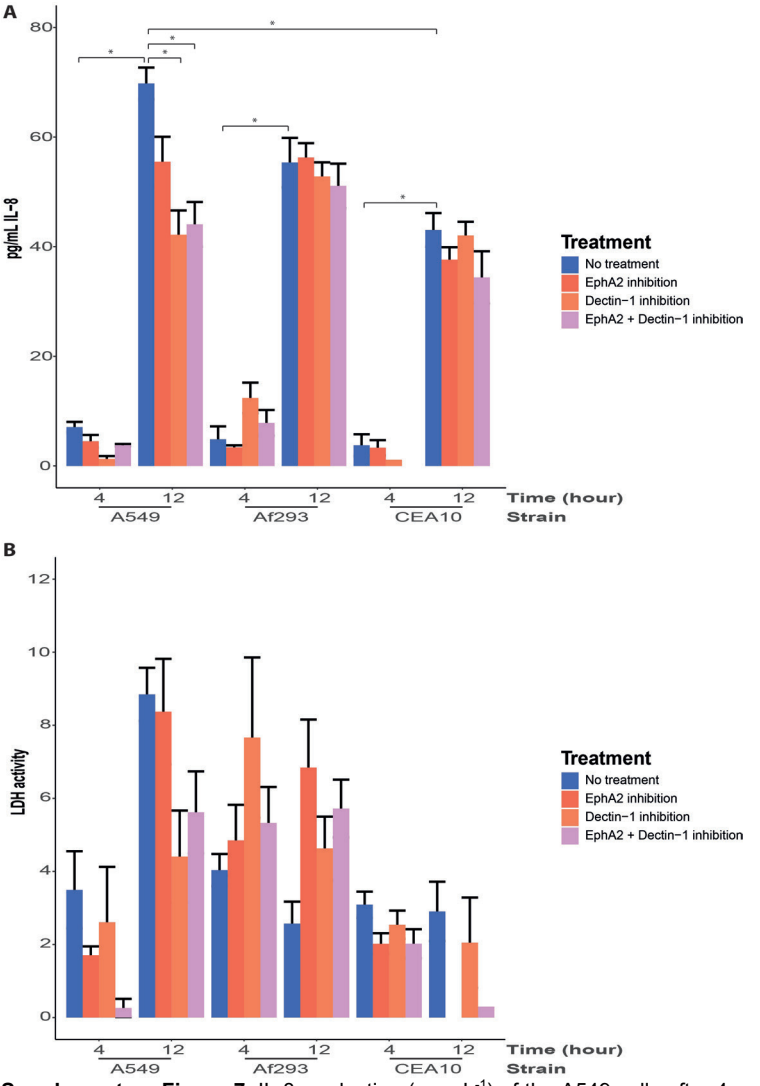
Supplementary Figure 4. Internalization and association of *A. niger* conidia, expressing the *dTomato* plasmid. A) Association of *A. niger* conidia after 4 h of infection, with (red bar) or without (blue bar) inhibition of the EphA2 receptor by dasatinib. B) Internalization of dormant, heat killed or swollen conidia after 4 h of infection, with (red bar) or without (blue bar) inhibition of the EphA2 receptor by dasatinib. Bars represent the average of three separate experiments consisting of 10 pictures per condition/strain with the standard error.



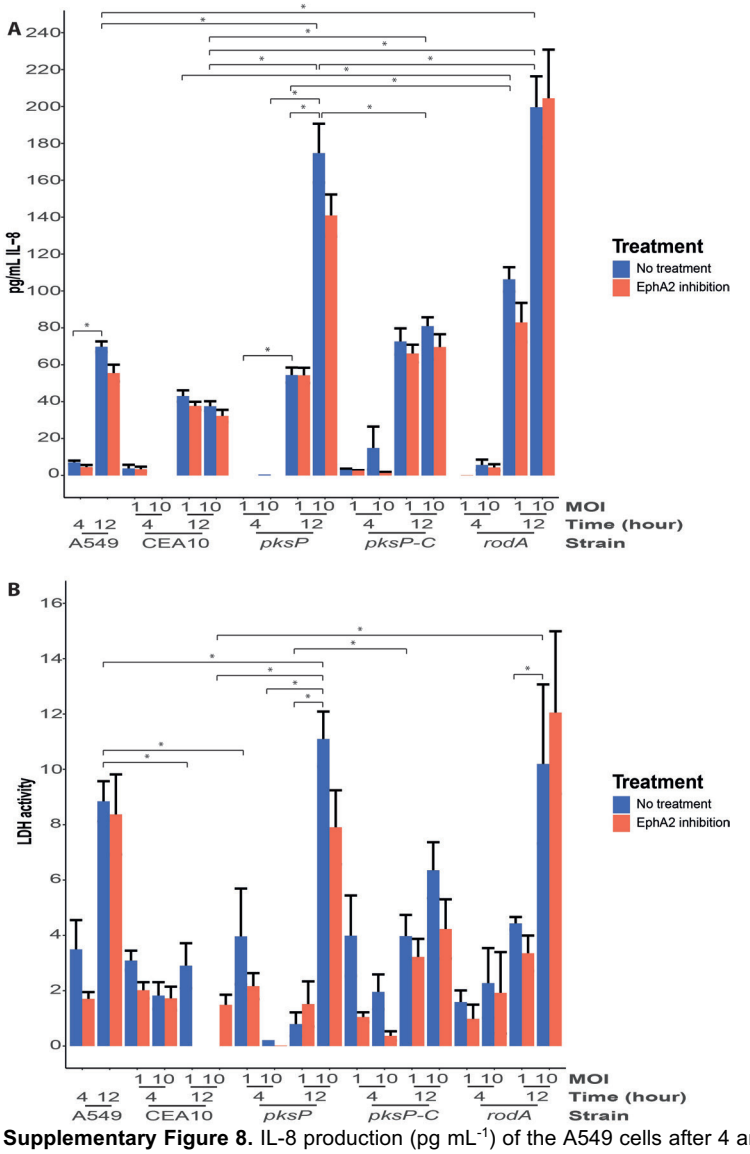
Supplementary Figure 5. β-glucan exposure of dormant, swollen and heat killed conidia of the Af293 and CEA10 wild-type strains and conidia of the *pksP* deletion and complementation strains and the *rodA* deletion mutant in the CEA10 background.



Supplementary Figure 6. Association(A) and internalization (B) of conidia of the CEA10 WT strain, the Δ*pksP* (lacking DHN-melanin) and Δ*rodA* (lacking the hydrophobin RodA) deletion strains, and the complemented Δ*pksP* strain after 4 h of infection with (red bar) or without (blue bar) inhibition of the EphA2 receptor by dasatinib. All conidia are stained with the *Aspergillus*-FITC antibody. Bars represent the average of three separate experiments consisting of 10 pictures per condition/strain with the standard error.



Supplementary Figure 7. IL-8 production (pg mL^{-1}) of the A549 cells after 4 and 12 hours of infections with *A. fumigatus* conidia of Af293 and CEA10 at a MOI of 1 (A). LDH activity of A549 cells after 4 and 12 hours of infections with WT *A. fumigatus* conidia of Af293 and CEA10 at a MOI of 1 (B). Blue bars represent cells which did not receive any treatment, EphA2 inhibition is shown by the red bars, Dectin-1 inhibition by the orange bar and dual inhibition of the EphA2 and Dectin-1 receptor is represented by the pink bar. Bars represent the average value of three independent experiments consisting of biological triplicates and technical duplicates.



Supplementary Figure 8. IL-8 production (pg mL^{-1}) of the A549 cells after 4 and 12 hours of infections with *A. fumigatus* of CEA10, the Δ*pksP* (lacking DHN-melanin) and Δ*rodA* (lacking the hydrophobin RodA) deletion strains, and the complemented Δ*pksP* (A). LDH activity of A549 cells after 4 and 12 hours of infections with of CEA10, the Δ*pksP* (lacking DHN-melanin) and Δ*rodA* (lacking the hydrophobin RodA) deletion strains, and the complemented Δ*pksP* (B). Blue bars represent cells which did not receive any treatment and EphA2 inhibition is shown by the red bars. Bars represent the average value of three independent experiments consisting of biological triplicates and technical duplicates.

3

The protective role of DHN-melanin against hydrogen peroxide revisited: no role in protection of conidia of the opportunistic human pathogen *Aspergillus fumigatus*

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Abstract

Previously, DHN-melanin was described to protect *Aspergillus fumigatus* against hydrogen peroxide (H_2O_2) thereby protecting this opportunistic human pathogen from reactive oxygen species generated by the immune system. This was based on the finding that the ATCC46645 mutant that has mutations in the *pksP* gene of the DHN-melanin synthesis pathway showed increased sensitivity to reactive oxygen species when compared to the wild-type. Here it is shown that deletion of the *pksP* gene in *A. fumigatus* strain CEA10 did not affect sensitivity against H_2O_2 and superoxide in a plate stress assay. In addition, direct exposure of the dormant white conidia of the *pksP* deletion strains to H_2O_2 did not result in increased sensitivity to this reactive oxygen species. Moreover, complementation of the ATCC46645 *pksP* mutant strain with the wild type *pksP* gene did result in pigmented conidia but did not rescue the H_2O_2-sensitive phenotype observed in the plate stress assay. Genome sequencing of the ATCC46645 *pksP* mutant strain and its complemented strain revealed a mutation in the *cat1* gene, likely due to the UV mutagenesis procedure used previously, which could explain the increased sensitivity towards H_2O_2. Together, DHN-melanin is not involved in protection against H_2O_2 and superoxide and thus has no role in survival of conidia when exposed to these reactive oxygen species.

Introduction

Aspergillus fumigatus is a saprotrophic fungus, which feeds on organic material of either dead or living organisms (Krijgsheld et al., 2013). Asexual reproduction leads to production of massive amounts of conidia that are dispersed into the air. We inhale several hundred *A. fumigatus* conidia daily (Mullins et al., 1984) and due to their small size (<5 μM) (Brakhage & Langfelder, 2002) these conidia can reach the deeper parts of the respiratory tract (Moore et al., 2011). Here they can cause non-invasive or invasive infections, especially in immunocompromised patients (Kosmidis & Denning, 2015).

Reactive oxygen species (ROS) are oxygen-derived molecules that include radicals like superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) but also non-radicals like hydrochlorous acid and ozone (Panday et al., 2015). These molecules play an important role in immune defence and genetic defects affecting ROS production in individuals compromises health (O'Neill et al., 2015). ROS damage many cellular components such as DNA and proteins and results in lipid peroxidation (Philippe et al., 2003). The ROS are produced by the NADPH-oxidase complexes that are part of the NOX family (Panday et al., 2015). These complexes are also expressed in type II epithelial and ciliated lung cells (Leiva-Juárez et al., 2018). A NOX-mediated ROS release is called an oxidative burst and together with reactive nitrogen species it enables microbial killing (Missall et al., 2004). The importance of the oxidative burst is shown in patients with chronic granulomatous disease (CGD) caused by a defect in the NOX2 enzyme complex. These patients are characterized by recurrent fungal and bacterial infections (Goldblatt & Thrasher, 2000). CGD mouse models also show high susceptibility to *A. fumigatus* infections (Pollock et al., 1995). Next to the defence against microbial pathogens, ROS also serve as messengers for the innate and adaptive immune response (Schieber & Chandel, 2014). Activation of TLR1, TLR2 and TLR4 need ROS for optimal activity in macrophages (West et al., 2011). The activation of the adaptive immune response by B and T cells is dependent on the production of ROS (Chaudhri et al., 1986; Wheeler & Defranco, 2012).

The green pigment 1,8-dihydroxynaphthalene (DHN)-melanin was reported to protect *A. fumigatus* conidia against peroxide (Jahn et al., 1997). However, deletion of *abr2* that is involved in early steps in the DHN-melanin pathway did not alter the peroxide sensitivity compared to the wild-type strain. This indicates that intermediates of DHN-melanin are sufficient to protect the conidia against peroxide (Sugareva et al., 2006). L-DOPA melanin was shown to protect against hydroxyl radicals in the human pathogenic dimorphic fungus *Cryptococcus neoformans* (Wang & Casadevall, 1994) and is also produced by *Aspergillus nidulans* (Gonçalves et al., 2012). Deletion of the polyketide synthase gene *wA* stops the production of L-DOPA melanin in this fungus and leads to increased sensitivity to H_2O_2 (Jahn et al., 2000).

Besides melanin, fungi have different types of enzymes that protect against ROS. Superoxides are converted to H_2O_2 by superoxide dismutases (SODs). The s*od1*-s*od4* genes of *A. fumigatus* encode such enzymes. Deletion of s*od1* and *sod2* leads to increased sensitivity to superoxides. Deletion of *sod3* did not alter the sensitivity to superoxides, while the role of *sod4* needs to be determined as it is an essential gene and can therefore not be deleted (Lambou et al., 2010). H_2O_2 is converted to water and oxygen by enzymes such as catalases, peroxidases,

peroxiredoxins and the thioredoxin system (Aguirre et al., 2006). There are three different catalase genes known in *A. fumigatus*; the conidial catalase *catA* and the two mycelial catalases *cat1* and *cat2*. Deletion of *catA* increases the sensitivity of the conidia to H_2O_2 but does not alter killing by murine alveolar macrophages (Paris et al., 2003). Deletion of *cat1* does not alter the sensitivity of germinated conidia of the fungus to H_2O_2, as determined in a metabolic assay (Calera et al., 1997), nor did it affect survival of conidia in a direct H_2O_2 exposure assay or exposure to polymorphonuclear (PMN) cells (Paris et al., 2003). Mycelia of a *cat2* deletion strain also did not show increased sensitivity to direct exposure of H_2O_2. However, the mycelium of a *cat1/cat2* double deletion strain is slightly more sensitive for H_2O_2 but no difference in killing by PMN cells is observed as compared to its wild type strain. Nonetheless, this double mutant develops slower in lungs of a rat model system (Paris et al., 2003). It was concluded that both mycelial catalases are required to scavenge H_2O_2 *in vitro* and *in vivo* but only provide partial protection against PMN cells. Whether additional catalases protect against these cells or that other ROS are more important for killing the fungus needs to be determined.

The *prx1*, *prxB* and *prxC* genes of *A. fumigatus* encode peroxiredoxins present in the cytosol (Prx1) or the mitochondria (PrxB and C). Purified Prx1 and PrxC are able to decompose H_2O_2 (Rocha et al., 2018). Deletion of each of these three genes in *A. fumigatus* increases sensitivity to paraquat and menadione that generate H_2O_2 and superoxides via redoxcycling with Δ*prx1* being most sensitive. Remarkably, the three single deletion strains do not show increased sensitivity to H_2O_2 in a plate stress assay (Rocha et al., 2018). In contrast, deletion of the fungal allergen peroxiredoxin Asp f3 does result in increased H_2O_2 sensitivity as determined in a plate stress assay. Absence of Asp f3 also results in an increased sensitivity for superoxides and inhibited hyphal growth. Furthermore, Asp f3 is essential for virulence in an immunocompromised mice model (Hillmann et al., 2016).

In this study we addressed the role of DHN-melanin in protection against H_2O_2 and superoxides generated via menadione. We show that deletion of the *pksP* gene in strain CEA10 did not affect sensitivity against H_2O_2 and superoxide in a plate stress assay, in contrast to the ATCC46645 *pksP* mutant strain (Jahn et al., 1997; Sugareva et al., 2006). Whole genome sequencing and single nucleotide polymorphism (SNP) analysis showed that the increase in H_2O_2 sensitivity might be due to a mutation in the *cat1* gene. Together, we here show for the first time that DHN-melanin is not involved in protecting conidia against hydrogen peroxide and superoxide.

Materials and methods

Strains and culture conditions

Strains used in this study are listed in Table 1. *A. fumigatus* and *Penicillium roqueforti* were grown on potato dextrose agar (PDA, Difco) at 37 °C and 25 °C, respectively, for production of conidia. These conidia were harvested with 0.85 % (w/v) NaCl with 0.005 % Tween 20 (VWR International). *A. fumigatus* and *P. roqueforti* were grown on minimal medium (MM; 6 g L^{-1} $NaNO_3$, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.2 mL L^{-1} Vishniac, and 20 mM glucose) or transformation medium (TM; MM with 25 mM glucose, 5 g L^{-1} yeast extract (Difco) and 2 g L^{-1} casamino acids (Difco)) for reactive oxygen sensitivity assays and genomic DNA isolation.

Table 1. Strains used in this study.

Strain	Description	Reference
A. fumigatus		
ATCC46645 *	*Aspergillus fumigatus*, clinical isolate	(Jahn et al., 1997)
ATCC46645*pksP*⁻ *	*pksP*⁻ derivative of ATCC46645 obtained via UV mutagenesis	(Jahn et al., 1997)
KL1.1 *	*pksP*⁻*::pksP* in ATCC46645*pksP*⁻	(Langfelder et al., 1998)
CEA10	*Aspergillus fumigatus*, clinical isolate	(Fedorova et al., 2008)
CEA10Δ*ku80*	*pyrG*::ku80 in CEA10	(da Silva Ferreira et al., 2006)
CEA10Δ*pksP*	Δ*pksP*::hph in CEA10Δ*KU80*	(Keizer et al., 2020; **Chapter 2**)
CEA10*pksP*C	*pksP::pksP* derivative of CEA10Δ*pksP*	(Keizer et al., 2020; **Chapter 2**)
P. roqueforti		
LCP96 04111	*Penicillium roqueforti* wild-type	(Punt et al., 2020)
PT34.2	*pksA*⁻ in LCP96 04111	(Seekles et al., 2020)

*Strains were kindly provided by Prof. dr. Axel A. Brakhage from HKI-Jena.

Peroxide and menadione sensitivity assays

Plate stress assay

Peroxide and menadione sensitivity assays were performed as described (Lessing et al., 2007; Sugareva et al., 2006). Briefly, 5 10^7 *A. fumigatus* conidia were mixed with 5 mL MM agar without a carbon source and poured onto MM agar plates (6 cm in diameter, containing 10 ml MM medium). For *P. roqueforti*, 10^8 conidia were mixed with 10 mL MM agar without carbon source and poured onto MM agar plates (10 cm in diameter, containing 20 mL MM medium). A hole of 10 mm was punched in the middle of the plate and filled with 100 µL 500 mM H_2O_2 (Sigma Aldrich) or 100 µL 1 mM menadione (Sigma Aldrich). Plates were incubated at 37 °C for 16 h after which the inhibition zone was measured.

Direct exposure stress assay

Conidia were harvested from 3-day-old PDA grown colonies and incubated for 0, 2, 4 or 6 h in TM. Resting and swollen conidia were diluted in Milli-Q to a concentration of 10^6 mL^{-1} and incubated for 30 min at room temperature with 0, 25 or 200 mM H_2O_2. Conidia were subsequently diluted in Milli-Q to a concentration of 10^3 mL^{-1} and 100 µL was plated onto PDA. Colony forming units were counted after overnight incubation at 37 °C and survival percentages were calculated. The colonies counted in the plate with 0 mM H_2O_2 was set as 100 % survival.

Chromosomal DNA isolation

Conidia were inoculated in TM and 50 µg mL^{-1} ampicillin (Sigma Aldrich), to prevent bacterial contamination, and grown overnight at 37 °C and 200 rpm. Mycelium was collected by filtering over a double layer of Miracloth (Merck Millipore) and was lyophilized overnight. Part of the lyophilized mycelium (~30 mg) was homogenized with a Tissuelyser (Qiagen) using 2 metal balls (4.76 mm in diameter) for 2 min at 25 Hz. DNA was isolated from the homogenized mycelium with the Qiagen DNeasy PowerPlant Pro kit, following manufacturer's protocol for problematic samples. Qubit® was used to check DNA quality and concentration. DNA samples were stored at -20 °C.

DNA sequencing and SNP analysis

Whole genome sequencing was performed by the Utrecht sequencing facility (USEQ). Libraries were prepared using Truseq DNA Nano library and sequenced on an Illumina NextSeq500 with 150 bp pair end mid output configuration. Quality of the reads was checked using fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqqc/). Cleaning and trimming of the reads was performed using the Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were mapped to the genome of reference strain Af293 for the reads from the ATCC derived strains (release 42 from FungiDB https://fungidb.org/fungidb/) or reference strain CEA10 for the CEA10 derived strains (release 42 from FungiDB https://fungidb.org/fungidb/) with bowtie2 v2.2.9 using options end to end and very sensitive (Langmead & Salzberg, 2012). For further quality control SAMtools v1.3 was used. Freebayes v.0.9.10-3 (Garrison & Marth, 2012) with the ploidy option set to 1 was used for variant calling. Vcfilter ("qual > 20", depth 5x) was used for post filtering of the obtained vcf file. SNPeff v4.3 was used to predict the effect of the variants (Cingolani et al., 2012). SNPs with a high (gain or loss of a stop codon, splice region variant) or moderate (missense) effect were used for analysis.

Cat1 SNP confirmation

The SNP in the *cat1* gene of the ATCC46645 *pksP*- strain and the *pksP* complementation strain was confirmed by sequencing the *cat1* PCR product amplified with primers *Cat1*_confirm PCR_fw (CGGTTCGGCAAGACTGTT) and *Cat1*_confirmPCR_rv (CTTTAGCCCGTCCGTCAG) and Q5 polymerase (New England Biolabs Inc.) according to the manufacturer's instructions. The sequences of the PCR products were aligned with the ATCC *cat1* gene using the Clustal Omega webtool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical analysis

Differences in inhibition zone after H_2O_2 or menadione exposure were analysed using a one-way ANOVA ($p \leq 0.05$). Differences in survival percentage of conidia after peroxide treatments were analysed using a non-parametric Kruskal-Wallis test ($p \leq 0.05$).

Data availability

Next generation sequencing (NGS) data of strains ATCC46645 and CEA10Δ*ku80* is available at NCBI Sequence Read Archive (SRA) under code PRJNA670081. NGS data from ATCC46645*pksP*-, KL1.1 and CEA10Δ*pksP* is available at the NCBI SRA under code PRJNA680589.

Results

Sensitivity of DHN-melanin mutants to oxidative stress

Conidia of the ATCC46645 UV mutant strain that contains mutations in the *pksP* gene are white and thereby do not produce melanin. These conidia were more sensitive to H_2O_2 as compared to its parental WT strain in the H_2O_2 plate stress assay (Figure 1A, grey bars), which is in accordance with (Sugareva et al., 2006). Deletion of the *pksP* gene by replacing the gene with the hygromycin resistant cassette in the CEA10Δ*ku80* background also resulted in a white phenotype but did

not lead to an increased sensitivity against H_2O_2 (Figure 1A, black bars). Notably, complementation of the mutated *pksP* gene in the ATCC46645 UV mutant resulted in green conidia but did not rescue the H_2O_2 sensitivity to wild-type levels. The complemented ATCC46645 strain had a similar sensitivity as the *pksP* mutant strain. In contrast, complementation of the CEA10Δ*ku80 pksP* deletion strain with a wild-type *pksP* gene resulted in green conidia and the strain showed similar resistance to to H_2O_2 as the wild-type strain. These results show that DHN-melanin is not involved in protection against H_2O_2 as was previously concluded (Jahn et al., 1997). Indeed, deletion of *pksA* resulting in a DHN-melanin deficient strain in *P. roqueforti* resulted in white conidia (Seekles et al., 2020) but again did not alter sensitivity to H_2O_2 as shown in the plate assay (Supplementary Figure 1).

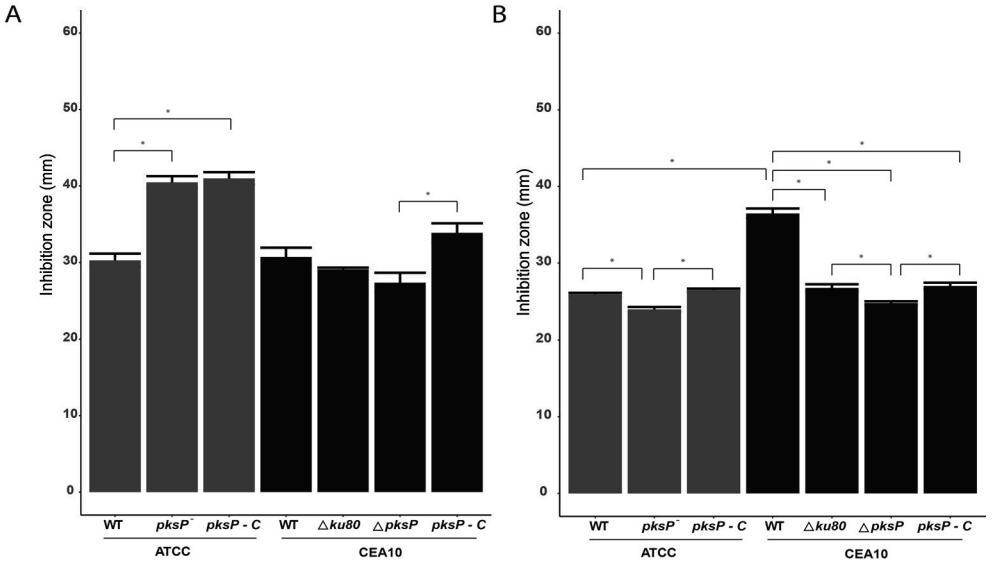

Figure 1. Peroxide (A) and menadione (B) sensitivity of *A. fumigatus* strains in the ATCC46645 background with the wild-type, *pksP*- and complementation strains (grey) and the CEA10 background with the Δ*ku80,* Δ*pksp* and complementation strains (black). Bars represent the average inhibition zone based on biological and technical triplicates with the standard error.

Dormant conidia were exposed for 30 min to 0, 25 and 200 mM H_2O_2. A decrease in survival of conidia was observed for all strains when the concentration H_2O_2 was increased from 25 mM to 200 mM (Figure 2A). Deletion or complementation of the *pksP* gene in the CEA10 background and mutations in the *pksP* gene and complementation in the ATCC46645 background did not significantly alter the sensitivity of dormant conidia to the different concentrations of H_2O_2 (Figure 2A). Next, conidia were incubated in medium for 2, 4 and 6 h prior to a 30 min exposure to 0, 25 and 200 mM H_2O_2. 2 h swollen conidia of the ATCC46645 *pksP*- strain and the CEA10Δ*ku80* strain seemed to be more resistant to the direct H_2O_2 exposure compared to the wild-type strains (Figure 2B). This resistance disappeared after 4 and 6 h swelling of the conidia (Figure 2 C, D). Together, the removal or complementation of DHN-melanin in an ATCC or CEA10 background did not alter the sensitivity of dormant or swollen conidia to direct H_2O_2 exposure.

Next, we investigated sensitivity of DHN-melanin mutants to menadione, which induces production of superoxides (Criddle et al., 2006). The ATCC46645 *pksP*- mutant and its complemented strain did not show altered resistance to menadione as compared to the wild-type strain (Figure 1B, grey bars). The CEA10 wild-type strain appeared more sensitive to menadione as compared to the ATCC46645 wild-type strain. Interestingly, deletion of the *ku80* gene in the CEA10 wild-type strain resulted in an increased resistance to menadione. Deletion of the *pksP* gene also increased the resistance to menadione in CEA10 background. On the other hand, complementation of the *pksP* gene decreased the resistance to the same level as the CEA10Δ*ku80* strain (Figure 1B, black bars). Together, these results show that DHN-melanin is not involved in resistance to superoxides. In fact, it slightly increased sensitivity.

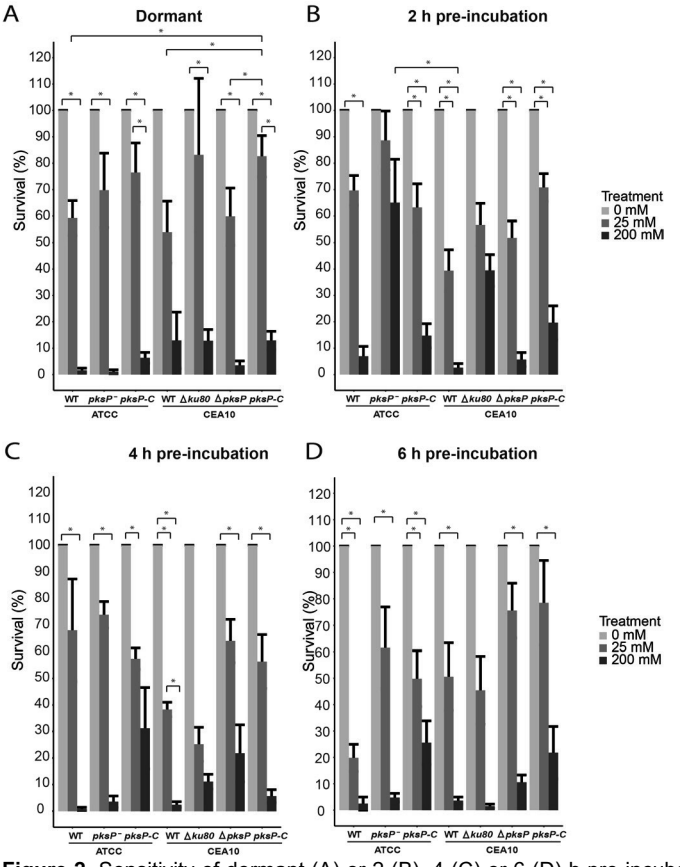

Figure 2. Sensitivity of dormant (A) or 2 (B), 4 (C) or 6 (D) h pre-incubated conidia to 0 mM, 25 mM or 200 mM H_2O_2. Bars represent the average survival based on biological triplicates with the standard error.

SNPs underlying melanin deficiency and H_2O_2 sensitivity

The ATCC46645 wild-type strain, the derived *pksP* mutant strain, the *pksP* complemented mutant strain, as well as strains CEA10Δ*ku80* and CEA10Δ*ku80*Δ*pksP* were sequenced to assess whether SNPs in genes other than *pksP* explain the increased H_2O_2 sensitivity of the *pksP* mutant strain and the

increased superoxide resistance of the CEA10Δ*ku80*Δ*pksP* strain. Reads from the ATCC46645 wild-type and derived strains were mapped back to the Af293 reference genome (Nierman et al., 2005), while reads from the CEA10 derived strains were mapped back to the CEA10 reference genome (Fedorova et al., 2008). A total of 272 SNPs was unique to the ATCC46645*pksP*$^-$ mutant strain (Figure 3), of which 6 in the *pksP* gene (Table 2, Figure 4). This resulted in 3 amino acid changes and 2 missing amino acids, due to deletion of codons, thus explaining the white phenotype of this strain. It is important to stress that complementation with the wild type *pksP* gene resulted in normal pigmentation of conidia, indicating the absence of mutations in the other genes involved in DHN-melanin biosynthesis. Indeed, this was confirmed by the genome sequence. Next, we focussed on SNPs in genes involved in ROS resistance (Supplementary Table 1) that were present in the ATCC46645*pksP*$^-$ mutant strain and its *pksP*$^-$ complemented strain, which are both ROS sensitive, and absent in the ATCC46645 wild-type strain. A SNP in the *cat1* gene resulting in a change of lysine 719 to glutamic acid was present in both the mutant and complemented strain (Supplementary Figure 2). This change in charged amino acids might affect the function of the Cat1 protein leading to an increase in H_2O_2 sensitivity.

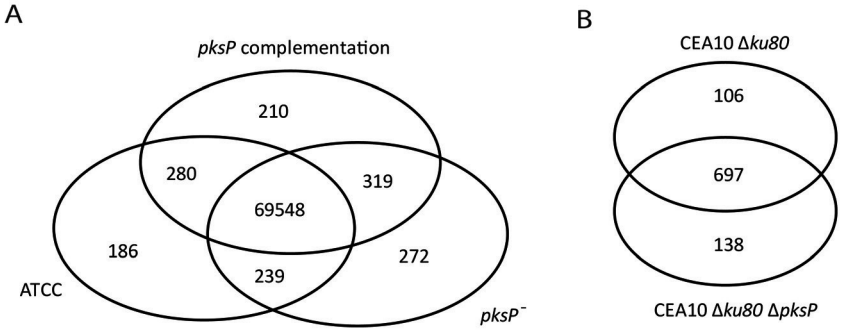

Figure 3. Venn diagrams of the SNPs present in the sequenced ATCC strains (A) mapped to the Af293 reference genome. The SNPs of the sequenced CEA10 strains (B) are mapped to the CEA10 reference genome.

SNPs in the CEA10Δ*ku80* genome were also compared to the CEA10 reference genome and three genes related to ROS sensitivity did contain a SNP. The first gene is AFUB_018600 and orthologs have been described that function in the oxidative stress response (fungidb.org). The second gene is AFUB_099260, which was reported to play a role in oxidation-reduction processes (fungidb.org) whereas orthologous of the third gene, AFUB_101360, play a role in the H_2O_2 response (fungidb.org). Mutations in these genes might be responsible for the decreased sensitivity of the CEA10Δ*ku80* strain for menadione, but this requires more research.

Table 2. Amino acid changes resulting from SNPs in the *pksP* gene in strain ATCC46645*pksP*$^-$.

Position	Amino Acid Change
187	Asparagine → Aspartic Acid
1578	Aspartic Acid → Asparagine
1580	Isoleucine deletion
1581	Isoleucine deletion
1607	Serine → Phenylalanine

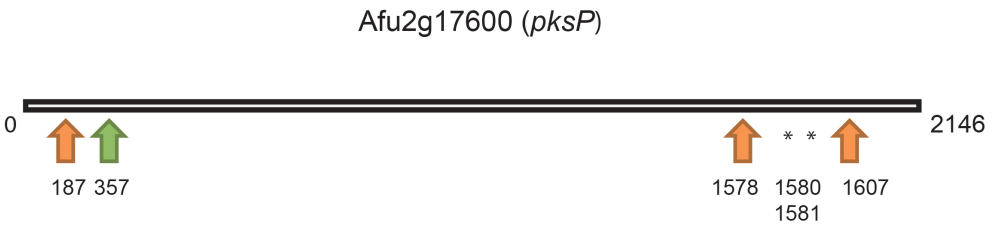

Figure 4. SNPs found in the ATCC46645*pksP*- mutant strain. Orange arrows indicate SNPs that lead to an amino acid change, the green arrow indicates a SNP with no amino acid change and the asterisks indicates SNPs that led to an amino acid deletion.

Discussion

We have shown by deletion of the *pksP* gene and its subsequent complementation that DHN-melanin does not protect against H_2O_2 and superoxides in strain CEA10 of *A. fumigatus* and *P. roqueforti* strain LCP96 04111. These results are in contrast with results obtained with the ATCC46645*pksP*- mutant strain (Jahn et al., 1997; Sugareva et al., 2006). Therefore, the latter strain was complemented by introducing *pksP* (Langfelder et al., 1998). This restored DHN-melanin formation but not H_2O_2 resistance, implying that another mutation is responsible for H_2O_2 sensitivity. Indeed, a mutation was found in the *cat1* gene encoding a secreted catalase. Catalases and peroxiredoxins play a role in the detoxification of H_2O_2 and are both regulated by Yap1 (Lessing et al., 2007; Rocha et al., 2018). *Yap1* is identified as a master-regulator of genes against ROS, and deletion of this transcription factor increases the sensitivity towards H_2O_2 and menadione (Lessing et al., 2007). Other catalase genes in the *A. fumigatus* genome as well as the peroxiredoxin genes and the y*ap1* gene did not have mutations. We therefore conclude that the increased sensitivity of the ATCC46645*pksP*- mutant strain is explained by the mutation in the *cat1* gene. This gene plays a role in ROS resistance in the mycelium and not in conidia but has no effect on virulence (Calera et al., 1997). Indeed, dormant and swelling conidia of the ATCC46645*pksP*- mutant strain did not show sensitivity to ROS. In contrast, microscopic analysis showed that only very few germinating conidia formed a germ tube (Supplementary Figure 3). Therefore, hyphae but not conidia of the ATCC46645*pksP*- strain are ROS sensitive. These results are in accordance with previously published research (Calera et al., 1997). The minimal role for *cat1* in virulence could mean that there are compensatory mechanisms upon deletion, which still protect the fungus against the H_2O_2 produced by immune cells. Alternatively, or in addition, other mechanisms of protection against ROS in the host are more important, e.g. the superoxide dismutases and peroxiredoxins.

Melanins have previously shown to protect against a variety of stress factors (Cordero & Casadevall, 2017). Absence of melanin increases the sensitivity of fungal species such as *C. neoformans* and *A. nidulans* against ROS like H_2O_2 (Jahn et al., 2000; Wang & Casadevall, 1994). It must be noted that both fungal species actually produce L-DOPA melanin, whereas *A. fumigatus* produces DHN-melanin. These melanins differ in their chemical structure (Supplementary Figure 4). L-DOPA melanin contains a dihydroquinone group (two hydroxyl groups attached to one ring structure), which can be converted to quinones and release 2 electrons that quench ROS radicals (Jacobson, 2000). Next to the dihydroquinone structure there is also

an indole structure (amine group in one ring structure) present in L-DOPA melanin, which can also quench free radicals produced by the ROS (Horstman et al., 2002). Both these structures are not present in the DHN-melanin, which could explain why this type of melanin does not confer protection against H_2O_2 and superoxide generated with menadione. The same seems to be the case for Asp-melanin. This type of pigment is produced by *Aspergillus terreus* instead of L-DOPA or DHN-melanin. Deletion of the genes encoding Asp-melanin, *melA* or *tyrP*, does also not alter the sensitivity towards H_2O_2 (Geib et al., 2016).

The CEA10*Δku80*Δ*pksP* deletion strain was more resistant to menadione than the CEA10*Δku80* strain, which could be due to mutations in unknown genes that play a role in superoxide protection. Interestingly, deletion of the *ku80* gene in the CEA10 background results in an increase in resistance towards menadione. ROS induce DNA damage and therefore we expected that deletion of *ku80* would lead to a further decrease in menadione resistance since this strain cannot repair DNA damage by non-homologous end joining (da Silva Ferreira et al., 2006). Possibly, inactivation of *ku80* was accompanied by a mutation resulting in decreased menadione sensitivity.

Together, we demonstrated that DHN-melanin does not protect against H_2O_2 which has long been recognized as an important protecting compound. Instead, we show a possible role for the mycelial catalase encoded by the *cat1* gene in protection of hyphae against extracellular H_2O_2. We propose that the absence of dihydroquinone and an indole structure in DHN-melanin explains the difference with L-DOPA melanin which is able to quench free radicals generated via ROS.

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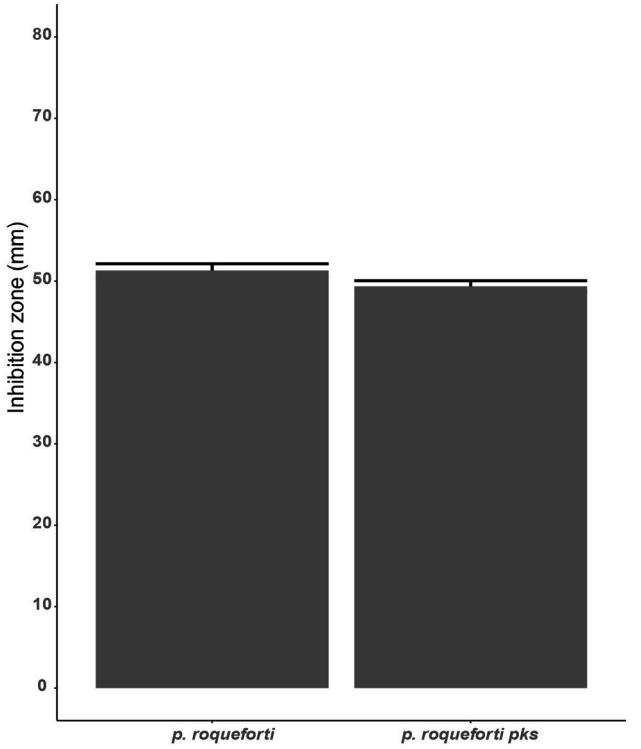
Supplementary data

Supplementary Table 1. Genes involved in ROS protection of conidia and hyphae of *A. fumigatus*.

Gene	GeneID	Function	Reference
yap1	Afu6g09930	Transcription factor that regulates catalase expression	(Lessing et al., 2007)
catA	Afu6g03890	Conidial catalase	(Paris et al., 2003)
cat1	Afu3g02270	Mycelium catalase	(Calera et al., 1997; Paris et al., 2003)
cat2	Afu8g01670	Mycelium catalase	(Paris et al., 2003)
pksP	Afu2g17600	DHN-melanin synthesis	(Jahn et al., 1997; Sugareva et al., 2006)
aspf3	Afu6g02280	Fungal allergen and has peroxidase activity	(Hillmann et al., 2016)
rgsD	Afu5g00900	Regulator of G protein signaling	(Kim et al., 2019)
aox	Afu2g05060	Mitochondrial enzyme alternative oxidase. Decreases damage caused by oxidative stress / minimize production ROS	(Grahl et al., 2012)
cycA	Afu2g13110	Cytochrome C, part of the respiratory chain to generate ATP	(Grahl et al., 2012)
pab1	Afu1g04190	Cytoplasmic messenger ribonuclear protein	(Wang et al., 2016)
sod1 (A)	Afu5g09240	Superoxide dismutase, expressed in conidia, Cytoplasmic Cu/Zn SOD	(Lambou et al., 2010)
sod2 (B)	Afu4g11580	Superoxide dismutase, expressed in conidia, Mitochondrial Mn SOD	(Lambou et al., 2010)
prx1	Afu4g08580	Cytosolic peroxiredoxin	(Rocha et al., 2018)
prxB	Afu5g15070	Mitochondrial peroxiredoxin	(Rocha et al., 2018)
prxC	Afu8g07130	Mitochondrial peroxiredoxin	(Rocha et al., 2018)
gstA	Afu3g10830	Glutathione peroxidase	(Burns et al., 2005)
gstC	Afu4g14530	Glutathione peroxidase	(Burns et al., 2005)
sakA	Afu1g12940	Putative mitogen-activated protein kinase (MAPK) with predicted roles in the osmotic and oxidative stress responses	(Day & Quinn, 2019)
phkA	Afu3g12550	Class X histidine kinase	(Day & Quinn, 2019)
atfA	Afu3g11330	bZIP-type transcription factor	(Day & Quinn, 2019)
bir1	Afu1g14070	Inhibitor of apoptosis protein 1	(Shlezinger et al., 2017)
hyr1	Afu3g12270	Putative glutathione peroxidase; peroxiredoxin	(Fan et al., 2016)
skn7	Afu6g12522	Putative transcription factor and response regulator of a two-component signal transduction system	(Lamarre et al., 2007)
mpkA	Afu4g13720	Mitogen-activated protein kinase	(Valiante et al., 2008)
mpkC	Afu5g09100	Putative mitogen activated protein kinase (MAPK)	(Bruder Nascimento et al., 2016)
pkaR	Afu3g10000	cAMP-dependent protein kinase regulatory subunit	(Zhao et al., 2006)
gliK	Afu6g06700	Gamma-glutamyl cyclotransferase gliK	(Gallagher et al., 2012)
sskA	Afu5g08390	Putative response regulator, part of a two-component signal transduction system	(Hagiwara et al., 2014)
pbsB	Afu3g05900	MAP kinase kinase (MAPKK)	(Hagiwara et al., 2014)
sho1	Afu5g08420	Putative transmembrane osmosensor with homology to *S. cerevisiae* Sho1p	(Ma et al., 2008)
rho1	Afu6g06900	Putative Rho-type GTPase	(Zhang et al., 2018)
pkcA	Afu5g11970	Protein kinase C, involved in cell wall integrity pathway	(Rocha et al., 2015)
sebA	Afu4g09080	Putative transcription factor	(Dinamarco et al., 2012)



Supplementary Figure 1. Peroxide sensitivity of *P. roqueforti* with or without (Δ*pks*) DHN-melanin based on inhibition zones. Bars represent the average inhibition zone based on biological and technical triplicates, with the standard error.

Afu3g02270 (*Cat1*)

```
Af293       GATCTTAAGGAGGGCCTTAGAACTTTTAAGTTTTTGGACCGGTTTCCCGTGGATCACTAG   180
ATCC        GATCTTAAGGAGGGCCTTAGAACTTTTAAGTTTTTGGACCGGTTTCCCGTGGATCACTAG   151
pksP-C      GATCTTAAGGAGGGCCTTAGAACTTTTGAGTTTTTGGACCGGTTTCCCGTGGATCACTAG   151
ΔpksP       GATCTTAAGGAGGGCCTTAGAACTTTTGAGTTTTTGGACCGGTTTCCCGTGGATCACTAG   150
            ************************** ********************************
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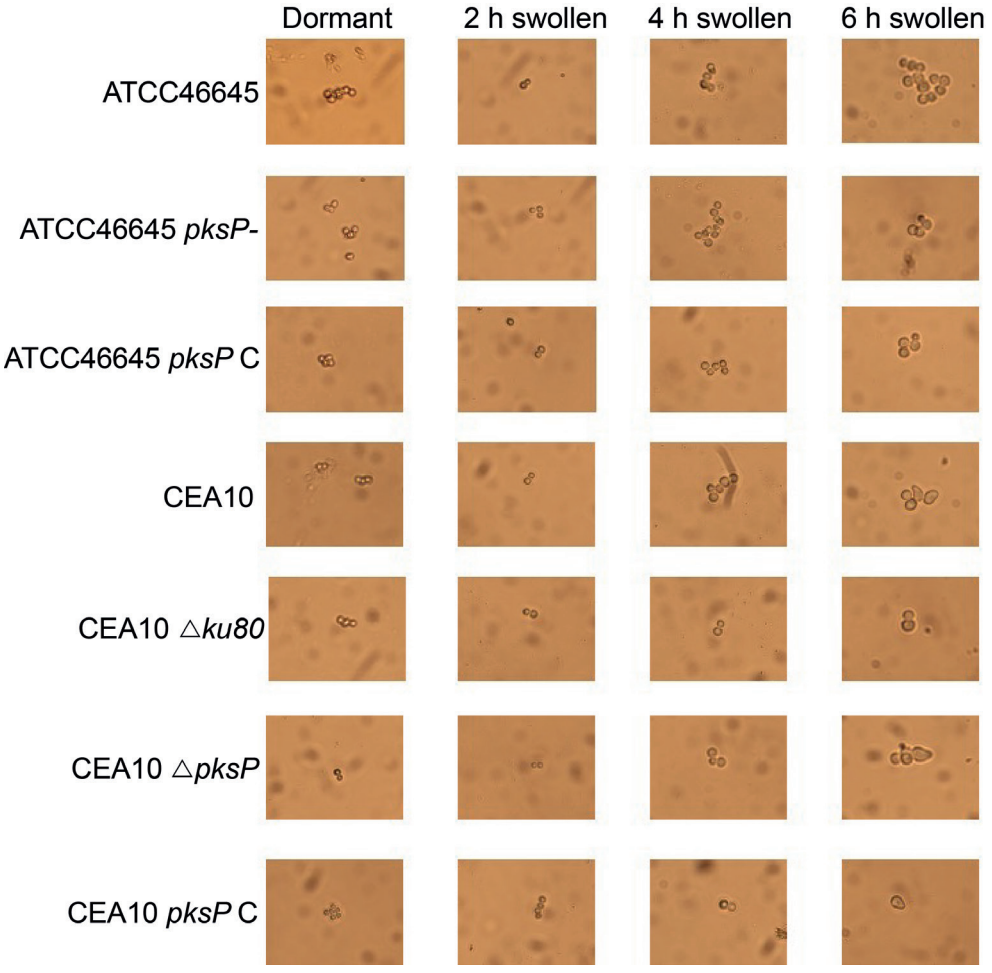
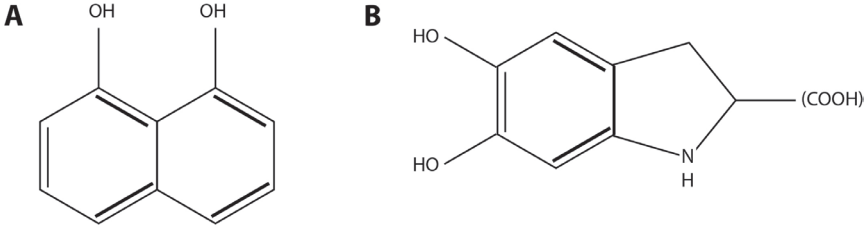
Supplementary Figure 2. PCR confirmation of the SNP in the *Cat1* gene present in the ATCC*pksP*- and the ATCC *pksP* complementation strain.



Supplementary Figure 3. Microscopic pictures of 0, 2, 4 and 6 h swollen conidia.



Supplementary Figure 4. Structure of DHN-melanin (A) and L-DOPA melanin (B). Adapted from (Eisenman & Casadevall, 2012).

4

LaeA-dependent production of small molecules of *Aspergillus niger* that interact with immune receptors

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Abstract

Microorganisms secrete a variety of compounds into their environment such as proteins, carbohydrates, and secondary metabolites. These molecules play diverse roles in the interaction of microbes with their abiotic and biotic environment. For instance, bacterial pathogens use extracellular compounds for immune evasion enabling a successful infection process. Little is known about secreted fungal molecules mediating immune evasion. Here we screened culture media of 3 aspergilli to assess whether these fungi secrete molecules that can compete with specific antibodies that bind to human immune receptors. Culture media of *A. fumigatus* Af293*, A. tubingensis* CBS 133792 and the non-acidifying mutant strain *A. niger* D15#26 contained components that showed competition for binding to a total of 13 receptors, of which CD162, CD181, and CD182, were shared between the 3 species. Filtration experiments showed that most, if not all, interacting components were ≤ 3 kDa. The *A. niger* D15#26 components that competed with antibodies for binding to CD162, CD181, CD182, and CD88 were thermostable and hydrophilic. Production of the components that competed with antibodies to bind to CD88 and CD182 was shown to be regulated by LaeA. The component(s) that competed for binding to CD181 was not only produced in the non-acidifying strain *A. niger* D15#26 but also in the non-acidifying *oahA* deletion strain of *A. niger*. Together, these data show that *Aspergillus* species can produce small molecules that interact with human immune receptors. These molecules that are regulated by different pathways may function in immune evasion strategies and may have therapeutic activity.

Introduction

Microbial compounds known as pathogen-associated molecular patterns (PAMPs) induce a host innate immune response by binding to pattern recognition receptors. Dendritic cells (DCs), macrophages, neutrophils (PMNs), natural killers (NKs), and monocytes express a large repertoire of soluble and membrane-bound receptors (also called CD receptors) that in interaction with their ligand initiate the immune response (Fietta et al., 1984; Kan & Bennett, 1991; Levitz & Farrell, 1990; Li et al., 2013). These immune cells are for example active on the lung epithelial layer and adjacent tissue to remove micro-organisms like fungal propagules (Mircescu et al., 2009; Shoham & Levitz, 2005). Cell wall molecules (e.g. chitin, mannan, glucan) are the best described fungal PAMPs. For instance, the polysaccharide β(1,3)-glucan was shown to interact with complement receptor 3, Dectin-1, EphA2, and ficolin-2. These interactions trigger a variety of responses like ROS production, neutrophil migration, and production of cytokines (Brown, 2006; Swidergall et al., 2018; Werner et al., 2009).

Microbes secrete molecules that mediate immune evasion. Various *Staphylococcus aureus* proteins involved in immune evasion have been identified by screening culture media in a competition binding assay using immune receptors (Bestebroer et al., 2007). For example, the secreted SSLs family proteins of *S. aureus* were shown to block the interaction between PSGL-1 and P-selectin inhibiting neutrophil (PMN) recruitment. This human pathogen also secretes a virulence factor described as chemotaxis inhibitory protein (CHIPS) that inhibits PMN responses by binding to the formyl peptide receptor (FPR) and the C5a receptor (C5aR; also known as CD88). Secreted *A. fumigatus* proteases such as aspartic protease (Pep1p), metalloprotease (Mep1p), and alkaline protease (Alp1) have also been described to mediate immune evasion (Behnsen et al., 2010; Shende et al., 2018). Possibly, opportunistic fungal pathogens such as *A. niger*, *A. fumigatus*, and *A. tubingensis* secrete also immune evasive compounds because of the large variety of molecules they release into their environment. The secretome of *Aspergillus* includes enzymes, secondary metabolites, sugars and organic acids (Brakhage & Schroeckh, 2011; Machida et al., 2005; Medina et al., 2005; Punt et al., 2002; Tsang et al., 2009). The number and type of secreted compounds vary depending on the culture conditions (Giorni et al., 2008; Lu et al., 2010; Sriranganadane et al., 2010; Wongwicharn et al., 1999). By combining *in silico* predictions and shotgun proteomics it was estimated that *A. niger* can secrete over 200 proteins (Braaksma et al., 2010). These proteins are predicted to serve functions in cellular communication, immunity and pathogenesis, degradation of substrates, morphogenesis, and cell proliferation (Tsang et al., 2009). A similar number of secondary metabolites (i.e. 145) have been isolated and / or detected from cultures of black aspergilli (*Aspergillus* section *Nigri)* (Nielsen et al., 2009). Secondary metabolites are low molecular weight molecules with various functions. Some metabolites are mycotoxins (e.g. fumonisin B2, ochratoxin A) causing food spoilage and being a threat for human health. Yet, for many fungal secondary metabolites a biological role has not been elucidated.

The *prtT* and *laeA* genes of *A. niger* have been shown to play a crucial role in the production of extracellular compounds (Bok & Keller, 2004; Budak et al., 2014; Punt et al., 2008; Sharon et al., 2011). Strains with *prtT* mutations exhibit 1 - 2 % of the extracellular protease activity when compared to the parental strain *A. niger*

AB4.1 (Punt et al., 2008). This gene encodes a Zn(II)2Cys6 transcription factor and controls the expression of 6 secreted proteases including aspergillopepsin A (PepA) and aspergillopepsin B (PepB) (Punt et al., 2008). Transcriptional and proteomic analysis of an *A. fumigatus prtT* mutant indicated that PrtT also regulates genes involved in iron uptake, ergosterol biosynthesis, and production of secondary metabolites (Hagag et al., 2012; Sharon et al., 2009). Gene *laeA* encodes a putative methyltransferase domain protein and is a global regulator of secondary metabolite gene clusters in *Aspergillus*. Amongst others, synthesis of gliotoxin, sterigmatocystin, penicillin, and lovastatin is being regulated by LaeA (Bok & Keller, 2004). Notably, secondary metabolite production of the *A. niger* Δ*laeA* strain differed when compared to the wild-type, with both an increase and decrease in metabolite levels (Niu et al., 2015). This indicates that this protein can also act as a repressor of secondary metabolite genes. Deletion of *laeA* also leads to a decrease in acidification of the culture medium (Niu et al., 2015), which is due to production of citric acid, gluconic acid and / or oxalic acid. Production of these organic acids is pH dependent (Andersen et al., 2009). For example, optimal production of citric acid in *A. niger* occurs at pH 2 (Karaffa & Kubicek, 2003), while oxalic acid production is optimal between pH 5 to 8 (Ruijter et al., 1999).

The global regulator LaeA was proposed to regulate secondary metabolite synthesis via chromatin remodelling and regulates amongst others secondary metabolites produced via polyketide synthases (PKS) or non-ribosomal peptide synthases (NRPS) (Brakhage, 2013). Both of these synthesis pathways require post-translational modification for activation. The 4'phosphopantetheinyl transferase (PPTase) activates PKS and NRPS. Deletion of this PPTase leads to a defect in secondary metabolite production in *A. fumigatus*, *A. nidulans* and *A. niger* (Johns et al., 2017; Jørgensen et al., 2011; Keszenman-Pereyra et al., 2003; Márquez-Fernández et al., 2007). Other fungal secondary metabolite pathways are the mevalonate or shikimic acid pathway, which are involved in the production of terpenes or aromatic secondary metabolites, respectively (Dewick, 2009; Keller et al., 2005).

Identification of secreted immune-reactive molecules might unravel new immune evasion strategies and may reveal novel therapeutic agents. In this study we show that culture media of *A. niger*, *A. fumigatus*, and *A. tubingensis* contain thermostable molecules ≤ 3 KDa that compete with monoclonal antibodies in their interaction to receptors involved in immune recognition. It is also shown that LaeA of *A. niger* is a repressor of the production of the compounds that compete with binding to CD88 and CD182, while secretion of the compound binding to CD181 is increased under non-acidifying medium conditions.

Materials and methods

Strains, growth conditions, isolation of culture medium, and extracellular fractions

Strains (Table 1, Figure 1) were grown for 3 days at 37 °C in minimal medium (MM; 6 g L^{-1} $NaNO_3$, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.2 mL L^{-1} Vishniac; pH 6.0) supplemented with 25 mM glucose and 1.5 % agar to obtain conidia. Conidia were harvested with 0.005 % (v/v) Tween-80 in 0.85 % (w/v) NaCl. A total number of 10^{10} conidia was used to inoculate 250 mL transformation medium (TM; MM supplemented with 5 g L^{-1} yeast extract (Difco) and 2 g L^{-1} casamino acids

(Difco)) in a 1 L Erlenmeyer. Cultures were grown for 16 h at 30 °C and 250 rpm in MM with 25 mM xylose or maltose as carbon source. Mycelium was harvested by filtration over 3 layers of Miracloth (Merck Millipore) and washed with 50 mL PBS (137 mM NaCl, 2.7 mM KCl, 3.8 mM $Na_2HPO_4{\cdot}2H_2O$, 1.5 mM KH_2PO_4). 10 g wet weight mycelium was transferred to a 500 mL Erlenmeyer containing 150 mL MM supplemented with either 25 mM xylose or maltose. Cultures were grown for 3 days at 30 °C and 250 rpm. Pellet formation was followed using light microscopy (Axioskop 2 plus, Carl Zeiss) and their surface area was measured after 72 h using Image J (https://imagej.nih.gov/ij/). Mycelium and mycelial fragments were removed by filtering over 3 layers of Miracloth and a 0.22 µm filter (Carl Roth). Xylose- and maltose-culture media were mixed 1:1 for analysis. In order to obtain small secreted molecules, supernatants were filtered using a 3 kDa cut-off Amicon ultra centrifugal filter (Merck Millipore). Culture media and ≤ 3 KDa fractions were lyophilized and suspended in PBS.

Strains lacking the *pptA* gene were grown in MM medium with either xylose or maltose as carbon source, which was mixed 1:1 with siderophore medium and 10 mM lysine (Sigma Aldrich). Siderophore medium was made by growing *A. niger* N402 in liquid MM in the absence of Fe^{2+}, with 5 mM glutamine (Sigma Aldrich) as a nitrogen source and 50 mM glucose as carbon source. *A. niger* was cultured for a total of 48 h at 200 rpm and after the first 24 hours of growth, fresh glutamine (5 mM) was added. Mycelium was removed from the medium by filtering over 3 layers of Miracloth, the pH was set to 6.0 and the siderophore culture medium was autoclaved (Márquez-Fernández et al., 2007).

Table 1. Strains used in this study.

Strain name	Parent	Relevant genotype	Reference
A. niger			
N402		Short conidiophore mutant of NRRL3	(Bos et al., 1988)
D15#26	N402	An UV-generated *pyrG*$^-$ *prtT*$^-$, *laeA*$^-$ strain isolated as a non-acidifying mutant (ac$^-$)	(Niu et al., 2015)
JN21.1	D15#26	pAO4_13	(Niu et al., 2015)
JN22.7	D15#26	pAO4_13-*laeA*	(Niu et al., 2015)
JN23.1	AB4.1	pAB4.1 pyrG+	
JN24.6	AB4.1	Δ*laeA*::AopyrG	(Niu et al., 2015)
AB1.13	AB4.1	*pyrG*$^-$ *prtT*$^-$	(Mattern et al., 1992)
AB1.13Δ*oahA*#76	AB1.13	Δ*oahA*::pyrG	(Li et al., 2012)
JP1.1	N402	Δ*pptA*::AopyrG	(Jørgensen et al., 2011)
MA870.1	JN24.6	Δ*laeA,* Δ*pptA*::hygR	This Chapter
AF11#56	AB4.1	Δ*prtT*::pyrG	(Franken et al., 2017)
MA306.1	MA234.1	Δ*crzA*::hygR	This Chapter
A. fumigatus			
Af293		Clinical isolate from lung tissue	(Pain et al., 2004)
A. tubingensis			
CBS 133792		Clinical isolate from an immuno-compromised patient suffering from osteomyelitis	(Bathoorn et al., 2013)

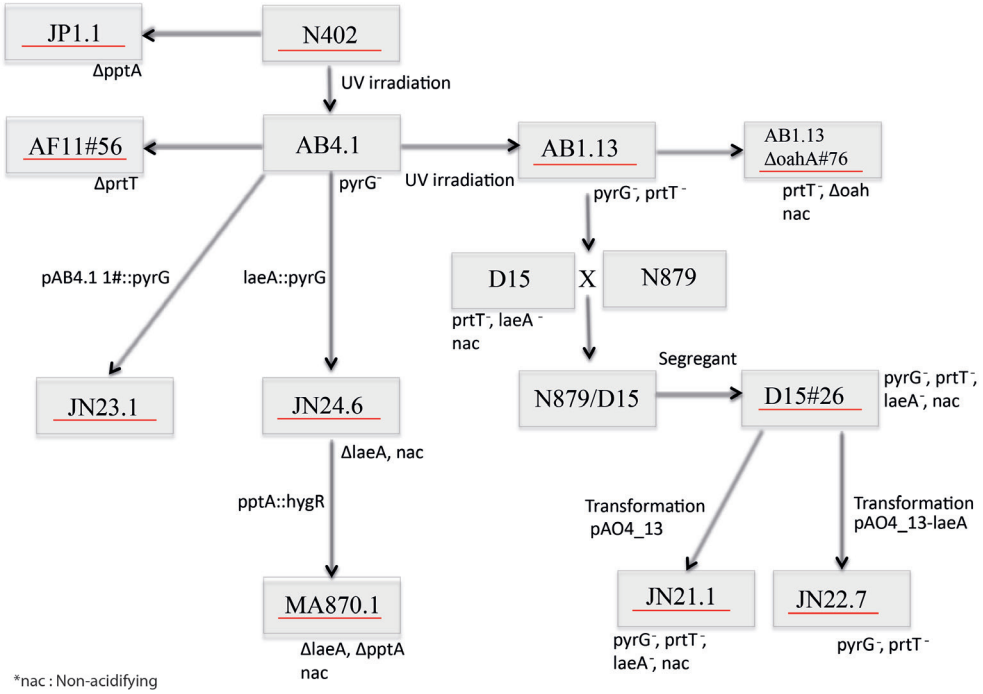

Figure 1. Schematic overview of strains used in this study (underlined in red). Adapted from (Niu et al., 2015). nac: non acidifying.

SDS PAGE

Proteins from culture media were precipitated with 4 volumes of acetone (Merck) for 16 h at 20 °C. Samples were centrifuged at 10,000 g for 15 min, re-suspended in 2 x SDS sample buffer (20 % glycerol (LPS Benelux), 4 % SDS (JT Baker), 100 mM Tris-HCl pH 6.8 (Roche), 0.01 % bromophenol blue (Acros Organics) and 5 % β-mercaptoethanol (Sigma-Aldrich)), and heated for 10 min at 100 °C. Samples and Low Molecular Weight Marker (14.000-70.000 Da) (Sigma-Aldrich) were loaded on 12 % SDS-PAA gels and stained with 0.1 % Coomassie Brilliant Blue G250 (Sigma-Aldrich) in 25 % methanol (Merck) and 10 % acetic acid (Merck).

PMNs, and PBMCs isolation and competition for receptor binding assay

Polymorphonuclear neutrophils (PMNs) and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy donors following the Histopaque-Ficoll gradient protocol (Bestebroer et al., 2007). Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands). Competition for receptor binding assay (CBA) (Supplementary Figure 1) was performed using commercial phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, and allophycocyanin (APC)-conjugated antibodies (Supplementary Table 1) as described (Bestebroer et al., 2007) with some modifications. Briefly, 100 µL of PMNs and 150 µL PBMCs (each 10^7 cells mL^{-1}) were mixed and centrifuged for 5 min at 7,000 g. The pellet was resuspended in 1 mL of fungal culture medium supernatant. If necessary, pH of the samples was adjusted to 7 using 0.1 M NaOH. After incubation for 15 min at 4 °C, 35 µL of the

mixture was incubated with antibodies (concentrations in Supplementary Table 1) at 160 rpm for 45 min at 4 °C in 96-well U-plates (Corning). Cells were washed with 150 µL RPMI medium (Life Technologies) containing 0.05 % human serum albumin (Sanquin) and centrifuged at 1200 g for 8 min at 4 °C. Cells were fixed with 1 % paraformaldehyde (Sigma-Aldrich) and fluorescence was measured by flow cytometry (FACSVerse, BD). Geometric mean fluorescence from neutrophils, monocytes, and lymphocytes was determined using FlowJo software (version V10.1, TreeStar) to gate each cell population. Reduction in fluorescence due to competition for binding with molecules within the culture media was calculated by dividing the mean signal by that of buffer-treated cells. Values were inverted and receptors with geometric means ≥ 2 were considered positive. Reproducibility of the assay was confirmed by using biological triplicates with culture media and blood cells from independent cultures and donors, respectively. Receptors that scored positive were re-measured in ≥ 3 independent experiments, receptors that scored positive in at least 2 experiments were scored as responsive. Data was visualized using R software (https://www.r-project.org/) and boxplots were generated with the ggplot2 package (http://ggplot2.org). Data from neutrophils, monocytes, and lymphocytes were included in the same plot and used to calculate median and quartiles. Data outside the boxplot whiskers were taken as outliers.

Characterization of D15#26 3KDa supernatant

Heat stability was assessed by incubating samples at 100 °C for 60 min. Samples were lyophilized and re-suspended in PBS. (Poly)peptides were precipitated from the culture medium for 30 min at 4 °C after adding acetone in a 1:1 ratio. After centrifugation at 11,000 g for 15 min, the pellet was air-dried, while the supernatant was dried using a rotoevaportor-RE (Büchi). Fractions were resuspended in PBS and tested in CBA. Hydrophobic compounds were extracted from the culture medium by mixing with 3 volumes of ethyl acetate (Acros Organics). Aqueous phases were collected, and treatment was repeated twice. Ethyl acetate fractions were pooled and dried with a rotoevaporator, while aqueous fractions were lyophilized. Fractions were resuspended in PBS in 1/3 of the original volume and tested in CBA.

Purification of D15#26 3KDa supernatant

Sep-Pak C18 column purification

Ten C18 solid phase cartridges (Sep-pak) were used to load 50 mL of culture medium that had been lyophilized and resuspended in 50 mL of PBS. Columns were eluted stepwise with 0, 10, 30 50, 70 and 100 % methanol (v/v). Fractions with the same percentage of methanol were pooled, lyophilized, resuspended in PBS, and tested in CBA. Fractions obtained from elution with 10 - 50 % methanol were pooled and concentrated 100-fold for LC-MS analysis.

LC-MS and *Preparative HPLC*

LC-MS was performed on a Shimadzu SCL-10A controller system (Shimadzu Cooperation) coupled to a Shimadzu pump LC10-AD and a Shimadzu CTO-10AS column oven. A Reprosil-Pur C18-AQ column (Particle size = 5 µm, Pore size = 120Å, 250 x 4,6 mm; Reprosil) was loaded with 50 µL 100-fold concentrated 10 - 50 % methanol pooled fraction. A 0 - 100 % gradient elution (Supplementary Table 2) with water and acetonitrile (JT Baker, HPLC grade) was used for separation of molecules during 60 min. The flow rate was 1 mL min^{-1} and compounds were

detected with a UV detector at 214 nm (Shimadzu SPD-10A). Mass spectrometry was done using a Finnigan LCQ Deca XP Max (Thermo Electron).
Preparative HPLC was run on a Shimadzu SCL-10A controller system coupled with a Shimadzu LC-8a pump and a Shimadzu SPD-10A UV detector. A Reprosil-Pur C18-AQ column (particle size =10 µm, pore size= 120Á, 250 x 22 mm; brand) with a Reprosil-Pur C18-AQ guard column (particle size =10 µm, pore size= 120Á, 30x22 mm) was injected with 450 µL of concentrated 10 - 50 % methanol pooled fraction. For sample separation a 0 to 60 % gradient elution with water and acetonitrile over 100 min was used with a flow rate of 12.5 mL min^{-1}. Ninety-five 13 mL fractions were collected using a Gilson Liquid Handler 215. Fractions were pooled in equal ratios (1 mL each fraction), obtaining 16 fractions that were dried in a SpeedVac, resuspended in 1 ml of PBS, and subjected to CBA.

Prediction of *A. tubingensis* SM clusters

A. tubingensis CBS 134.48 genome v 1.0 (de Vries et al., 2017) (http://genome.jgi.doe.gov/Asptu1/Asptu1.download.html) was used to predict the number of genes clusters involved in biosynthesis of secondary metabolites. Analysis was performed using anti-SMASH parameters (http://antismash.secondarymetabolites.org). By using the "homologous gene cluster" tool, gene clusters were identified with similarity to gene clusters in the *A. niger* ATCC1015 (http://genome.jgi.doe.gov/Aspni5/Aspni5.download.html) and *A. fumigatus* Af293 (http://genome.jgi.doe.gov/Aspfu1/Aspfu1.download.html) genomes.

Results

Characterization of fungal cultures

Protein profiles of *Aspergillus* culture media were monitored after 24, 48, and 72 h of growth. To this end, samples of maltose and xylose media were mixed in a 1:1 ratio, precipitated, and analysed by SDS-PAGE (Figure 2). Protein profiles showed a high variation between the different strains. *A. niger* N402 showed bands > 68 kDa that were reduced in intensity in *A. niger* D15#26. *A. tubingensis* that also belongs to the *Aspergillus* section *Nigri* showed a protein profile different from the two *A. niger* strains. *A. fumigatus* showed also a distinct profile containing high (55 - 80 kDa) and low molecular weight bands (15 - 30 kDa). The *A. niger* D15#26 strain does not acidify the culture medium. Indeed, pH of the culture medium had increased to 7 after 72 h of growth (Table 2). In contrast, *A. niger* N402 and *A. tubingensis* had lowered the pH to 5.5, while the pH of the culture medium of *A. fumigatus* had increased to pH 8. All cultures showed pelleted growth, but morphology was different (Table 2). The *A. niger* strain D15#26 produced smaller pellets mixed with dispersed growth when compared to wild-type *A. niger* N402 (Supplementary Figure 2).

Table 2. pH of *Aspergillus* culture media after 24, 48, and 72 h of growth and mycelium morphology after 72 h. The pH of culture medium at 0 h was 6.

Strain	pH			Growth characteristic after 72 h of growth
	24 h	48 h	72 h	
A. niger N402	4.5	5.5	5.5	Dense pellets (± 450,000 μm^2)
A. niger D15#26	5.5	6.5	7	Pellets (± 50,000 μm^2) with some dispersed growth
A. tubingensis	4.5	5	5.5	Dense pellets (± 230,000 μm^2)
A. fumigatus 293	7	7	8	Pellets (± 22,500 μm^2) with dispersed growth

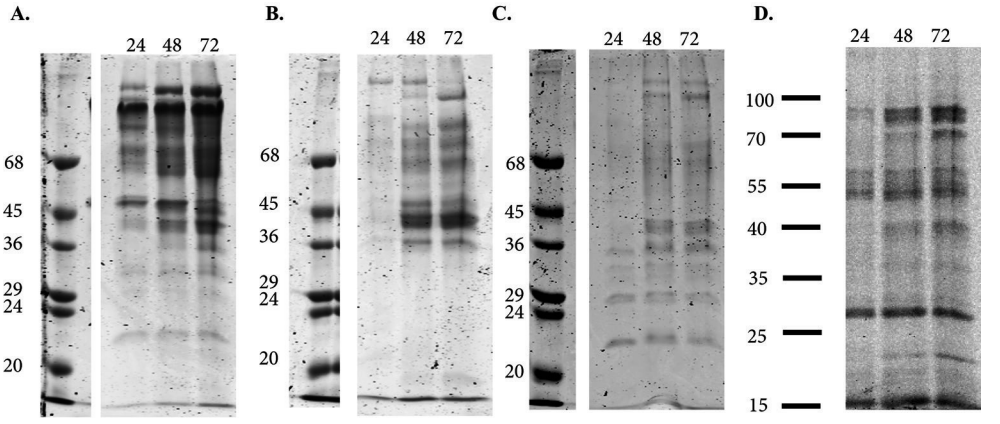

Figure 2. CBB stained SDS PAA gels of mixed maltose and xylose culture media of *A. niger* N402 (A), *A. niger* D15#26 (B), *A. tubingensis* (C), and *A. fumigatus* 293 (D) after 24, 48, and 72 h of growth.

Mixed maltose and xylose culture media were used to challenge PMNs and PMBCs in a competition binding assay (CBA) in order to detect secreted compounds that interact with human immune receptors. A component(s) in the culture medium of *A. niger* N402 (Figure 3A) competed with monoclonal antibodies directed against CD162 in several of the experiments but variation was too high to reach a median ≥ 2 fold signal when compared to the control. Possibly, the lack of response with other receptors was due to extracellular proteases degrading binding proteins. We therefore used a derivative of N402, strain D15#26, that has low protease activity due to a *prtT* mutation. Culture media of *A. niger* D15#26 indeed contained a component(s) that competed for binding to CD141, while receptors CD162, CD181, CD182, and CD47 were responsive in several experiments but had a median < 2 (Figure 3B). Anion / cation chromatography was performed as a first step for protein purification and fractions were tested in CBA. However, competition for binding to receptor molecules was not observed in any of the fractions (Supplementary Figure 3). The purification procedure included a dialysis step with membranes with a 12 kDa cut off. To assess whether small molecular weight molecules were responsible for the CBA response culture media were filtered using 3 kDa filters and fractions were tested with a subset of receptors. Responses of the ≤ 3 kDa fraction of D15#26 were stronger when compared to whole culture medium (see boxplot median Figure 4A). CD162, CD181, and CD182 showed a strong response, while response of CD88 was also close to 2-fold. The N402 ≤ 3 kDa fraction resulted in signals of receptors CD162, CD181, and CD182 just below the 2-fold response threshold (Figure 4B). CBA was also performed with the complete set of receptors using whole culture media (Figure 5) and ≤ 3 kDa fractions (Figure 6) of clinical *A. tubengensis* and *A. fumigatus* strains. Culture media of these strains contained components that competed with binding of a total of 11 receptors, of which CD162, CD181, CD182, CD192, CD47, CD13, and CD99 were shared between both clinical isolates. In most cases activity was present in the ≤ 3 kDa fractions (Figure 6).

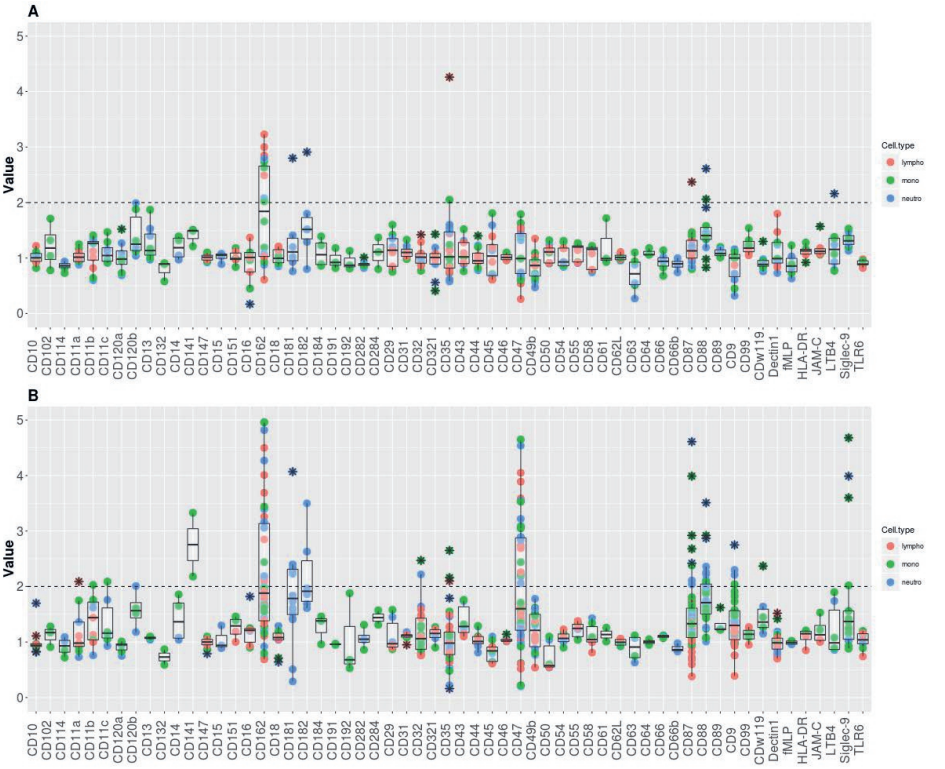

Figure 3. Competition binding assay of culture media of *A. niger* N402 (A) and *A. niger* D15#26 (B). Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represents outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.

Characterization D15#26 ≤ 3 kDa fraction

Incubation at 100 °C for 60 min did not affect competition activity of molecules within the ≤ 3 kDa fraction of D15#26 to receptors CD162, CD181, CD182, and CD88 (Supplementary Figure 4). Activity was also not reduced by removing proteins by precipitation with acetone. In agreement, competition activity was absent in the protein fraction. Extraction with ethyl acetate also did not affect activity in the aqueous phase (Supplementary Figure 5). These results suggest that competing molecules are hydrophilic. Next, the ≤ 3 kDa fraction of D15#26 was loaded onto a C18 Sep-pak column and molecules were eluted using a methanol gradient. Both flow-through as well as fractions eluted with 10 - 50 % methanol contained components with competition activity to receptors CD162, CD181, CD182 and CD88 (Supplementary Figure 6). Analysis of the D15#26 ≤ 3 kDa fraction by LC-MS indicated that it contained around 30 peaks (Supplementary Figure 7). CBA of the fractions collected from the LC-MS analysis was performed using the same subset of receptors used to test the ≤ 3 kDa fractions. Competing activity was not found in the tested pooled fractions. Loss of activity could be explained due to a partial binding of active components to C18-AQ column and therefore a decrease of the sample concentration or the inability of the hydrophilic compounds to bind to the C18-AQ column.

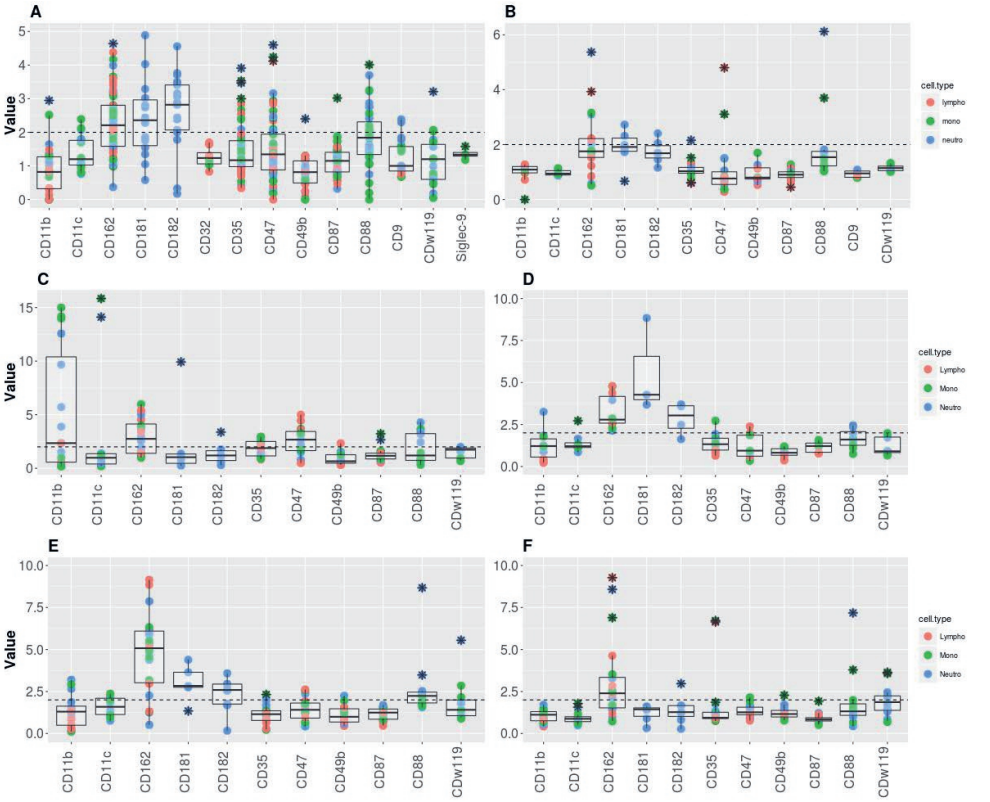

Figure 4: Competition binding assay using ≤ 3kDa fractions of culture media of D15#26 (A), N402 (B), JN22.7 (C), JN21.1 (D), JN24.6 (E), and JN23.1 (F). Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represents outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.

LaeA is involved in production of CD181, CD182, and CD88 binding compounds

D15#26 is a strain resulting from UV mutagenesis (Figure 1) carrying mutations in *pyrG, prtT,* and *laeA* (Niu et al., 2015). Here, it was addressed whether *laeA* impacts the production of immune receptor competing molecules in *A. niger.* To this end, the ≤ 3 kDa fractions from the culture media of D15#26, a *laeA* complemented derivative of D15#26 (JN22.7), and its control (JN21.1) that only has a *pyrG* complementation (Figure 1) were tested in the CBA using a subset of receptors including CD162, CD181, CD182, and CD88 that were found to be responsive with secreted molecules of D15#26 (Figure 4A, B). The ≤ 3kDa fraction of the *laeA* complemented strain was positive for CD11b, CD47, and CD162, but not for receptors CD88, CD181, and CD182 (Figure 4C). The control strain JN21.1 behaved as D15#26 (Figure 4D) showing that complementation of *pyrG* did not affect production of the small competing compounds.

Gene *laeA* was previously inactivated in strain AB4.1 (Figure 1) by homologous recombination with a *pyrG* gene cassette (Niu et al., 2015). The ≤ 3 kDa fractions of the Δ*laeA* deletion strain (JN24.6) and its control (JN23.1, *pyrG* complementation of AB4.1) were tested in the CBA. JN24.6 produced competing activity with CD88, CD162, CD181, and CD182 (Figure 4E), while its control strain only produced competing activity with CD162 (Figure 4F). These results show that the absence of LaeA is correlated with production of CD88, CD181, and CD182 competing components. Considering that these strains contain an intact *prtT* gene, the production of competing activity with receptors CD88, CD162, CD181, and CD182 is not related with *prtT* mutations.

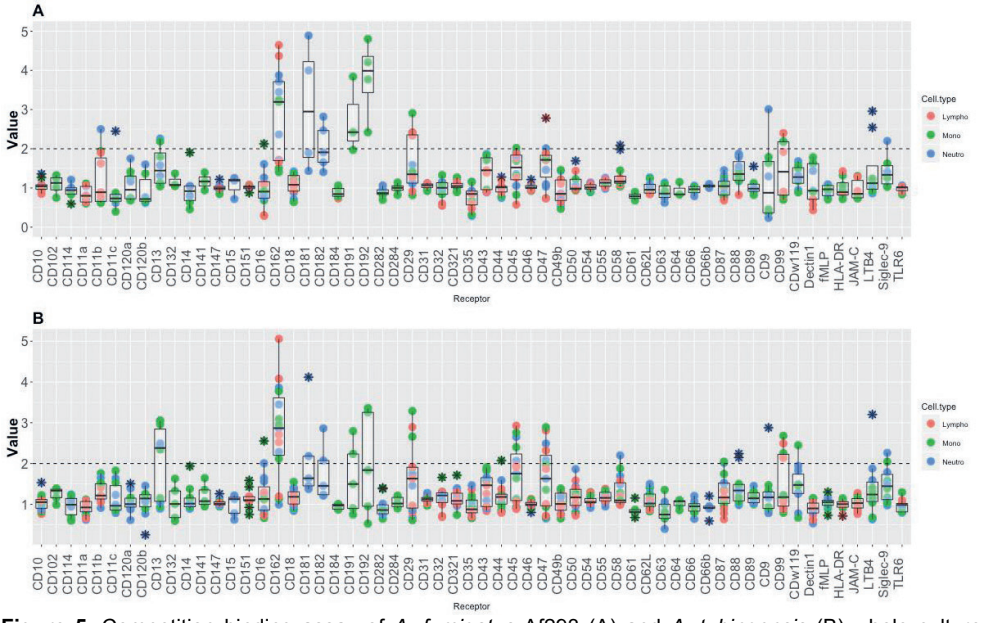

Figure 5. Competition binding assay of *A. fumigatus* Af293 (A) and *A. tubingensis* (B) whole culture media. Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.

Medium acidification and production of immune-reactive components

Inactivation of *laeA* affects production of the organic acids citric acid, gluconic acid, and oxalic acid. Indeed, acidification was absent in the medium of 72 h-old cultures of strains lacking LaeA (Table 3). In contrast, the *laeA* complemented strain JN22.7 acidified the medium to pH 3, while pH dropped to 4-5 in the case of JN23.1 and AB4.1. Inactivation of the oxaloacetate hydrolase gene *oahA* in strain AB1.13 containing an intact *laeA* copy also results in absence of acidification of the culture medium (Niu et al., 2015). The ≤ 3 kDa fraction of the Δ*oahA* strain contained molecules with activity to CD162 and CD181 in the CBA (Figure 7). Taken together, CD181 reactive components are produced by the *A niger* strain when medium is not acidified.

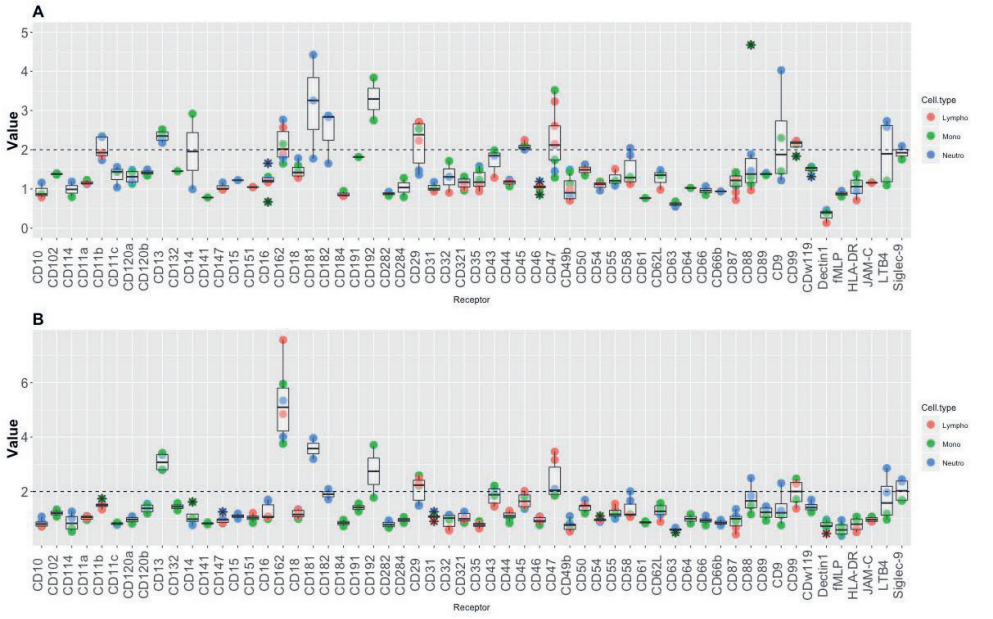

Figure 6. Competition binding assay of *A. fumigatus* Af293 (A) and *A. tubingensis* (B) ≤ 3 kDa fractions. Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.

Effect of PptA deletion on production of immune-competing components

We next investigated if molecules competing for binding with immune receptors produced by JN24.6 are synthesized via the NRPS or PKS pathway. Inactivation of *pptA* abolishes the production of secondary metabolites via the NRPS and PKS pathway. Strains lacking *pptA* require culture medium containing siderophores for growth, as deletion of *pptA* leads to impaired siderophore biosynthesis (Oberegger et al., 2003). In subsequent experiments siderophore medium was used to culture the Δ*pptA* strains as well as the other strains. Absence of *pptA* in N402 (JP1.1) did not affect medium acidification after 72 hours of growth in siderophore medium, while absence of medium acidification was observed for the Δ*laeA* strain (JN24.6) and the Δ*laeA*Δ*pptA* strain (MA870.1) (Table 3).

Table 3. pH of the medium of 72-h-old *A. niger* cultures grown in MM in the presence of xylose or maltose. Strains JP1.1, JN24.6 and MA870.1 were grown in siderophore medium

Strains		Medium addition	pH Maltose	pH Xylose
D15#26	Δ*pyrG*, Δ*prtT*, Δ*laeA*		7	7
JN21.1	Δ*pyrG*, Δ*prtT*, Δ*laeA*		7	7
JN22.7	Δ*pyrG*, Δ*prtT*		3	3
JN23.1			5	4
JN24.6	Δ*laeA*		7	7
AB1.13	Δ*pyrG*, Δ*prtT*		4.5	4.5
AB1.13Δ*oahA*#76	Δ*pyrG*, Δ*prtT*, Δ*oahA*		7	7
JP1.1	Δ*pptA*	Siderophore medium	5	5.5
JN24.6	Δ*laeA*	Siderophore medium	7	7
MA870.1	Δ*pptA*, Δ*laeA*	Siderophore medium	6	7

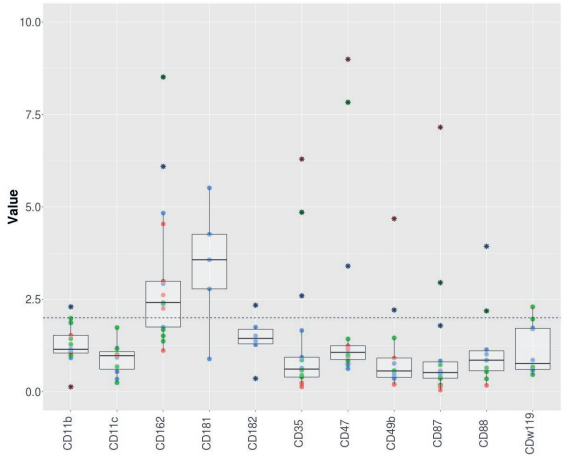

Figure 7. binding assay of the ≤ 3kDa fraction of the culture medium of strain AB1.13Δ*oahA*#76. Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.

When grown in regular minimal medium, molecules competing for binding for the CD162, CD88, CD181 and CD182 receptor were secreted into the culture medium by JN24.6 (Figure 4E). However, when this strain was grown in siderophore medium molecules competing for binding to the CD162 receptor were detected but no molecules competing for binding to the CD88, CD181 and CD182 receptor were produced (Figure 8B). Also, in strains lacking *pptA* (JP1.1) and both *pptA* and *laeA* (MA870.1) no competing molecules for CD88, CD181 and CD182 were detected (Figure 8A and C, respectively). Furthermore, the protein profile of the strains lacking *pptA* is rather different as compared to the control JN24.6. For example, strain JP1.1 produces proteins around 70 and 55 kDa that appear to be absent in the other strains (Figure 9). These results suggest that the use of siderophore medium inhibits the production of competing molecules for CD88, CD181 and CD182 receptor, while competing molecules for CD162 were not influenced by the presence of siderophore medium (Figure 8).

Discussion

Here we showed that culture media of *A. fumigatus* Af293, *A. tubingensis* and *A. niger* N402 and the non-acidifying *A. niger* strain D15#26 contain ≤ 3 kDa compounds that compete for binding with antibodies to human cellular receptors that have been related to immune recognition, activation, or modulation. Components within the culture media with competing activity were identified for the CD13, CD29, CD45, CD47, CD88, CD99, CD141, CD162, CD181, CD182, CD191, CD192, and Siglec-9 receptors. The competing activity for CD162, CD181, and CD182 was shared between the four strains. These receptors play a role in the recruitment of leukocytes (CD162) and neutrophils (CD181 and CD182) to the site of infection. The immunological role of these and the other responsive receptors is well described (Supplementary Table 3) but to our knowledge none of them have been associated to fungal infections. The results of the CBA were in general quite variable, which is

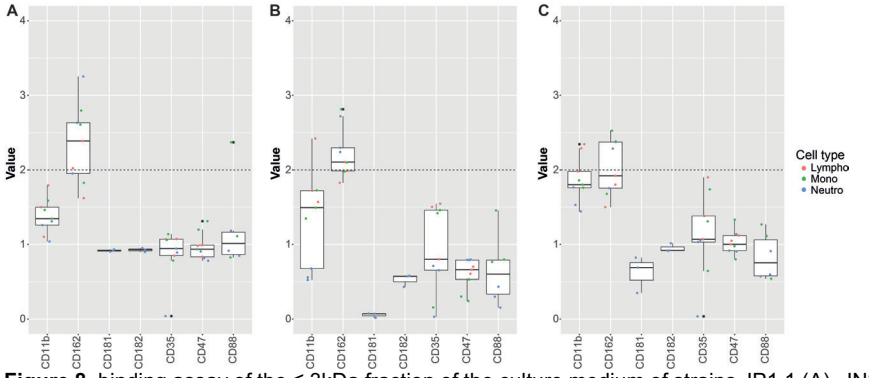

Figure 8. binding assay of the ≤ 3kDa fraction of the culture medium of strains JP1.1 (A), JN24.6 (B) and MA870.1 (C). Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.

most likely due to the fact that for each experiment cells from different donors were used.

Initially, we hypothesized that fungal proteins were responsible for the competing activity with these receptors as has been described for *S. aureus* (Bestebroer et al., 2007). Furthermore, a group of well-studied proteins in fungi were described as fungal immunomodulatory proteins (FIPs). Up to date more than 38 FIPs have been identified in different fungal species. FIPs are subdivided in 5 different groups, of which the Fve-type FIPs, small proteins around 13 kDa, and Cerato-type FIPs are most studied. The two groups can be identified by Pfam domains PF09259 and PF07249, respectively (Liu et al., 2020). The genome of *A. niger* contains a Cerato-type FIP gene (the secreted serine protease An02g01550) but no Fve-type FIPs genes. A more in-depth analysis of the genome of *A. niger* could identify more FIPs. Nevertheless, no competing activity was recovered after protein purification using affinity chromatography. Filtration studies showed that molecules ≤ 3 kDa were involved. Such molecules can be peptides, carbohydrates, or secondary metabolites. The fact that we mainly detect small molecules suggests that *Aspergillus* might use also a different strategy for immune evasion. Further characterization and purification was performed using the *A. niger* D15#26 ≤ 3 kDa fractions. The molecules could not be heat inactivated. They bound to a C18 Sep-pak column and could be eluted with 10 - 50 % methanol, but were not extracted using ethyl acetate, suggesting that these molecules were moderately hydrophilic. The latter fractions contained a variety of low molecular weight molecules as shown by LC-MS.

Next to the characteristics of the small molecules produced, the mutations in the D15#26 responsible for the productions of the small molecules binding to the immune receptors CD88, CD162, CD181 and CD182 were assessed. Experimental data showed that strains with an inactive *pyrG* and / or *prtT* but with an intact *laeA* were not producing binding compounds except for CD162. Preliminary CBA data with a Δ*prtT* strain (AF11#56, a derivative of AB4.1) underscored that deletion of PrtT did not lead to the production of CD88, CD162, CD181, and CD182 binding compounds (Supplementary Figure 8). In contrast, gene *laeA* was shown to have a role as a repressor of production of competing compounds of CD88, CD181 and

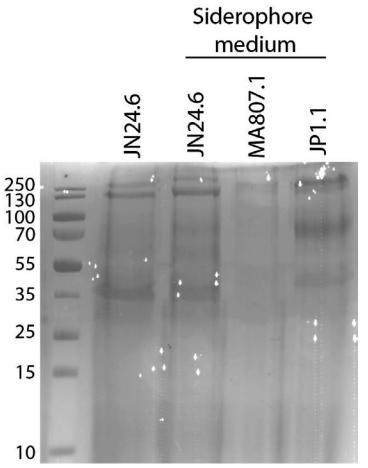

Figure 9. CBB stained SDS PAA gels of mixed maltose and xylose culture media after 72 h of growth of *A. niger* JN24.6 and of JN24.6, MA871.1 and JP1.1 after growth in siderophore medium.

CD182. A Δ*laeA* strain did not acidify the culture medium like D15#26 and produced CD88, CD162, CD181, and CD182 competing activity. On the other hand, the acidifying *laeA* complemented D15#26 strain only produced competing activity with CD162, showing that the global regulator of secondary metabolism LaeA is a repressor of production of the competing activities to CD88, CD181, and CD182 (Figure 9). The finding that LaeA can act as a repressor was previously reported in *A. nidulans* and *A. niger*. These *laeA*-deficient strains showed increased secretion of an uncharacterized secondary metabolite (Bok & Keller, 2004) and aspernigrin (Niu et al., 2015). *LaeA* is also an activator of secondary metabolism. For instance, loss of *laeA* inhibits synthesis of sterigmatocystin and penicillin in *A. nidulans*, lovastatin in *A. terreus*, and gliotoxin in *A. fumigatus* (Bok et al., 2006; Brakhage, 2013).

Medium acidification specifically affects the production of competing molecules to CD181. This was based on the fact that two non-acidifying strains, D15#26 and Δ*oahA* produced components that interacted with this receptor. Similar results were obtained in preliminary experiments with the non-acidifying *crzA* mutant of *A. niger* in an AB4.1 background (Supplementary Figure 9). CrzA is a transcription factor of the calcium / calcineurin pathway, involved in fungal morphogenesis, virulence, and ion tolerance (de Castro et al., 2014). This shows that competing activity for CD181 depends indirectly on LaeA, as it is secreted under all non-acidifying conditions tested, while the mechanism underlying repression of CD182 and CD88 competing activity is linked to the presence of LaeA (Figure 10).

Immune receptors CD181 and CD182 are better known as CXCR1 and CXCR2. They are chemokine receptors belonging to the G-protein-coupled receptor (GPCR) family. Ligands binding to CXCR1 and CXCR2 include IL-8, NAP-2, GCP-2, and GRO-α, β, γ (Ahuja & Murphy, 1996; de Castro et al., 2014; Lee et al., 1992). Activation of CXCR1 and CXCR2 mediates neutrophil migration and chemotaxis and favours angiogenesis (Stillie et al., 2009). Both receptors are present on neutrophils, are closely related, and generally interact with similar ligands, but not necessarily with the same affinity (Cerretti et al., 1993; Cummings et al., 1999; Wolf et al., 1998). The observation that the CD181-interactive compound produced under non-

acidifying conditions is not reacting with CD182 suggests the presence of two different compounds binding specifically to each receptor.

Together, our results show that small hydrophilic secondary metabolites, carbohydrates, and / or small peptides are responsible for competition for binding of antibodies to cellular receptors. *Aspergilli* secrete polyketides (PKS), non-ribosomal peptides (NRPS), terpenes, and indole alkaloids as main groups of secondary metabolites (Keller et al., 2005). Deletion of the *pptA* gene abolishes secondary metabolite production via the PKS and NRPS pathways but this strain needs siderophore medium to grow (Márquez-Fernández et al., 2007). Growth of the Δ*laeA* strain in siderophore medium did not alter medium acidification (Table 4), but the molecules competing for binding with the CD88, CD181 and CD182 were not produced (Figure 8B). This indicates that the addition of siderophore medium affects the production of these molecules. In line with these results, we also do not see the production of these molecules in the Δ*pptA* strain and Δ*laeA*Δ*pptA* strain (Figure 8A and C, respectively). We therefore could not determine if the small molecules were indeed produced via a PKS and / or NRPS pathways. Future research is needed to elucidate if the molecules competing for binding are indeed secondary metabolites and, if so, which synthesis pathway is involved.

A. niger D15#26 produces more responsive molecules when compared to its wild type strain N402. D15#26 contains a variety of mutations including a mutation in *laeA* that controls production of secondary metabolites like sterigmatocystin, penicillin, and lovastatin (Bok & Keller, 2004; Niu et al., 2015). The competing components produced by D15#26 are expressed in a LaeA-independent manner. This may well be the case because *A. niger* ATCC1015 is predicted to have 81 gene clusters associated with secondary metabolite production and 145 secondary metabolites have been identified from the *Aspergillus Nigri* section (Inglis et al., 2013). Many of these compounds (e.g. ochratoxin A, naptho-γ-pyrones, bocoumarins) are found in both *A. niger* and *A. tubingensis* (Nielsen et al., 2009). As described by (Inglis et al., 2013), *A. fumigatus* Af293 contains 39 secondary metabolite gene clusters, while at least 226 *A. fumigatus* secondary metabolites have been reported, some of them associated with virulence (Frisvad et al., 2009; Latgé, 1999). The fact that components produced by D15#26 (lacking LaeA) and *A. fumigatus* and *A. tubingensis* (both containing LaeA) compete for to the same set of receptors (CD162, CD181, and CD182) indicates that we are dealing with a variety of molecules that are regulated differently but might be produced by orthologous gene clusters. By using Anti-SMASH prediction software, we detected 88 gene clusters in the genome of *A. tubingensis* CBS134.48. Of these, 30 and 4 gene clusters showed homology ≥ 75 % and 50 % at the amino acid level when compared to *A. niger* ATCC1015 and *A. fumigatus* Af293, respectively. The 4 gene clusters having similarity between *A. tubingensis* CBS134.48 and *A. fumigatus* Af293 were also found in *A. niger* ATCC1015. In this case a similarity ≥ 57 % was found in the latter strains. Shared clusters were assigned and predicted in *A. tubingensis* as Cluster 4 (non-ribosomal peptide), Cluster 18 (type I polyketide synthase), Cluster 27 (terpene), and Cluster 79 (other). Cluster 4 showed similarity with *A. fumigatus* Afu1g10380 (nrps1 / pes1), while Cluster 18 had similarity with *A. fumigatus* Afu2g01290, Cluster 27 with No PKS or NRPS backbone 6 and cluster 79 with No PKS or NRPS backbone 2. These clusters are of interest for further analysis with respect to immune receptor binding.

Identification of the small compounds secreted by *A. niger laeA* mutant strains might result in novel therapeutic agents. Furthermore, absence of medium acidification might also explain the secretion of immune-modulatory components of the pathogen *A. fumigatus*. In this study it was shown that this species produced components ≤ 3 kDa that compete with antibodies for interaction with receptors CD162, CD181, CD182, CD192, CD99, CD45, CD47, and CD29. Interestingly, *A. fumigatus* increases the pH of the culture medium to 8. This increase may be responsible for the production of the binding compounds. Notably, (Kniemeyer, 2011; Sriranganadane et al., 2010) reported that secretion of serine protease (Alp1), metalloproteases (Mep1), and leucine aminopeptidases (Lap1 and Lap2) by *A. fumigatus* were favoured at pH between 7 - 7.5 but undetected at pH 3.5 (Kniemeyer, 2011; Sriranganadane et al., 2010). Secretion of Alp1 is related with immune evasion as it degrades human complement proteins C3, C4, and C5 (Behnsen et al., 2010). Possibly, PacC that is required for alkaline adaption and implicated as another global regulator of secondary metabolite production (Brakhage, 2013; Tilburn et al., 1995) plays an important role in the production of these compounds.

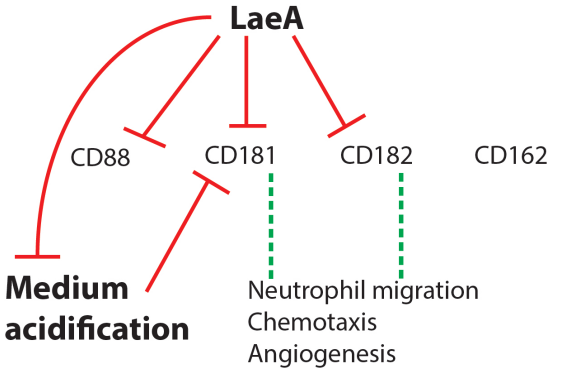

Figure 10. Schematic overview of the effectors and their role in the production of components competing with binding to the CD88, CD181, CD182 and CD162 receptor.

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Supplementary Table 1. Volume per well used in the CBA. * Pre-dilutions (CD15 1:10, fMLP 1:100, CD32 1:10, CD44 1:5, CD16 1:40, CD45 1:10). The cell types where receptors are expressed are indicated by neutrophils (N), monocytes (M), and lymphocytes (L).

Well	FITC	uL	Cell type	Clone/brand	PE	uL	Cell type	Clone/brand	APC	uL	Cell type	Clone/brand
1	CD11a	0,5	N,M,L	HI111, BD	CD32*	1	N,M,L	7.3 /Fitzgerald	CD10	1,5	N,M,L	MEM-78, Invitrogen
2	CD15*	1	N	MMA, BD	CD35	2,5	N,M,L	E11/ BD	CD11c	4	N,M	B-ly6, BD
3	CD147	1,5	N,M,L	HIM6, BD	CD44*	1	N,M,L	515, BD	CD11b	4	N,M,L	ICRF44, BD
4	CD18	1	N,M,L	6.7, BD	CD47	3	N,M,L	B6H12, BD	CD13	4	N,M	WM15, BD
5	CD31	1,5	N,M,L	WM59, BD	CD54	2	N,M,L	HA58, BD	CD14	4	N,M	M5E2, BD
6	CD46	1	N,M,L	E4.3, BD	CD58	1,5	N,M,L	1C3, BD	CD29	1,5	N,M,L	MAR4, BD
7	CD99	3	M	Tü12,BD	CD321	0,75	N,M,L	M.Ab.F11, BD	CD16*	1,5	N,M,L	3G8, BD
8	CD102	2	M	aa191-218, BioCon	CD87	5	N,M,L	VIM5, BD	CD45*	2	N,M,L	HI30, BD
9	CD61	1,5	M	10.1, DB	CD89	2	N,M	Mip8a, Abd-Serotec	CD55	1	N,M,L	IA10, BD
10	CD62L	1,5	N,M,L	Dreg-56, BD	CD63	1	N,M	CLB-gran/12, ImmunoTech	CD50	0,3	N,M,L	CBR-IC3/1, BD
11	CD64	1,5	M	VI-PL2, BD	CD114	1,5	N,M	LMM741, BD	JAM-C	2,5	M,L	208212, R&D
12	CD66b	1,5	N	G10F5, BD	CDw119	1	N,M	GIR-208, BD	Siglec-9	0,75	N,M	191240, R&D
13	CD43	1	N,M,L	6D269, Santa Cruz	CD132	2	M	TUGh4, BD	CD141	3	M	1A4, BD
14	CD66	1	N,M	B1.1/CD66, BD	CD151	1,5	M,L	14A2.H1,BD				
15	CD184	4	M	20102, R&D	CD162	2	N,M,L	KPL-1, BD				
16	fMLP*	1,5	N,M	Rea169, MilBio	CD191	4	M	53504, R&D				
17	HLA-DR	2	M	G46-6,BD	CD192	2	M	48607, R&D				
18	LTB4R	2	N,M	202/7B1, Abd-Serotec	CD181	1,5	N	42705.111, R&D				
19	CD284	4	M	HTA125, AbDSerotec	CD182	2,5	N	48311.211, BD				
20	CD120a	4	N,M	16803.161, R&D	CD88	0,3	N,M	S5/1, Biolegend				
21	CD120b	3	N,M,L	22235.311, R&D	CD282	0,5	N,M	T2.5, EBioscience				
22	CD9	2	N,M,L	M-L13, BD	CD49b	1	N,M,L	12F1, BD				
23	TLR6	8	N,M	86B1153.2, invitrogen	Dectin-1	4	N,M	15E2, BD				

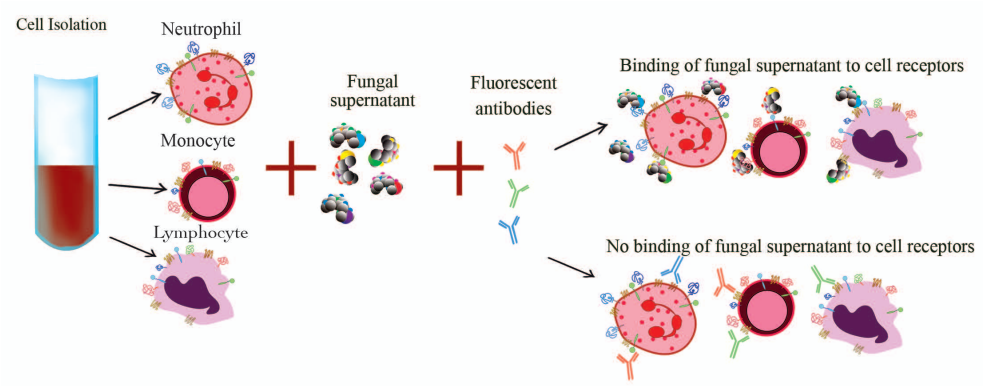
BD (Becton, Dickinson and company, Le-Pont-De-Claix, France), Santa Cruz (Dallas, USA), Abd-Serotec (MorphoSys ABD GmbH ABD Serotec, Düsseldorf, Germany), R&D (R&D Systems Inc, Minneapolis, USA), Fitzgerald (Fitzgerald Industries International, Acton, USA), ImmunoTech (Immunotec Inc, Vaudreuil-Dorion, Canada), Biolegend (San Diego, USA), EBioscience (San Diego, USA), Invitrogen (Carlsbad, USA), MilBio (Miltenyi Biotec, Bergisch Gladbach, Germany), BioCon (BioConnect Life Sicence, Huissen, The Netherlands).

Supplementary Table 2. LC-MS and preparative HPLC gradient elution profile. Buffer A: 95 % MiliQ water, 5 % acetronitrile, and 0.1 % trifluoroacetic acid. Buffer B: 5 % MiliQ water, 95 % acetronitrile and 0.1 % trifluoroacetic acid.

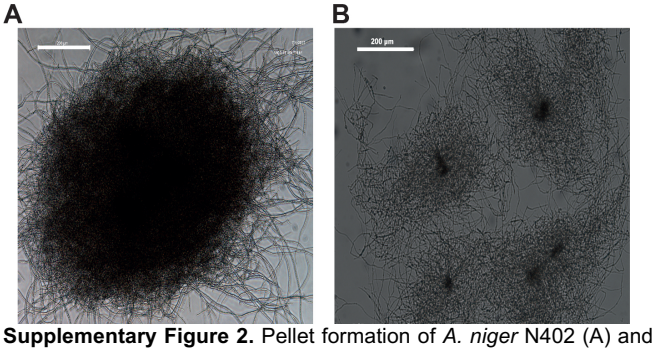
Time (min)	Percentage Buffer B
0-5	0
5-80	0-60
80-85	60-100
85-90	100
90-95	100-0
95-100	0

Supplementary Table 3. Function and description of cellular receptors to which molecules from *Aspergillus* spp. supernatants bound.

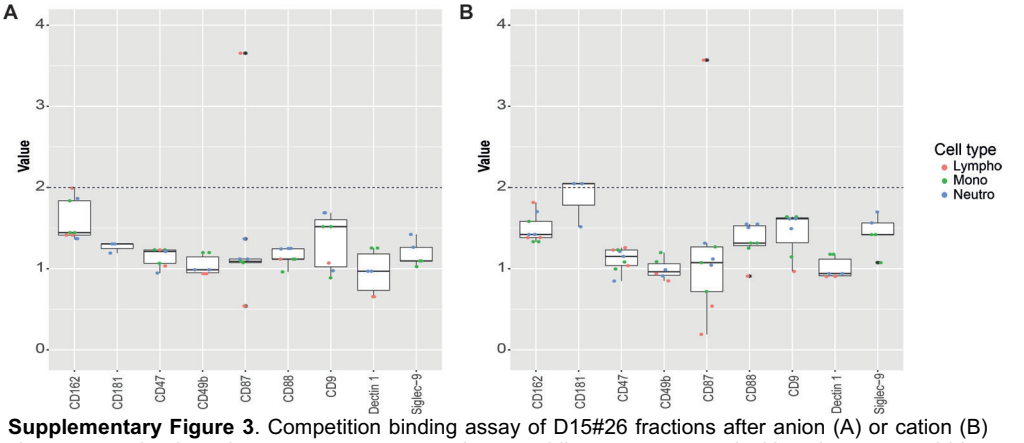
Receptor	Synonyms	Encodes	Function
CD162	P-selectin ligand	Glycoprotein-receptor for the cell adhesion molecules P- E- and L-selectins.	Leukocyte rolling and trafficking during inflammation. Activates platelets.
CD181	CXCR1	seven-transmembrane G-protein (67-70 kD)	Receptor to interleukin-8. Activation and chemotaxis of neutrophils.
CD182	CXCR2	seven-transmembrane G-protein (67-70 kD)	Receptor to interleukin-8. Activation and chemotaxis of neutrophils.
CD88	C5aR	Single chain protein with seven membrane-spanning regions (43 kD).	Binds to complement component 5; stimulating chemotaxis, granule enzyme release, intracellular calcium release and superoxide anion production.
Siglec-9	Sialic acid-binding Ig-like lectin-9	Member of the Siglec subgroup of the immunoglobulin superfamily.	Negative regulation of T cell activation. Affects neutrophil apoptosis
CD99	MIC2	Single chain transmembrane protein (32 kD)	Involve in T-cell adhesion and spontaneous rosette formation with erythrocytes.
CD47	Rh-associated protein	Integrin-associated protein (IAP). Member of the immunoglobulin superfamily (42-52 kD).	Cell addition and modulation of integrins. Associates with SIRP1a; involved in migration and phagocytosis.
CD45	Leukocyte Common Antigen (LCA)	Single chain type I membrane glycoprotein, tyrosine-protein phosphatase C (180 – 240 KD)	Regulator of T-cell coactivation upon binding to DPP4. Also regulates cell growth, differentiation, cell cycle, and oncogenic transformation.
CD13	Aminopeptidase N	Type II transmembrane glycoprotein, aminopeptidase N.	Antigen processing and cleavage of chemokines.
CD191	CCR1	C-C chemokine receptor type 1 (41 kD).	Inflammation and susceptibility to viruses and parasites.
CD192	CKR2, CCR2A	Transmembrane glycoprotein and member of the G-protein coupled receptor 1 family.	Role in inflammation. Induce infiltration of macrophages. Associated with obesity.
CD141	Thrombomodulin, TM	Single chain, type I membrane glycoprotein, also known as thrombomodulin. (75 kD)	Activation of protein C and reduction of thrombin. Anticoagulation function and embryonic and atherosclerotic plaque development.
CD29	Integrin β_1 chain, ITGB1	Type I glycoprotein (130 kD)	Acts as a fibronectin receptor and is involved in cell-cell and cell-matrix interactions.



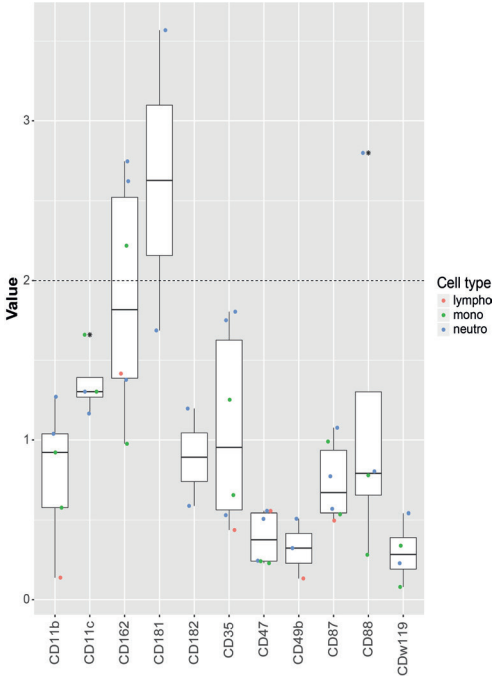
Supplementary Figure 1. Competition of binding assay. PMNs and PBMCs are isolated from blood of healthy donors and mixed with the fungal culture supernatant. After incubation the cells are mixed with fluorescently labelled antibodies against cell-surface receptors of the immune cells. Binding of the fungal culture supernatant is determined by the reduction of fluorescence compared to a control sample. Fluorescence was measured by flow cytometry. Adapted from (Escobar et al., 2016).



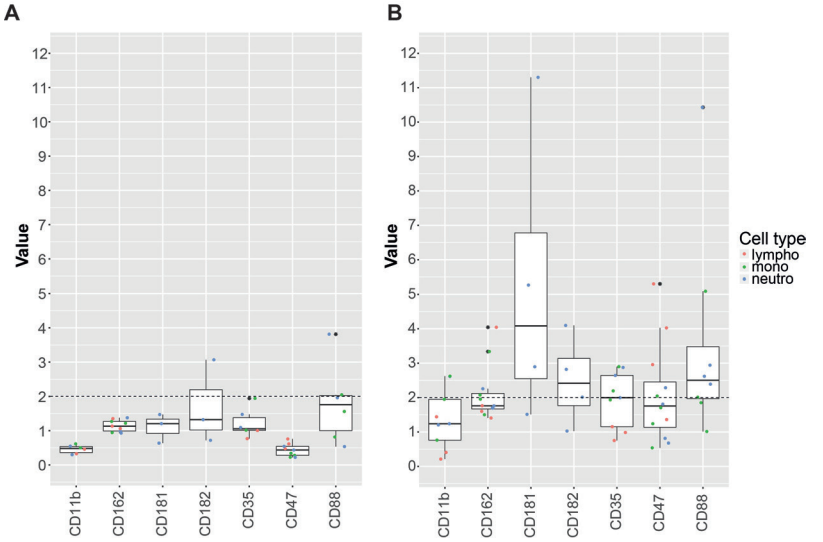
Supplementary Figure 2. Pellet formation of *A. niger* N402 (A) and *A. niger* D15#26 (B) after growing for 72 h in MM supplemented with 25 mM xylose. Scale bare represents 200 µm.



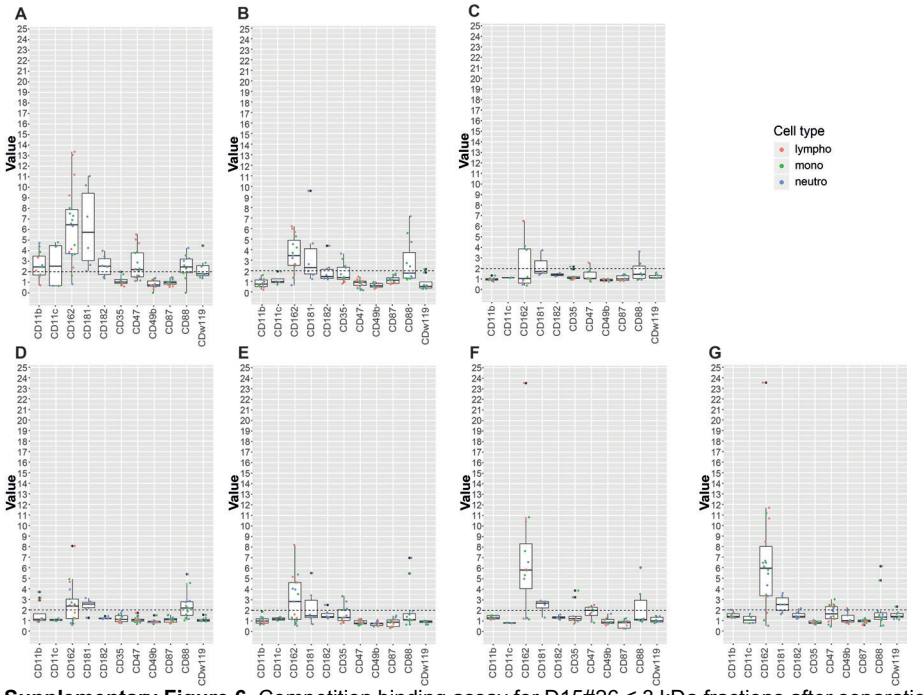
Supplementary Figure 3. Competition binding assay of D15#26 fractions after anion (A) or cation (B) chromatography. Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.



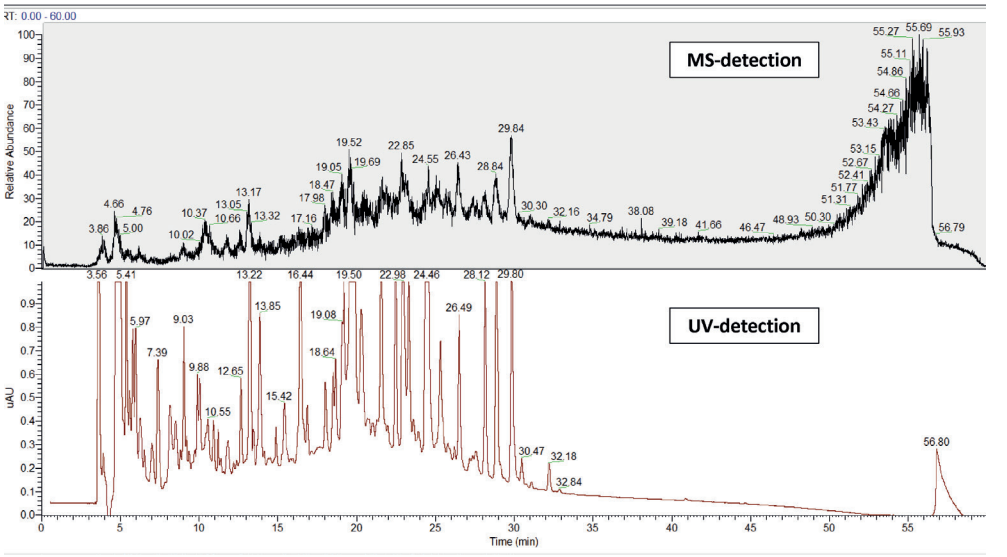
Supplementary Figure 4. Competition binding assay of 60 min - 100°C treated D15#26 ≤ 3 kDa fractions. Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.



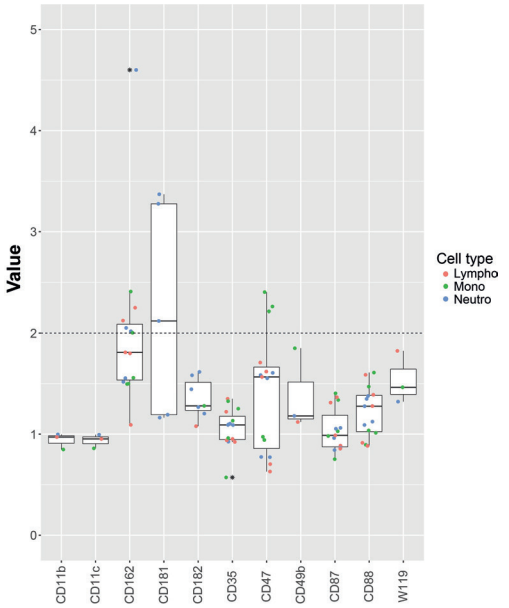
Supplementary Figure 5. Competition binding assay for D15#26 ≤ 3 kDa fractions after extraction with ethyl acetate (A) and the corresponding aqueous phase (B). Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.



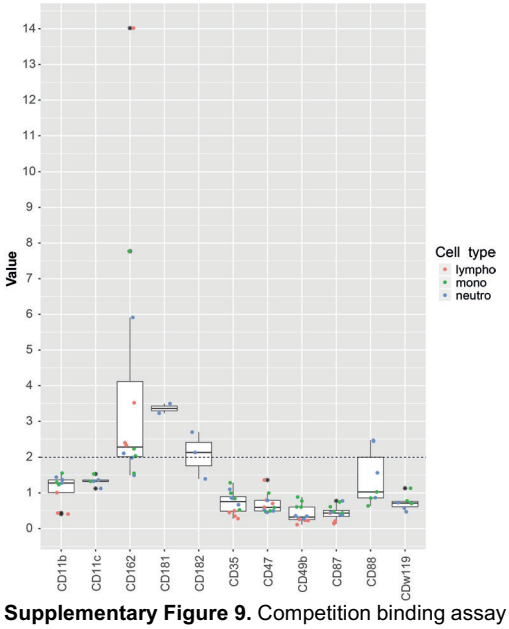
Supplementary Figure 6. Competition binding assay for D15#26 ≤ 3 kDa fractions after separation on a C18 Sep-pack column and elution with a gradient of methanol. Fractions used in the CBA: flowthrough (A), 0 % methanol (B), 10 % methanol (C), 30 % methanol (D), 50 % methanol (E), 70 % methanol (F) and 100 % methanol (G). Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectivelym * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.



Supplementary Figure 7. LC-MS analysis of 10-50 % pooled methanol fractions after chromatography using a C-18 Sep-Pak column. Retention times were detected with MS detector and UV 214 nm.



Supplementary Figure 8. Competition binding assay for *A. niger* AF11#56. Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.



Supplementary Figure 9. Competition binding assay for *A. niger* Δ*crzA* ≤ 3 kDa fractions. Lymphocytes, monocytes, and neutrophils are represented with red, green, and blue dots, respectively, * represent outliers. Y-axis represent the inverted geometric mean of fluorescence, the X-axis represent the used receptors in the CBA, data points above the dotted line (2) are scored as positive for binding of molecules from the culture medium.

5

Comparison of virulence and genomic differences of five *Aspergillus fumigatus* isolates shows that Af293 is less virulent

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Submitted

Abstract

Conidia of *Aspergillus fumigatus* are inhaled by humans on daily basis. As a consequence, these conidia can cause infections that differ in severity ranging from allergic bronchopulmonary aspergillosis to invasive aspergillosis. In this study virulence of two *A. fumigatus* strains isolated from a non-invasive sino-nasal aspergillosis (DTO271-B5 and DTO303-F3) and three strains from an invasive aspergillosis (Af293, ATCC46645 and CEA10) infection was assessed using type II A549 lung epithelial cells, *Protostelium aurantium* amoebae, *Galleria melonella* larvae and zebrafish embryos as infection models. No major differences were shown between virulence of the strains in the lung epithelial cells. In contrast, strain ATCC46645 was most virulent in the amoeba and zebrafish model, whereas it was much less virulent in the *Galleria* infection model. DTO303-F3 was most virulent in the latter model. In general, reference strain Af293 was less virulent when compared to the other strains. Genome sequence analysis showed that this strain differs from the other four strains with 136 SNPs in virulence-related genes, one of which in *ftmD* (Afu8g00200) that is part of the fumitremorgin gene cluster. The fact that Af293 would be unable to produce this mycotoxin could be the reason for its lower virulence. In addition, increased sensitivity to ROS and reduced germination can contribute to reduced virulence. Together, virulence of individual strains can differ between model systems, but we did not observe an overall difference in virulence between strains originating from an invasive or non-invasive infection.

Introduction

Aspergillus fumigatus is a saprotrophic fungus, feeding on dead or living organic matter (Krijgsheld et al., 2013). The 2 - 3 µm wide conidia (Brakhage & Langfelder, 2002) are dispersed via the air and hundreds of these conidia are inhaled by humans daily (Mullins et al., 1984). Upon inhalation, these conidia can cause lung infections that differ in severity. Allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and *Aspergillus* bronchitis are classified as non-invasive aspergillosis infections that can occur in immune-competent patients. ABPA is the result of sensitization to *A. fumigatus* allergens triggering an allergic inflammatory response in the bronchial airways (Kurup et al., 1998). For instance, it occurs in about 2.5 % of all patients with asthma and in 1 - 15 % of patients with cystic fibrosis (Denning et al., 2013; Stevens et al., 2003). CPA manifests as an aspergilloma, a chronic cavity pulmonary aspergillosis, or as a chronic fibrosing pulmonary aspergillosis (Denning, 2009). Less than 1 % of asthma patients and around 5 % of cystic fibrosis patients develop CPA combined with ABPA (Denning et al., 2011; Denning et al., 2013). Finally, *Aspergillus* bronchitis is characterized by a superficial, but chronic infection of the airways. It is for instance found in a small proportion (1.6 %) of CF patients (Brandt et al., 2018). Beside lung infections, *A. fumigatus* is also a causative agent of non-invasive infections in sino-nasal areas in humans and animals (Mohammadi et al., 2017). In dogs, *A. fumigatus* is the most commonly identified fungal species in sino-nasal aspergillosis (SNA) (Peeters & Clercx, 2007).

The most severe infection caused by *A. fumigatus* is an invasive pulmonary aspergillosis (IPA). IPA develops in immune-compromised patients in particular. Hyphae resulting from inhaled conidia invade the respiratory mucosa, which can be followed by invasion of other organs (Naaraayan et al., 2015). Stem cell recipients are the highest risk group for these infections (Kontoyiannis et al., 2010), followed by solid organ transplant recipients (Pappas et al., 2010). The high risk is caused by the severe immune suppression caused by medication which is needed for these transplantations (Sasaki et al., 2015).

Genome-wide comparative analysis and single nucleotide polymorphism (SNP) calling revealed that 101 *A. fumigatus* isolates from clinical and environmental origin were distributed in four clusters. 0.3 % and 0.2 % of the reference genomes of the clinical strains Af293 and A1163 (CEA10) were identified as a SNP, respectively (Garcia-Rubio et al., 2018). Significant genetic and phenotypic variability related to pathogenicity and stress tolerance was observed between 16 clinical *A. fumigatus* isolates (Hagiwara et al., 2018). However, no correlation was found between the observed genetic and phenotypic variabilities and disease phenotype in humans. A comparison between the clinical isolates Af293 and CEA10 revealed that the former is more susceptible to hypoxic conditions than the latter (Kowalski et al., 2016). Interestingly, this study also revealed a correlation between reduced fitness at low oxygen and reduced virulence in an immune-suppressed murine IPA model. Virulence of Af293 and CEA10 was also compared in a zebrafish infection model. These studies showed that the faster growing CEA10 strain is more virulent than the slower growing Af293 strain (Rosowski et al., 2018). Together, differences have been observed between virulence of different *A. fumigatus* strains. Yet, no extensive systematic comparisons have been made in infection models.

In this study, we have compared virulence of five *A. fumigatus* isolates that originate from invasive and non-invasive infections and relate the virulence to SNPs. To this end, type II A549 epithelial lung cells, amoebae, larvae of *Galleria melonella*, and zebrafish embryos were used as models of infection. Type II A549 epithelial lung cells have been used extensively to study the lifestyle of *Aspergillus* species but in most studies only a limited set of strains were compared (Escobar et al., 2016; Escobar et al., 2018; Wasylnka et al., 2005). In the study of (Wasylnka & Moore, 2002) this system revealed no major differences in binding, uptake and germination between *A. fumigatus* strains ATCC13073 and CHUV. In addition, expression analysis of Af293 and CEA10 during infection of A549 cells did not reveal a similar response in both strains. It was proposed that these differences were due to the different backgrounds of the strains and not the presence of the A549 cells (Watkins et al., 2018).

Previous research showed that amoebae and innate immune cells can trigger similar responses to fungi such as *Cryptococcus neoformans* and partially also to *A. fumigatus* (Casadevall, 2006). Upon interaction with the model amoeba *Dictyostelium discoideum*, conidia are internalized with a similar efficiency as during phagocytosis by human monocytes (Hillmann et al., 2015). Fungivorous amoeba such as *Protostelium aurantium* can feed on conidia of *A. fumigatus* (Radosa et al., 2019). This suggests that amoebae can be used as an early evolutionary model for human immune cells.

The innate immune system is also the main defence against microbial pathogens in insects such as *Galleria melonella*. Due to this similarity, larvae of *G. melonella* can be used as an infection model. The haemolymph of the larvae contains anti-microbial peptides and haemocytes, similar to human phagocytes, to clear microbial pathogens (Kavanagh & Reeves, 2004). A correlation between gliotoxin production and virulence of *A. fumigatus* isolates was suggested in this model (Reeves et al., 2004). In addition, *A. fumigatus* siderophore mutants show strongly reduced virulence in *G. melonella* larvae and a high correlation with survival of mice upon infection (Slater et al., 2011). Similar results were observed with mutants of the dimorphic pathogen *Candida albicans* (Brennan et al., 2002). Comparison of three wild-type *A. fumigatus* strains in *G. melonella* larvae showed differences in survival of the larvae. Conidia of ATCC46645 were most virulent, while conidia of D141 and Af237 had a similar virulence (Slater et al., 2011).

Transparent zebrafish embryos are a good model to study microbial infections as well. The hindbrain of the embryos consists of an epithelial cell layer into which the conidia of *A. fumigatus* can be injected (Koch et al., 2019). The zebrafish embryo has a functional innate immune system, with macrophages and neutrophils functioning at 30 hours post fertilization (Lieschke et al., 2001). In contrast, the adaptive immune response only develops 20 days post fertilization (Page et al., 2013). A recent study showed that zebrafish embryos can be used to investigate the efficacy and mechanisms of antifungal drugs. Treatment of the zebrafish embryos with voriconazole showed that this anti-fungal is able to kill hyphae but macrophages are needed to slow down initial fungal growth (Rosowski et al., 2020). Here we show no specific differences in these four infection models in virulence between strains that were isolated from invasive aspergillosis infections in humans or from non-invasive canine sino-nasal infections (Valdes et al., 2020). In general, reference strain Af293 was less virulent when compared to the other strains. Analysis of SNPs in virulence-related genes, showed that Af293 differs from the

other four strains in 136 SNPs in these genes, one of which in *ftmD* (Afu8g00200) which is part of the fumitremorgin gene cluster. The absence of production of this mycotoxin could explain why Af293 is less virulent when compared to the other strains.

Materials and Methods

Strains and growth conditions

Strains used in this study (Table 1) were grown for 3 days on potato dextrose agar (PDA) (Difco) at 37 °C. Conidia were harvested with 0.85 % (w/v) NaCl with or without Tween 20 (0.005 %) (VWR international) depending on the type of experiments and filtered through 3 layers of sterile Miracloth (Merck Millipore) to remove mycelium and hyphae. Conidia were counted using a Bürker counting chamber.

Table 1. Strains used in this study

Strain	Description	Origin	Reference
Af293.1	pRG3AMA1-RFP	Human, invasive infection	(Leal et al., 2010)
CEA10	Wild type	Human, invasive infection	(Girardin et al., 1993)
ATCC46645	Wild type	Human, invasive infection	ATCC *
DTO271-B5	Wild type	Canine, non-invasive infection	(Valdes et al., 2018)
DTO303-F3	Wild type	Canine, non-invasive infection	(Valdes et al., 2018)

*the strain was kindly provided by Prof. Dr. Axel A. Brakhage from HKI, Jena, Germany

Peroxide and menadione sensitivity assays

Conidia were harvested in 0.85 % (w/v) NaCl with 0.005 % Tween 20. Peroxide and menadione sensitivity assays were performed as described previously (Lessing et al., 2007; Sugareva et al., 2006; **Chapter 3**). Briefly 5 10^7 conidia were mixed in 5 mL minimal medium (MM; 6 g L^{-1} $NaNO_3$, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 mL L^{-1} Vishniac) with 1 % agar and poured onto MM agar plates (6 cm in diameter with 10 mL MM) with 20 mM glucose (Sigma Aldrich). A 10 mm-wide hole was punched in the middle of the plate and filled with 100 µL 500 mM H_2O_2 (Sigma Aldrich) or 100 µL 1 mM menadione (Sigma Aldrich). Plates were incubated at 37 °C for 16 h after which the inhibition zone was measured.

Size of conidia

The size of conidia was determined as described (van den Brule et al., 2020). In short, conidia were diluted to an amount of 10^5 conidia mL^{-1} ISOTON II diluent (Beckman Coulter). 100 µL conidia solution was used to determine the size distributions using a Beckman Coulter Counter Multisizer 3 equipped with a 70-µm aperture tube. 10^3 datapoints were extracted and used for analysis. Objects with a diameter < 2 and > 6 µm were excluded from the dataset.

Cell culture and fungal infection

The human lung carcinoma epithelial cell line A549 (ATCC, CCL-185) was maintained by serial passage in Dulbecco's modified Eagle medium (DMEM) (Ref. code: 11995-065, Gibco) supplemented with 10 % fetal bovine serum (FBS) (Gibco). Fungal infections were performed as described (Keizer et al., 2020; **Chapter 2**). In short, cells were seeded at a concentration of 2 10^5 cells mL^{-1} and cultured at 37 °C with 5 % CO_2 until a confluent monolayer was formed. Cells in 12, 24 or 48 wells

plates (Corning®, Costar®) were challenged with 2 10^6 conidia mL^{-1} DMEM + 10 % FBS, resulting in a multiplicity of infection (MOI) of 1. Cells were cultured in 48 wells plates containing 8 mm glass coverslips (ThermoFisher Scientific) for internalization, association and germination experiments, in 12 wells plates for the IL-8 secretion or in 24 wells plates containing 12 mm glass coverslips (VWR International) for the identification of apoptotic and necrotic cells (see below).

Internalization, association and germination of conidia with A549 cells

Internalization and association experiments were performed as described (Keizer et al., 2020; **Chapter 2**). In short, A549 lung epithelial cells were grown on 8 mm glass coverslips (ThermoFisher Scientific) until a confluent layer was formed. Conidia of CEA10, ATCC46645, DTO271-B5 and DTO303-F3, which did not express a fluorescent protein such as *RFP* in Af293.1, were stained with 20 µg mL^{-1} *Aspergillus* FITC labelled antibody (ThermoFisher Scientific) for 60 min at room temperature before addition to the A549 cells. Unbound antibody was not removed by washing to avoid loss of conidia during pelleting. Conidia were added to A549 cells at an MOI of 1 and incubated for 2 h. Unbound conidia were removed by washing three times with pre-warmed DMEM + 10 % FBS and incubation was continued for 2 h, resulting in a total infection time of 4 h. Conidia adhering to the A549 cells were visualized with 1 % calcofluor white (CFW) (Sigma Aldrich) in DMEM + 10 % FBS. CFW was added for 10 min at 37 °C followed by one washing step with DMEM + 10 % FBS. The cells and conidia were fixed with 4 % paraformaldehyde (PFA) (VWR international) for 5 min at 4 °C and 20 min at room temperature. PFA background fluorescence was quenched by a 20 min incubation at room temperature with 20 mM NH_4Cl (Acros Organics). A549 cells were visualized with 1 µg mL^{-1} Hoechst (BD Biosciences). Coverslips were mounted to a glass slide using FluorSave™ (Merck Millipore) and dried overnight. The number of conidia associated to the A549 cells (number of conidia per A549 cell) was determined by analysing 10 randomly chosen fields at the coverslip for imaging. Internalization of the conidia was determined by analysing z-stacks made at 10 randomly chosen fields of the coverslip. Conidia which were red or green were counted as internalized, whereas conidia that also stained with CFW were classified as adhering non-internalized conidia since CFW is not able to penetrate into the A549 cells. Internalization values were expressed as the percentage of the total conidia that associated with the cells. Experiments were performed as biological triplicates, with pictures taken at 10 random places on the slide. At least 100 conidia were counted per strain in each replicate.

Germination experiments were based on experiments described (Escobar et al., 2016). A549 cells were grown on 8 mm glass coverslips (ThemoFisher Scientific) until a confluent layer was formed. Conidia were added to the A549 cells at an MOI of 1 and incubated for 2 h. Unbound conidia were then removed by washing three times with pre-warmed DMEM + 10 % FBS. Incubation then continued for another 10 h, making the total incubation time of the conidia with the A549 cells 12 h. Associated conidia and hyphae were stained with 1 % CFW in DMEM + 10 % FBS for 10 min at 37 °C followed by one washing step with pre-warmed DMEM + 10 % FBS. Cells and conidia were then fixed with 4 % PFA for 5 min at 4 °C and 20 min at room temperature. PFA background fluorescence was quenched by a 20 min incubation at room temperature with 20 mM NH_4Cl. Conidia and hyphae that did not express a reporter protein (CEA10, ATCC46645, DTO271-B5 and DTO303-F3),

such as *RFP* in Af293.1, were visualized using antibodies. To this end the cells were first blocked by a 1 h incubation with 0.3 % bovine serum albumin (BSA) (Sigma Aldrich) in PBS. The primary anti-*Aspergillus* antibody (Rabbit anti-*Aspergillus*, ab20419 Abcam) was 1000-fold diluted in PBS and incubated with the cells for 2 h. Cells were washed once with PBS, after which the secondary antibody (Goat anti-Rabbit AlexaFluor 488, #4412 Cell Signaling Technology), which had been 1000-fold diluted in PBS, was added to the cells for 1 h. Coverslips were mounted to the glass slide using FluorSave^{tm} and dried overnight. The percentage of germinated conidia was determined by analysing z-stacks made at 10 randomly chosen fields on the coverslip. Hyphae and conidia that were green or red and stained with CFW were counted as outside of the A549 cells. Germination values were expressed as the percentage of germinated conidia from all associated conidia to the A549 cells. Biological triplicates were used with pictures taken at 10 random places on the slide. At least 100 conidia or hyphae were counted in each replicate.

Quantification of IL-8 secretion

Confluent cell layers of A549 cells were challenged in 12 wells plates (Corning®, Costar®) with conidia for 2 h, after which the unbound conidia were removed by washing three times with pre-warmed DMEM. Exposure of the A549 cells to the conidia was continued for another 2 or 10 h. The culture medium was added to 96 wells IL-8 ELISA plates (ThermoFisher Scientific) according to the manufacturer's instructions. Experiments were performed using biological triplicates and technical duplicates.

Identification of apoptotic and necrotic cells

A549 cells were grown on 12-mm glass coverslips (VWR international) until a confluent cell layer was formed. The A549 cells were challenged with conidia for 2 h, after which unbound conidia were removed by washing three times with pre-warmed DMEM + 10 % FBS. Exposure of the A549 cells to the conidia continued for another 2 or 10 h and washed twice with PBS. Staining of the apoptotic and necrotic cells was based on (Baskić et al., 2006). In short, 100 µg mL^{-1} acridine orange (AO) (VWR International) and 100 µg mL^{-1} ethidium bromide (EB) (Sigma Aldrich) was added to PBS for each experiment and added to the cells. The cells were immediately mounted on a coverslip with FluorSave^{tm} and examined by fluorescence microscopy within 15 min after adding the dye. The experiments were performed using biological triplicates with pictures taken at 10 random places on the slide. At least 100 cells per strain were analysed per replicate.

A549 cell damage

Confluent layers of A549 cells in 24 wells plates (Corning®, Costar®) were challenged with conidia for 2 h, after which unbound conidia were removed by washing three times with pre-warmed DMEM. Exposure of the A549 lung cells to the conidia was continued for another 2 or 10 h. Cell damage at both time points of challenging (4 and 12 h in total) was measured by lactate dehydrogenase (LDH) released into the medium. The medium was added to a transparent 96 wells plate (Corning®, Costar®) and LDH activity was measured using an LDH activity kit (Sigma Aldrich) according to manufacturer's instructions. A549 cells not challenged with conidia served as control. Experiments were done using biological triplicates and technical duplicates.

Galleria melonella infection experiments

Galleria melonella larvae were injected in the hindleg with 10 µL PBS containing 10^5, 10^6 or 10^7 conidia mL^{-1} and placed in the dark in a 37 °C incubator. Survival of the larvae was monitored for 7 days after infection. Larval death was assessed by the lack of movement and the presence of a black pigment in the larvae. Fungal survival was monitored for 3 days after infection. At day 0, 1, 2 and 3 the haemolymph of three larvae for each condition was collected in an Eppendorf tube by bleeding. The haemolymph was homogenized and diluted in PBS and plated onto PDA agar plates containing 50 µg mL^{-1} ampicillin (Sigma Aldrich) to prevent bacterial growth. Plates were incubated overnight at 37 °C and fungal survival was determined by the colony forming units (CFU). All experiments were done using biological triplicates.

Protostelium aurantium growth and fungal infection

Protostelium aurantium var. *fungivorum* was grown as described (Radosa et al., 2019) at 22 °C in Petri dishes with phosphate buffer (PB: 26.8 g L^{-1} $Na_2HPO_4 \cdot 7H_2O$, 2.4 g L^{-1} KH_2PO_4, pH 6.4) and with the pigmented yeast *Rhodotorula mucilaginosa* as food source. Fungal survival and phagocytosis by the amoebae were determined as described (Ferling et al., 2020; Radosa et al., 2019). For fungal survival the conidia were allowed to swell in Czapek-Dox medium (CZD) (Difco) for 4, 6 or 8 h at 37 °C. *P. aurantium* cells were washed three times with PB to remove residual *R. mucilaginosa* and added to the swollen conidia at an MOI of 10 and incubated for 18 h at 22 °C. Subsequently, the plates were transferred to 37 °C for 1 h to kill the amoebae and 0.002 % (w/v) resazurin (Sigma Aldrich) was added to the plate. Fungal survival was expressed as the percentage of resorufin formation by fungi confronted with *P. aurantium* and the resorufin formation by fungi alone. Resorufin fluorescence was measured after 15 h of incubation at 37 °C with an Infinite M200 Pro fluorescence plate reader (Tecan). Experiments were done using biological and technical triplicates.

For phagocytosis of conidia by amoebae, conidia were first stained for 30 min with 0.1 mg mL^{-1} FITC (Sigma Aldrich) in 0.1 M Na_2CO_3 on a rotary shaker at 50 rpm at room temperature. Excess FITC was removed by washing three times with PBS. Conidia were allowed to swell for 4.5 h in CZD at 37 °C. *P. aurantium* cells were treated as above, added to the swollen conidia at an MOI of 10 and incubated at 22 °C for 2 h. The growth medium was aspirated and replaced by 0.1 mg mL^{-1} CFW (fluorescent brightener 28) (Sigma Aldrich) in PBS. Excess CFW was removed by washing once with PBS. Amoebae and fungi were fixed using 3.7 % formaldehyde (Carl Roth) for 10 min prior to imaging. Experiments were done using three biological replicates, with at least 100 amoebae counted for each replicate.

To study germination of conidia after incubation with amoebae, conidia were stained for FITC as described above. Conidia were allowed to swell for 4, 6 or 8 h in CZD at 37 °C. *P. aurantium* cells were added to the swollen conidia at an MOI of 10 and incubated for 18 h at 22 °C. Fixation with formaldehyde and staining with CFW was carried out as above. Experiments were done using three biological replicates, and at least 100 conidia were counted for each replicate.

Zebrafish care and maintenance and infection experiments

Wild-type AB/TL zebrafish lines, adults and embryos, were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Breeding of the zebrafish adults was approved by the local animal welfare committee (DEC) of the University of Leiden under license number 10612 and in accordance with the international guidelines specified by the EU Animal Protective Directive 2010/63/EU. All zebrafish studies were performed on embryos before the free feeding stage and therefore the work did not fall under the animal experimentation law according to the EU Animal Protection Directive 2010/63/EU. Adult zebrafish were kept at 28 °C in an aquarium with a 14 h day and 10 h night cycle. Embryos were cultured at 28.5 °C in egg water (60 µg mL^{-1} sea salt; Seramarin) and were anesthetized in the same solution containing 0.02 % (w/v) buffered Tricaine (3-aminobenzoic acid ethyl ester) (Sigma Aldrich) before fungal microinjections.

Fungal microinjection into the hindbrain of the zebrafish was done as described (Koch et al., 2019). In short, conidia (10^8 conidia mL^{-1}) were taken up in PBS with 2 % polyvinylpyrrolidone (PVP). The injected dosage of approximately 100 conidia per larvae was microscopically checked at 40 times magnification. The conidia were injected into the hindbrain ventricle of zebrafish embryos via the otic vesicle at 36 h post fertilization (HPF). The survival of embryos was monitored until 5 days post fertilization (DPF). Experiments were done using biological triplicates, for each replicate 48 zebrafish were injected per strain. Zebrafish were scored as dead when there was no observable heartbeat.

Germination of conidia

Germtube formation was quantified after swelling conidia for 4, 6 or 8 h in CZD at 37 °C. For each time-point and strain at least 1000 conidia or germlings were counted.

Microscopy

Confocal images of internalized, associated and germinated conidia upon interaction with A549 lung epithelial cells were acquired with a Zeiss LSM 700 microscope using the Plan-Apochromat 63 x 1.40 oil DIC (WD=0.19) objective. Images were taken using the 405, 488 and 555 nm laser lines. Fluorescence emission of CFW and Hoechst was detected using the 400-490 nm spectral band. Red fluorescence emission of RFP was detected with the 560-700 nm spectral band and FITC fluorescence was detected with the 490-555 nm spectral bands.

Images of the apoptotic and necrotic cells were acquired with an Axioskop 2 plus at a 20 x magnification. The FITC filter with an excitation of 450-488 nm was used to image Acridine Orange with an excitation of 431 nm and the TRITC filter with an excitation of 540-570 nm was used to image the ethidium bromide with an excitation of 524 nm. Phagocytosis of the conidia by amoebae was visualized using a Zeiss Axio Observer 7 spinning disk confocal microscope using the 63 x oil objective. Images were taken using the bright field to image the amoebae, the 405 nm laser line was used to image the FITC stained conidia and the 488 nm laser line was used to image the CFW stained conidia.

All images were analysed and processed with the Fiji image processing package of ImageJ (www.fiji.sc).

Chromosomal DNA isolation

Conidia were inoculated in transformation medium (TM; MM supplemented with 5 g L^{-1} yeast extract (Difco) and 2 g L^{-1} casamino acids (Difco)) and 50 µg mL^{-1} ampicillin, to prevent bacterial contamination, and grown overnight at 37 °C and 200 rpm. Mycelium was collected by filtering over a double layer of Miracloth (Merck Millipore) and lyophilized overnight. Part of the lyophilized mycelium (~30 mg) was lyzed with a Tissuelyser (Qiagen) using 2 metal balls (4.76 mm in diameter) for 2 minutes at 25 Hz. DNA was isolated from the homogenized mycelium with the Qiagen DNeasy PowerPlant Pro kit following the manufacturer's protocol for problematic samples. Qubit® was used to check DNA quality and concentration. DNA samples were stored at -20°C.

Whole genome sequencing and virulence-related genes

DNA sequencing of strains ATCC46645 and CEA10 was performed by the Utrecht sequencing facility (Useq). Libraries were prepared using Truseq DNA Nano library and sequenced on an Illumina NextSeq500 with 150 bp pair end mid output configuration. Read quality was checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), cleaning and trimming was performed with the Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The genome sequence of Af293 (version 42 from FungiDB https://fungidb.org/fungidb/) was used as the reference to map reads using bowtie2 v2.2.9 (Langmead & Salzberg, 2012). Freebayes v0.9.10-3 (Garrison & Marth, 2012) was used for variant calling. Virulence-related genes have been described previously (Puértolas-Balint et al., 2019). Custom R scripts were used to find and filter the SNPs in these genes, effects of the SNPs were predicted using SnpEff v3.3k (Cingolani et al., 2012).

Phylogenetic tree

A set of published *A. fumigatus* genomes (Supplementary Table 1) was used. Alignment and variant calling of the genomes were performed as described above. The final set of variants of the strains used in this study and those described in Supplementary Table 4 were used to build a SNP-based tree as described (Lind et al., 2017). In short, SNPRelate V3.7 (Zheng et al., 2012) was used to filter biallelic SNPs with no missing data, a linkage disequilibrium threshold of 0.8 and a minor allele frequency of 0.03. In total 14100 SNP markers were aligned with MAFFT 7.310 using default options (Katoh et al., 2002). RaxML was used to obtain a maximum likelihood tree with the GTRGAMMA model with 1000 bootstrap replicates (Stamatakis, 2014). The best-scoring ML tree was used for analysis. Figtree was used to visualize and analyse the phylogenetic tree (Rambaut, 2018).

Statistical analysis

A one-way ANOVA with a p-value ≤ 0.05 was used to analyse differences in inhibition zones after peroxide or menadione exposure, conidial association to A549 cells, amount of IL-8 secretion, LDH release and hyphal length after co-incubation with A549 cells or amoebae. Values were scored as in or out and treated as binary data for the analysis of the internalization of conidia. In this case, differences were analysed using a Pearson chi-square test with p-values ≤ 0.05 considered significant. The non-parametric Kruskal-Wallis test was used to analyse differences in survival of conidia after incubation with the amoebae or after injection in the *G.*

melonella larvae with a P-value ≤ 0.05 considered significant. The same test was used to assess the number of amoebae with ingested conidia, the germination of the conidia after co-incubation with the amoebae and amoebae survival after co-incubation with conidia. Survival of the *G. melonella* larvae and zebrafish embryos was plotted using the Kaplan-Meier survival curve. Differences in survival between strains was determined using a non-parametric log rank test. P-values ≤ 0.05 were considered significant.

Data availability

Next generation sequencing (NGS) data of strains ATCC46645 and CEA10 is available at NCBI Sequence Read Archive (SRA) under the code: PRJNA670081. DTO271-B5 and DTO303-F3 DNA sequence data were extracted from BioProject PRJNA598656.

Results

Variable oxidative stress resistance among *A. fumigatus* strains

Strains Af293, ATCC46645, CEA10, DTO271-B5 and DTO303-F3 were compared for their resistance to oxidative stress *in vitro*. To this end, hydrogen peroxide and superoxide (menadione) stress were chosen. Both are reactive oxygen species (ROS) used by the immune system to kill microbial pathogens (Warris & Ballou, 2019). Af293 appeared most sensitive to hydrogen peroxide (Figure 1A) and menadione (Figure 1B), while ATCC46645 and DTO303-F3 were most resistant to both types of stress.

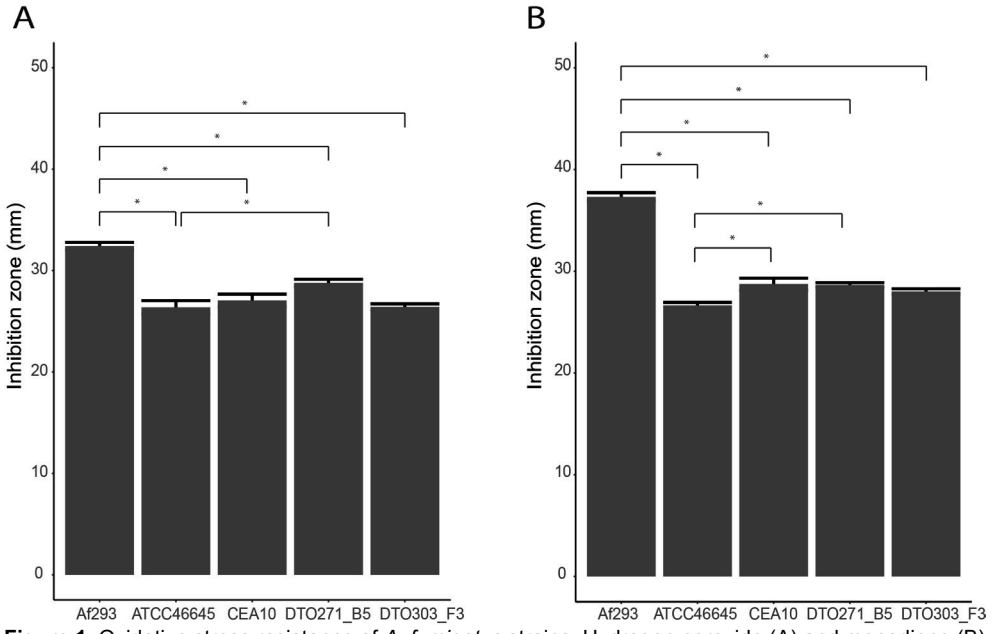

Figure 1. Oxidative stress resistance of *A. fumigatus* strains. Hydrogen peroxide (A) and menadione (B) sensitivity of five *A. fumigatus* isolates. Bars represent the average inhibition zone based on biological and technical triplicates (± SE). * indicates statistical significance.

Interaction of *A. fumigatus* strains with lung epithelial cells

Conidia were exposed to a monolayer of type II A549 lung epithelial cells. After 4 h of incubation, association of conidia of Af293 and CEA10 to the A549 lung epithelial cells was highest (Figure 2A; 0.19 and 0.17 conidia/cell, respectively). The conidia of ATCC46645, DTO271-B5 and DTO303-F3 associated with only 0.14, 0.11 and 0.12 conidia/cell, respectively. Internalization of conidia of the five strains hardly varied. The conidia of Af293 and DTO303-F3 were internalized slightly higher (82 % and 79%, respectively) whereas those of CEA10, DTO271-B5 (both 77 %) and ATCC46645 (74 %) were slightly less efficient internalized (Figure 2B).

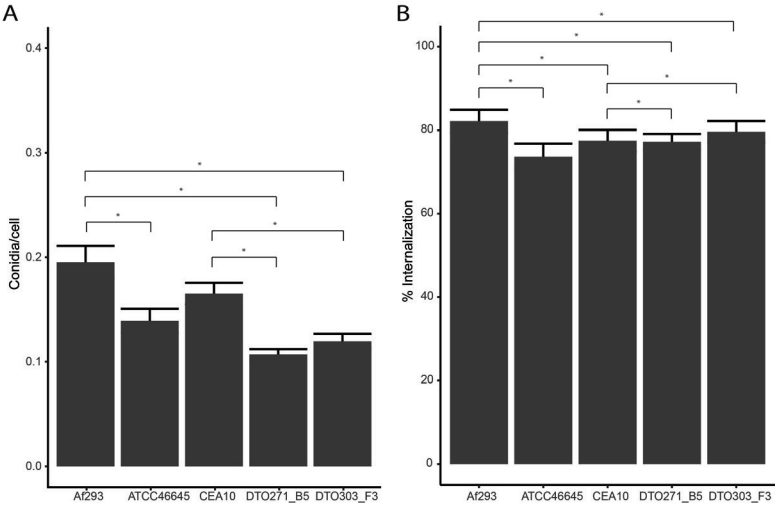

Figure 2. Association (A) and internalization (B) of conidia after 4 h of incubation with type II A549 lung epithelial cells. Bars represent the average (± SE) of three independent experiments. * indicates statistical significance.

After 12 h of incubation with A549 cells, the majority of the conidia of the five strains had germinated (Figure 3A). Most hyphal growth was observed for ATCC46645 (89 %), DTO271-B5 (89 %) and DTO303-F3 (85 %), while least growth was observed for CEA10 (75 %) and Af293 (67 %). Most conidia were internalized, whereas most hyphae resulting from these conidia were located outside of the cells and thus had grown outside of the cells (Figure 3B). Of the five strains, conidia of Af293 were most effectively internalized. More germination was observed among conidia outside of the A549 cells, indicating that germination of non-internalized conidia is more efficient then for internalized conidia.

Lactate dehyrogenase (LDH) activity in the medium was determined as measure for host cell damage after 4 and 12 h of co-incubation (Figure 4A). Conidia associated within the first 4 h, and a large part was internalized into A549 cells after 4 h, but no germination was observed. In contrast, after 12 h hyphae were observed, next to conidia, both in and outside the A549 cells. After 4 h, LDH activity in the culture supernatant was similar to the control of uninfected A549 cells for all 5 strains. After 12 h an increase in LDH release into the medium was found when compared to 4 h and to the A549 control at 12 h. A549 cells infected with conidia from DTO303-F3 released most LDH in the medium, indicating that these cells were damaged the most, while A549 cells infected with CEA10 released the least LDH.

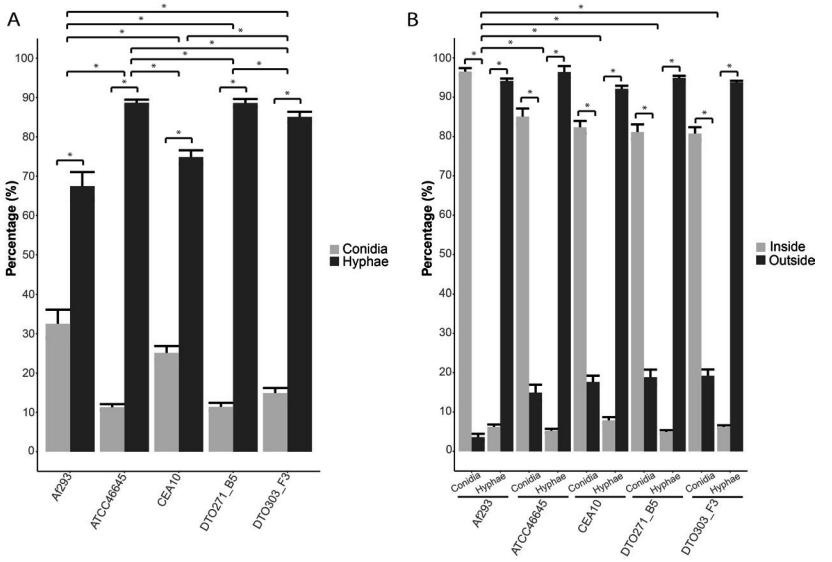

Figure 3. Germination of *A. fumigatus* during coincubation with epithelial cells. Percentage of conidia (light grey) and hyphae (dark grey) after 12 h of incubation with type II A549 lung epithelial cells (A). Internalized (light grey) or non-internalized (dark grey) conidia or hyphae after 12 hours of incubation with the A549 lung epithelial cells (B). Bars represent the average (± SE) of three independent experiments. * indicates statistical significance.

The initiation of the immune response in the A549 lung epithelial cells by conidia was determined by measuring interleukin 8 (IL-8) release in the medium. After 4 h of infection hardly any IL-8 release into the medium was found in any of the conditions (Figure 4B). In contrast, an increase in IL-8 release was found after 12 h, but we did not find a significant difference between the uninfected control and the A549 cells incubated with one of the five *A. fumigatus* strains. This shows that, even though most cell damage was induced after infection with DTO303-F3 conidia, this does not correspond to a higher initiation of the immune response measured by IL-8 release.

To determine if the integrity of the epithelial cells was compromised due to interactions with *A. fumigatus*, the percentage of apoptotic and necrotic cells was scored after 4 and 12 h using a dual acridine orange and ethidium bromide staining and microscopic analysis (Figure 4C and D). A similar distribution of apoptotic (5 - 11 %) and living cells was observed after 4 h of incubation of A549 cells with or without conidia, whereas virtually no necrotic cells were detected. In contrast, a clear increase in apoptotic cells was observed after 12 h co-incubation with conidia (Figure 4D). Conidia of Af293 and DTO271-B5 might induce a slightly higher apoptosis (20 % and 19 %, respectively) and necrotic (2 % and 1 %, respectively) incidence of A549 cells as compared to the other three strains (around 15 % apoptotic and 0.3 % necrotic) but these differences were not significant.

Together, it is concluded that a 12 h incubation of A549 with *A. fumigatus* conidia results in an increase in apoptotic cells, but the 5 different strains did not induce significant differences in the amount of apoptotic and necrotic cells. Moreover, no major differences in internalization, germination, cell damage or IL-8 release were found between strains isolated from an invasive infection (Af293, ATCC46645 and CEA10) and strains derived from a non-invasive infection

(DTO271-B5 and DTO3030-F3). The latter two strains did however associate to a lower extent to A549 cells. We did see that conidia of Af293 associated and internalized more to A549 cells but germinated less. However, induced cell damage did not significantly differ between the strains. The results show a rather similar lifestyle of *A. fumigatus* isolates on A549 cells and suggests that the type of infection these isolates caused in patients is merely a reflection of the location of the infection (lung or sinus) rather than differences in virulence traits between strains.

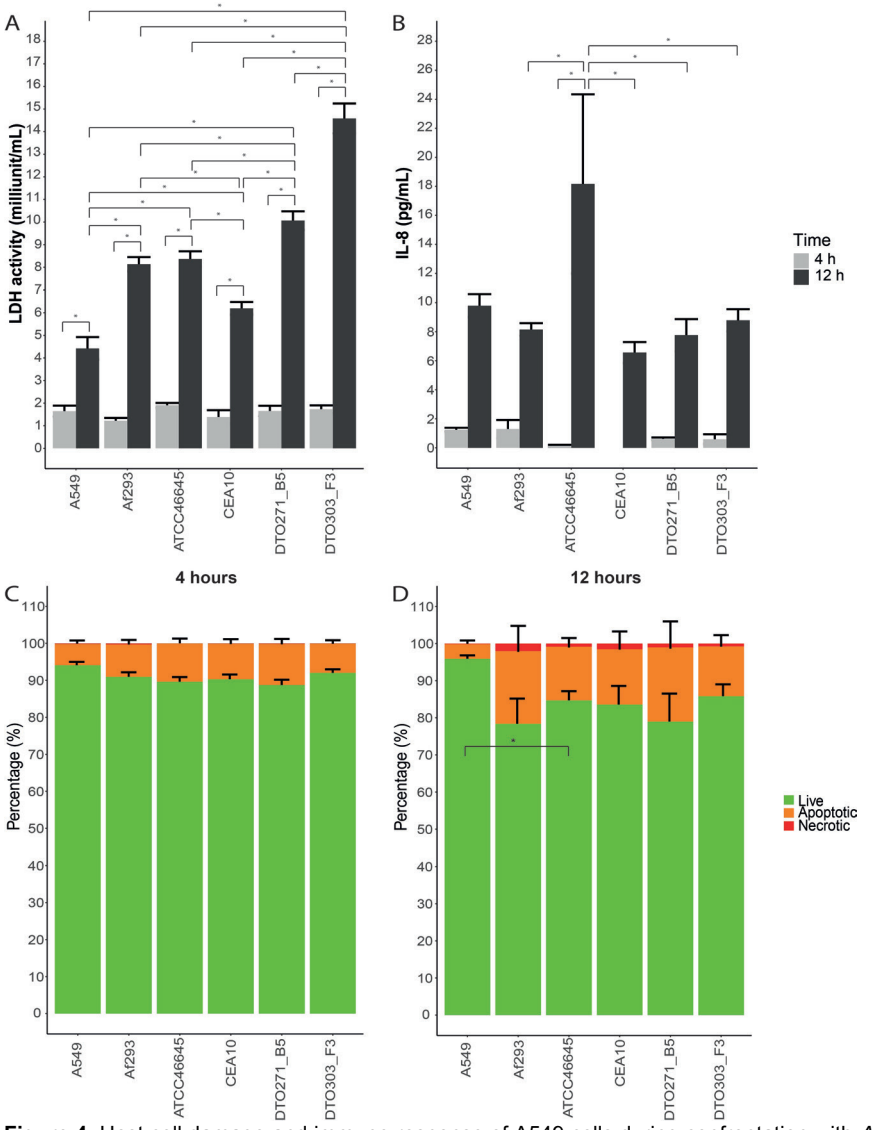

Figure 4. Host cell damage and immune response of A549 cells during confrontation with *A. fumigatus*. LDH release (mU mL^{-1}) (A) and IL-8 production (pg mL^{-1}) (B) of the A549 cells after 4 (light grey) and 12 (dark grey) h of infection. Percentage of cells which are alive (green), apoptotic (orange) or necrotic (red) after 4 (C) or 12 (D) h of infection with *A. fumigatus* conidia. Bars represent the average of three independent experiments (± SE). * indicates statistical significance.

Germination dynamics among *A. fumigatus* strains

Dormant conidia of *A. fumigatus* are surrounded by a protective layer of dihydroxynapthlene (DHN)-melanin and hydrophobin and stealth proteins. This layer is shed during germination. As *P. aurantium* primarily recognizes swollen conidia of *A. fumigatus,* its phagocytic efficiency is highly dependent on the dynamics of the fungal germination process. We therefore first compared the germination kinetics of the five strains by determining the number of germlings at 4, 6 and 8 h. Microscopic analysis of conidia in CZD medium revealed a marked difference in germination kinetics between the five strains (Table 2). Af293 and CEA10 produced relatively few germ tubes even after 8 h, when compared to conidia of ATCC46645 of which 54 % had formed germ tubes at this timepoint (Table 2). CEA10, DTO271-B5 and DTO303-D3 revealed intermediate dynamics with 39 %, 20 % and 25 % of the conidia forming germ tubes after 8 h, respectively. The results clearly demonstrated reduced germination kinetics for Af293.

Table 2. Percentage (± SE) of germ tube formation after 4, 6 or 8 h swelling in CZD medium at 37°C.

Strain	**4 h incubation**		**6 h incubation**		**8 h incubation**	
	Conidia	Germlings	Conidia	Germlings	Conidia	Germlings
Af293	100 % ± 0	0 % ± 0	99.25 % ± 0.22	0.75 % ± 0.22	97.37 % ± 0.26	2.63 % ± 0.26
ATCC46645	100 % ± 0	0 % ± 0	89.69 % ± 1.36	10.31 % ± 1.36	45.74 % ± 2.74	54.26 ± 2.74
CEA10	99.76 % ± 0.07	0. 24 % ± 0.07	96.7 % ± 0.39	3.3 % ± 0.39	61.03 % ± 1.2	38.97 % ± 1.2
DTO271-B5	99.64 % ± 0.18	0.36 % ± 0.18	97.87 % ± 0.33	2.13 % ± 0.33	79.98 % ± 2.9	20.02 % ± 2.9
DTO303-F3	99.25 % ± 0.1	0.75 % ± 0.1	96.41 % ± 0.87	3.59 % ± 0.87	75.04 % ± 0.74	24.96 % ± 0.74

Internalization and killing of *A. fumigatus* strains by *Protostelium aurantium*

Phagocytic uptake and survival of the fungus was monitored when exposed to the amoebae at different time points of its germination process. Internalization of conidia can be monitored due to quenching of FITC fluorescence in the acidic compartment of the phagolysosome of the amoebae (Figure 5A, B and C). At 4.5 h after initiation of germination, conidia of ATCC46645 were taken up with the highest efficiency, whereas uptake of Af293 conidia had the lowest efficiency (Figure 5D). The fact that Af293 uptake was approximately 50 % lower as compared to the other four strains reflected its less effective recognition by *P. aurantium* due to a delayed germination.

Next, survival of 4, 6 and 8 h pre-swollen conidia was quantified after exposure to amoebae. For all five strains, the early time-point of 4 h resulted in higher survival rates when compared to conidia that had been pre-incubated for 6 h, and thus underwent an extended swelling period (Figure 6A, B). However, this effect was not significant for the ATCC46645 strain. Highest survival rates were seen when the fungal conidia were pre-incubated for 8 h (Figure 6C). This is in line with the high number of germ tubes formed that will interfere with the phagocytic uptake an attenuate phagocytic killing (Table 2). Conidia of Af293 behaved different when compared to the other 4 strains. Survival of 4 h pre-swollen conidia was higher than for the other 4 strains, while 8 h pre-swollen Af293 conidia were killed more efficiently than any of the other four strains.

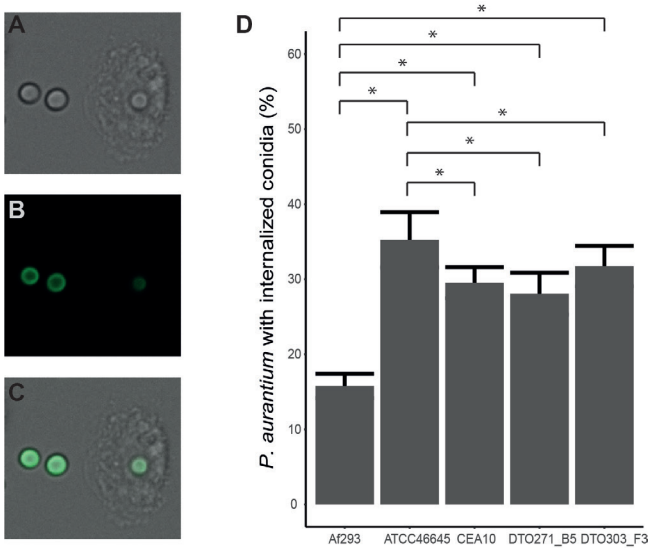

Figure 5. Uptake of swollen conidia by *P. aurantium* after 2 h of incubation. DIC (A) and FITC (B) channel of phagocytosed and non-phagocytosed pre-swollen conidia (4.5 h) and their merge (C). FITC signal of the phagocytosed conidia is lower due to the acidification of the phagolysosome. D) Percentage (± SE) of amoeba that had phagocytosed one or more conidia. Bar represent three individual experiments. * indicates statistical significance.

We further monitored germination and growth of 4, 6 and 8 h pre-swollen conidia after an 18 h incubation period with the amoebae. The number of conidia and hyphae were scored microscopically by the number and length of the hyphae. While for ATCC46645, DTO271-B5 and DTO303-F3 the number of hyphae increased with a prolonged period of pre-swelling, only few hyphae were found for Af293 and CEA10, regardless of how long these conidia had been pre-incubated (Figure 6D, E, F). For Af293, those hyphae that were found continuously grew in the presence of the amoebae (Figure 6G, H, I). For CEA10, conidia hardly germinated after an 18 h interaction with the amoebae (Figure 6F) and thus, essentially no hyphae were detected for this strain to monitor hyphal length (Figure 6G, H, I). Germination of DTO271-B5 and DTO303-F3 was also reduced in the presence of the predator, but only a slight retardation in germination was observed for ATCC46645. These results indicate that the amoebae can affect the germination process and the formation of germ tubes via an unknown mechanism. For Af293 the germination process was found to be relatively slow which was aggravated in the presence of *P. aurantium*.

Next survival of the amoebae was monitored by counting the amoebae before and after the 18 h incubation with conidia at different stages of germination. Survival of amoeba was virtually 100 % when exposed to 8 h pre-swollen conidia of Af293, CEA10 and DTO271-B5, whereas approximately 50 % of the amoebae survived incubations with 8 h pre-swollen conidia of ATCC46645 and DTO303-F3 (Supplementary Figure 1). These results indicate that ATCC46645 and DTO303-F3 had the highest amoebicidal activities. Remarkably, survival of the amoebae did not correlate with germination and hyphal length. Amoebae incubated with conidia of Af293, CEA10 and DTO271-B5 survived the best, while only the latter strain germinated efficiently (Supplementary Figure 1). Together, these results indicate a

marked difference between the behaviour of Af293 in the amoeba infection system in comparison with the other four isolates differing in efficiency of uptake, killing by amoebae and germination. The apparent low level of initiation of germination of Af293 most likely results in relative low uptake and high survival. In contrast, higher germination kinetics of the other four strains results in more efficient uptake and killing. Together, these results indicate that delayed germination, as demonstrated for Af293, may protect conidia during early confrontation with a phagocyte.

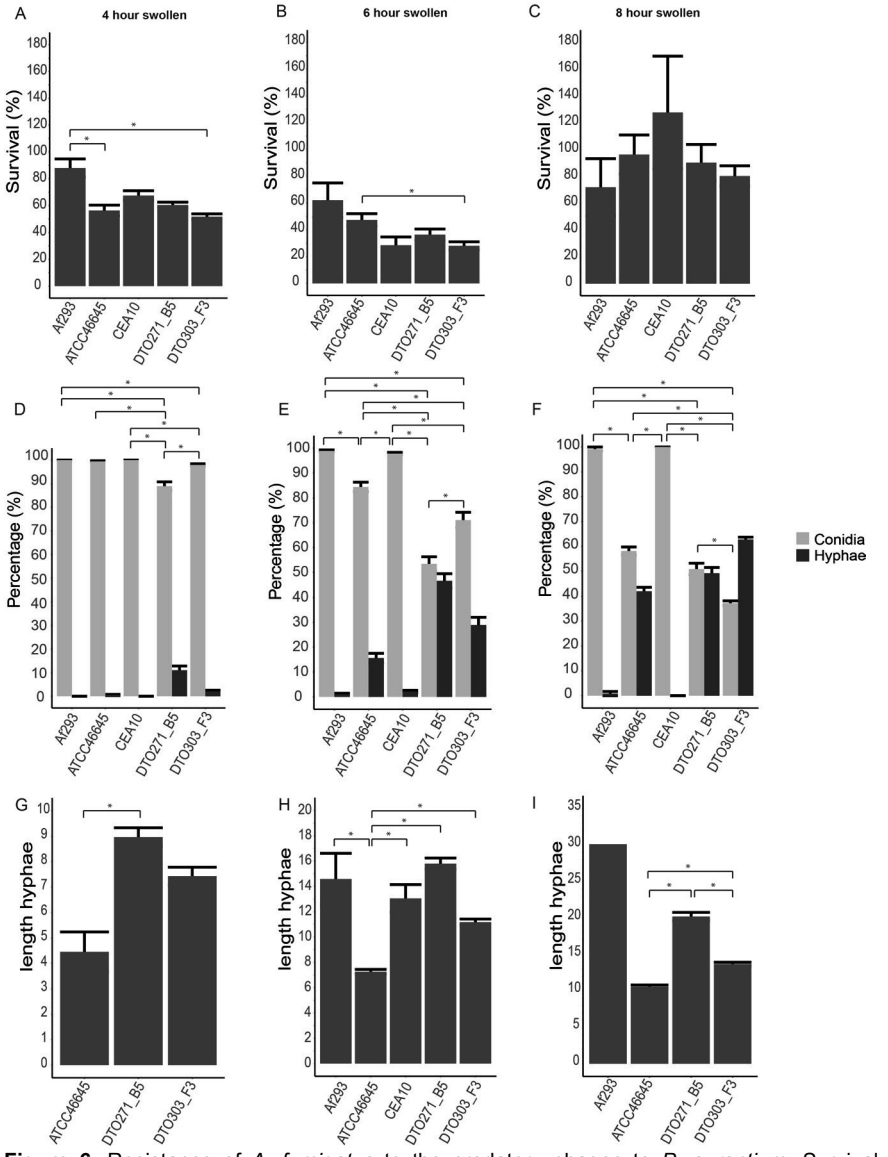

Figure 6. Resistance of *A. fumigatus* to the predatory phagocyte *P. aurantium.* Survival (A, B, C), germination (D, E, F) and hyphal length (G, H, I) of conidia following pre-swelling for 4 (A, D, G), 6 (B, E, H) or 8 h (C, F, I) and coincubation with *P. aurantium* for 18 h. Bars represent the average (± SE) of three independent experiments. * indicates statistical significance.

Galleria model

Virulence of the five *A. fumigatus* strains was assessed in *G. melonella.* To this end, larvae were injected in the hindleg with 10^5, 10^6 and 10^7 conidia mL^{-1} and incubated at 37 °C. Survival of the *A. fumigatus* strains was followed in the *G. melonella* larvae over the first 3 days of infection (Figure 7 A-C). In general, the number of conidia in the haemolymph of the larvae decreased, which was irrespective of the strain or spore concentration used, indicating that conidia exit the haemolymph quickly, and with the similar efficiencies to establish the infection elsewhere in the larvae. The slower germinating Af293 was found to be retained in the haemolymph at day 1, but only when larvae were challenged with an intermediate number of conidia. Also, CEA10 was retained for 24 h upon infection with highest conidial load.

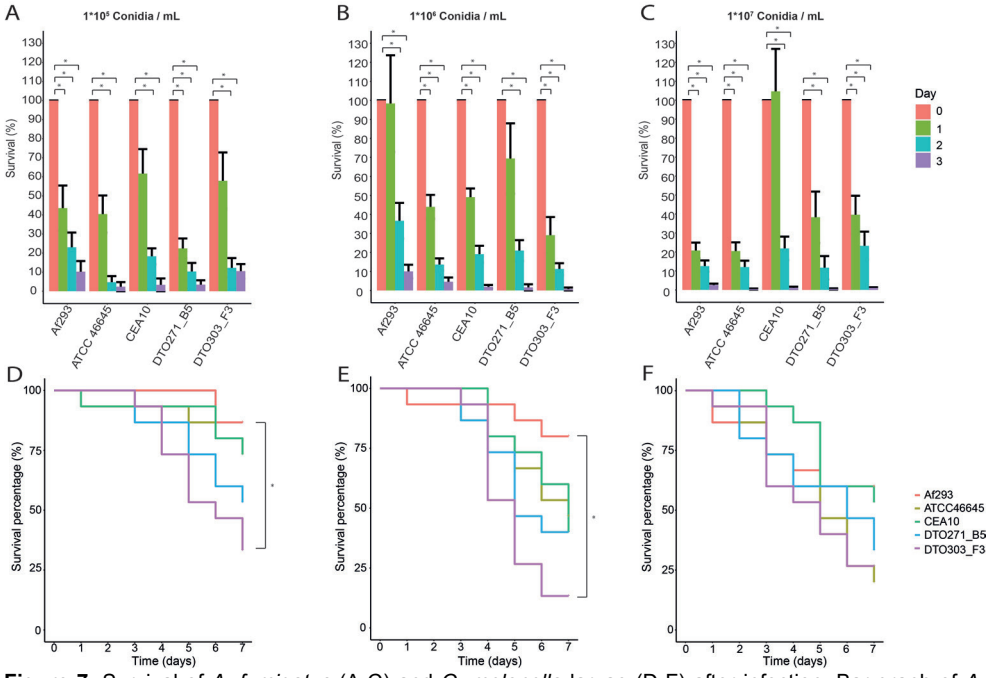

Figure 7. Survival of *A. fumigatus* (A-C) and *G. melonella* larvae (D-F) after infection. Bar graph of *A. fumigatus* survival and Kaplan-Meier survival plots of *G. melonella* over 3 or 7 days of incubation with 10^5 (A, D), 10^6 (B, E) and 10^7 (C, F) conidia mL^{-1}, respectively. Bars represent the average (± SE) of three independent experiments. Each line consists of three biological replicates. * indicates statistical significance.

Next, larvae survival was monitored for 7 days after the injection. At the highest dose of 10^7 conidia mL^{-1}, all strains were able to develop infections in the *Galleria* larvae killing 50 - 75 % of the larvae (Figure 7F). Larvae survived better when exposed to 10^6 conidia mL^{-1} of Af293, CEA10 and ATCC46645 (Figure 7E) and killing remained high for DTO271-B5 and DTO303-F3, even at the lowest infection dose of 10^5 conidia mL^{-1} (Figure 7C). Taking the results of the three spore amounts together, larvae injected with conidia of slower germinating Af293 survived the best when compared to the other four strains. However, virulence with *Galleria* was not entirely dependent on germination kinetics, as also the faster germinating

strain CEA10 showed reduced killing. Together, these results show that larvae were killed irrespective of the initial fungal load and killing probably followed germination of fungal conidia outside the haemolymph system. Overall killing of the larvae was most efficient for ATCC46645, DTO271-B5 and DTO303-F3 indicating more specific virulence determinants for these three strains.

Zebrafish model

Zebrafish embryos were the fourth model used to assess virulence between the five *A. fumigatus* isolates. Injection of 100 conidia into the hindbrain of the zebrafish embryos led to mortality of the embryos over a 96 h period. The highest and lowest survival was observed with Af293 and ATCC466645 conidia, respectively (Figure 8).

Conidia of *A. fumigatus* are between 2 - 3 µm in size (Brakhage & Langfelder, 2002). There are also indications that the size of the conidia can differ between strains of the same species (van den Brule et al., 2020). With the injection of the conidia in the hindbrain of the embryos damage is induced. This damage could become larger when the diameter of the conidia would be larger. As shown in Supplementary Figure 2, there is no significant difference in conidia size between the strains used in this study. This means that the observed differences in survival of the zebrafish embryos is due to the difference in virulence of the strains.

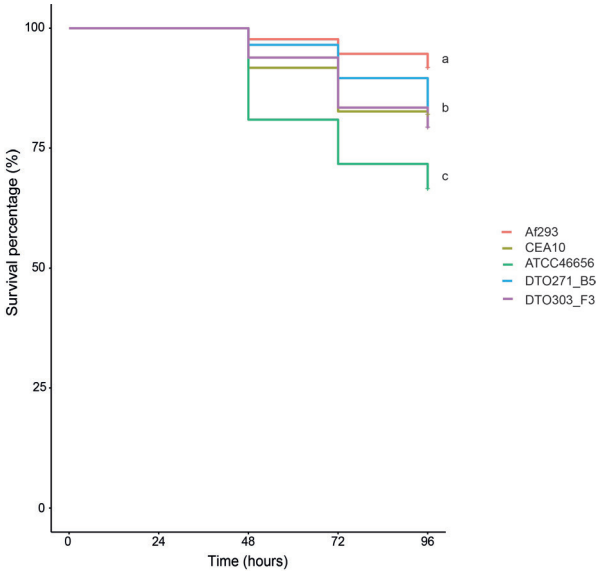

Figure 8. Kaplan-Meier survival plot of the zebrafish embryos injected with 100 conidia of different *A. fumigatus* isolates. Statistical different groups are indicated with a unique letter (a, b or c). Each line consists of three biological replicates.

Virulence-related genes

Results demonstrate that Af293 is less virulent in amoebae, *Galleria* and zebrafish infection models as compared to the other four strains. ATCC46445 and DTO303-F3 were most virulent in zebrafish and *Galleria* infection models, respectively. This variation in virulence might be related to absence or presence of virulence-related genes or mutations in these genes. Single Nucleotide

Polymorphisms (SNPs) were identified within the set of previously published virulence-related genes (Puértolas-Balint et al., 2019) (Figure 9). The virulence-related genes are divided in sub-groups of genes related with thermotolerance, resistance to the immune response, cell wall, toxins and secondary metabolites, allergens, nutrient uptake and signalling and regulation (Supplementary Table 2). Surprisingly, there were no high impact SNPs (defined as SNPs predicted to have a high impact on the protein such as loss of function, as predicted by Snpeff (Cingolani et al., 2012)) present in the selected genes. Strikingly, 136 SNPs were identified that were shared between the four strains, which indicates that Af293 differs with the other strains in 105 virulence-related genes. In addition, ATCC46645 stood out with 45 unique SNPs as compared to the other three strains, when aligned to the Af293 reference genome. CEA10, DTO271-B5 and DTO303-F3 had 17, 14, and 13 unique SNPs, respectively, when aligned to the Af293 reference genome. Strain specific mutations in virulence-related genes might contribute to difference in virulence between these strains. The 136 SNPs shared between ATCC46645, CEA10, DTO271-B5 and DTO303-F3 occurred in all groups of virulence-related genes. For instance, SNPs were identified in the fumagillin biosynthesis polyketide synthase fma-PKS (Afu8g00370), (Supplementary Table 3). *A. fumigatus* strains that do not produce fumagillin, due to a *fmaA* knock-out, cause less damage to pulmonary epithelial cells when compared to the wild-type strain (Guruceaga et al., 2018). The observed SNPs could alter the fumagillin production in ATCC46645, CEA10, DTO271-B5 and DTO303-F3 or especially in Af293 and affect virulence of the latter strain, but more analysis is required to confirm this.

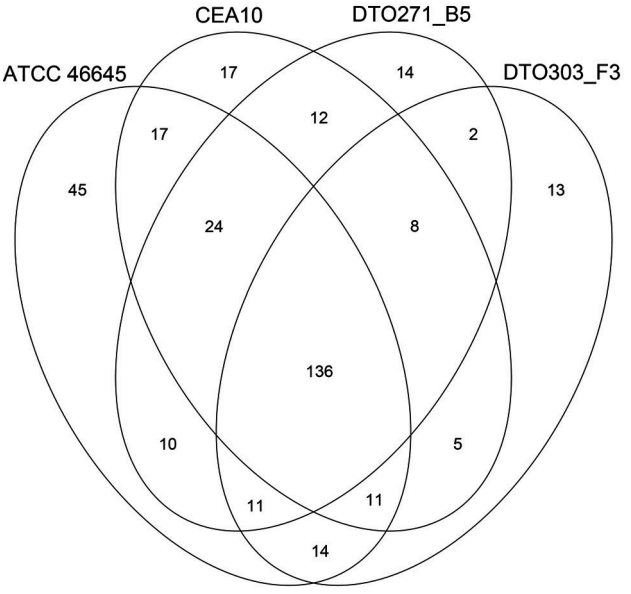

Figure 9. Venn diagram of all SNPs (low, moderate and high impact) in the virulence related genes listed by (Puértolas-Balint et al., 2019). The genome of Af293 is used as a reference genome.

Conidia of ATCC46645 and DTO303-F3 were the most virulent based on survival data of *G. melonella* larvae and zebrafish embryos. These strains share 14 SNPs (Figure 9; Supplementary Table 4) in genes belonging to resistance to the immune response, cell wall, toxins and secondary metabolites and allergens. For instance, there were SNPs in the superoxide dismutase gene *sod4* (Afu6g07210), the class I chitin synthase *chsA* (Afu2g01870), the Asp-hemolysin gene *aspHS* (Afu3g00590) and in a gene with predicted nucleic acid and zinc binding activities (Afu5g12760). Most SNPs were found in genes belonging to the fumigaclavine C (fga) biosynthesis cluster. In this gene cluster we see SNPs in *fgaFS* (Afu2g17970), *easM* (Afu2g18010), *fgaAT* (Afu2g18020) and *fgaCAT* (Afu2g18030). Fumigaclavine C has been reported to have anti-inflammatory effects (Yu et al., 2017) and reduces the expression of inflammatory cytokines (Wu et al., 2005). The two observed shared SNPs, which lead to an amino acid change in the genes related to the fumigaclavine C biosynthesis cluster could affect the anti-inflammatory effects of the fumigaclavine C. This could lead to an increase in the inflammatory response and cytokine production of the host and therefore leading to a more severe infection and killing of the host.

A phylogenetic tree based on all the SNPs in the genome of the strains listed in Supplementary Table 1 was made based on the Af293 reference genome (Figure 10). The *A. fumigatus* strains can be found in three clusters. Where Af293, CEA10, DTO271-B5 and DTO303-F3 are located in the same cluster, in this cluster CEA10, DTO271-B5 and DTO303-F3 are closest to each other. ATCC46645 is located in a separate cluster. The invasive and non-invasive isolates, as well as environmental isolates, did not cluster together. But we did see a cluster of non-invasive strains, that originate from samples taken at different time points from the same patient with a chronic pulmonary aspergillosis (Ballard et al., 2018). Strains isolated from dogs with a non-invasive sino-nasal aspergillosis (Valdes et al., 2018) generally also cluster when they had been isolated from the same dog.

Discussion

Previous studies have indicated that genetic and phenotypic variability between *A. fumigatus* isolates impact virulence (Garcia-Rubio et al., 2018; Hagiwara et al., 2018; Rosowski et al., 2018). In this study infection of five isolates of *A. fumigatus* that were isolated from patients with invasive (Af293, CEA10 and ATCC46645) or non-invasive aspergillosis (DTO271-B5 and DTO303-F3) was assessed in four infection models. An overview of the results is presented in Figure 11 and Supplementary Table 5. The most striking result is the observation that Af293 is less virulent when compared to the other strains. No differences were seen between virulence of the strains in type II A549 lung epithelial cells. In contrast, strain ATCC46645 was most virulent in the amoeba and zebrafish model, whereas DTO303-F3 was the most virulent in the *Galleria* infection model.

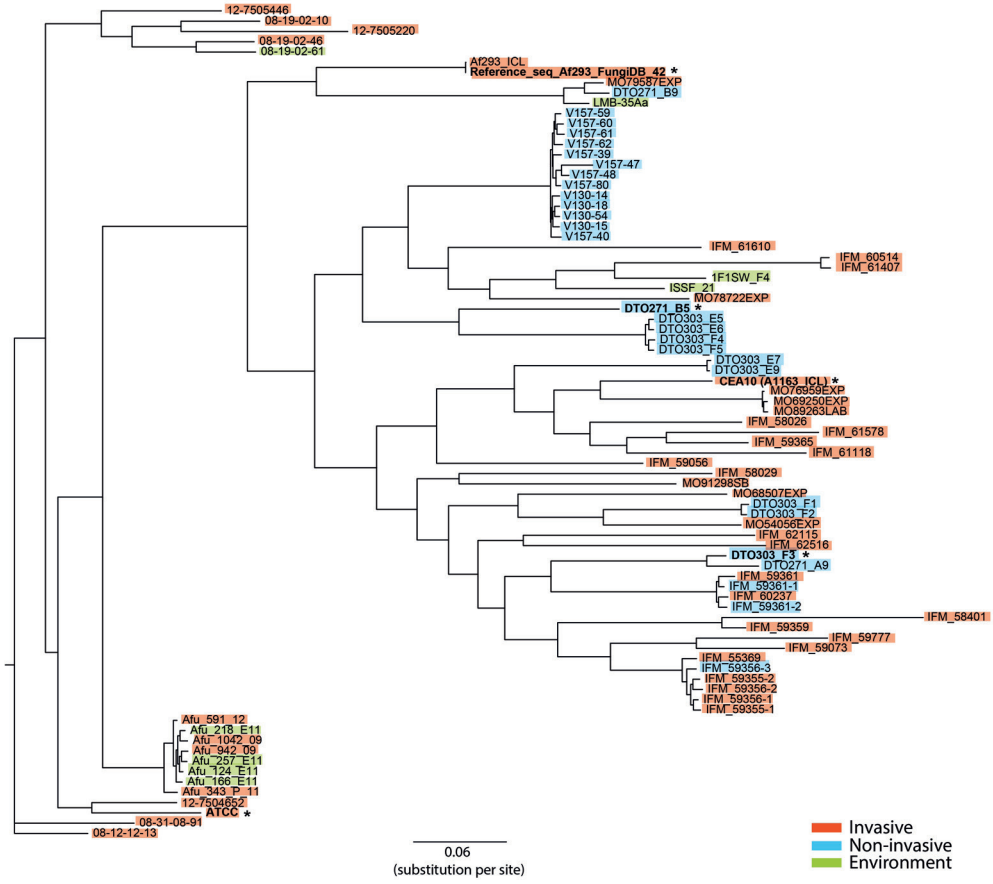

Figure 10. Phylogenetic tree based on SNPs compared to the Af293 reference genome. Strains that were isolated from an invasive infection are marked red, from a non-invasive infection marked blue and strains isolated from the environment marked green. Strains used for the experiments in this study are indicated in bold with an *.

The amoeba infection system is regarded as a model system for phagocytosis by macrophages. Reduced uptake and high survival can be regarded as evasion of phagocytosis. Interestingly, 4 and 6 h pre-swollen conidia of Af293 stand out as compared to the four other strains, surviving the best and having the lowest uptake. This would suggest that Af293 is actually better in evading the immune system as compared to the other four strains. The interaction studies with A549 lung epithelial cells indicate no major differences between the five strains, except for a slightly higher efficiency of association of Af293 conidia. Together with an internalization of 80 % this results in a slightly higher uptake of conidia in the A549 lung epithelial cells. Internalized conidia of *A. fumigatus* were not killed by amoebae and germinated much less in amoebae when compared to A549 lung epithelial cells. These internalized conidia therefore represent a potential reservoir of these fungal strains. In addition, delayed germination after uptake in epithelial cells, observed by (Escobar et al., 2016), can also be regarded as a strategy to escape immune surveillance in lungs, for example by alveolar macrophages at the lung surface.

Af293 behaves rather different in the four infection models when compared to the other four strains. It has reduced virulence and escapes immune recognition. This is in accordance with previous work showing that Af293 was less virulent as compared to CEA10 in an IPA murine infection model and in zebrafish (Kowalski et al., 2016; Rosowski et al., 2018). In this context it is interesting to notice that Af293 is clearly more sensitive to *in vitro* stress conditions induced by ROS like hydrogen peroxide and superoxide generated via menadione. Evading phagocytosis is a way to escape killing by ROS, so Af293 benefits especially by escaping uptake by phagocytic cells. This trait seems to be correlated with the efficiency of germination. Dormant conidia of Af293 hardly germinate in CZD medium, while conidia of Af293 and CEA10 hardly germinate in presence of amoebae. The absence of germination of CEA10 and Af293 and the reduced germination of DTO303-F3, DTO271-B5 and ATCC46645 in the presence of *P. aurantium* and type II A549 lung epithelial cells therefore appears to be an inhibitory mechanism induced by the host. Af293 stands out in this comparison as germination is very low and possibly the increased sensitivity of Af293 to hypoxic conditions is involved (Kowalski et al., 2016). Reduced availability of oxygen in the systems affects the radial growth on plate and the biomass production in a liquid culture and could also influence the ability to initiate germination. Germination in hypoxic condition could be influenced by changes in gene expression or metabolism (Wezensky & Cramer, 2011).

Absence of germination also results in reduced recognition by immune cells since surface components are more shielded by cell wall surface components. The presence of DHN-melanin, rodlets and the stealth protein CcpA are, in part, responsible for shielding conidial surfaces (Voltersen et al., 2018). Absence of CcpA was previously shown to reduce virulence in a neutropenic mouse infection model. This was correlated with enhanced recognition of 3 h swollen conidia of the *ccpA* deletion strain by primary human polymorphonuclear neutrophils (PMNs) and activation of human monocyte derived dendritic cells. In addition, absence of CcpA resulted in reduced cell damage of A549 lung epithelial cells, while uptake of swollen conidia by A549 lung epithelial cells was similar. We studied binding and uptake of dormant conidia of Af293 by A549 lung epithelial cells and observed a higher association as compared to the other four strains while only a slightly higher cell damage was induced after 12 h by Af293. Whether recognition of the dormant conidia by A549 lung epithelial cells is modulated by CcpA requires further study. Other research indicates that exposed β-glucan and DHN-melanin in dormant conidia are already sufficient for recognition (Brown & Gordon, 2001; Han et al., 2011; Stappers et al., 2018; Swidergall et al., 2018). On the other hand, recognition and uptake of conidia by amoebae does require pre-swelling of the conidia. We observed that uptake of pre-swollen Af293 conidia is 50 % reduced as compared to the other four strains. This observation suggests that Af293 has a modified conidial surface, for example due to differences in the amount of CcpA or that swelling of Af293 conidia is less efficient. The latter supposition is in agreement with the observed reduction in formation of germlings after 8 h incubation in CZD medium. Even though these characteristics of Af293 seem to be beneficial for its virulence, it was found the be the least virulent in *G. melonella* and the zebrafish infection model. This indicates virulence of the fungus in these models is multifaceted with host interactions at several levels of complexity, such as germination dynamics, recognition by host cells, as well as the site of primary infection in the host.

Trypacidin is produced by the polyketide synthase TynC and present on the conidia of *A. fumigatus*, except on CEA10 conidia, which due to a frameshift in the TynC gene cluster does not produce trypacidin (Throckmorton et al., 2016). Deletion of *tynC* abolishes production of the toxin trypacidin, increases phagocytosis by macrophages and the amoebae *Dictiostelium discodeum*, and increases survival of the amoebae after infection (Mattern et al., 2015). In our *P. aurantium* amoeba model we observed an increase in survival of the tested strains of 4 h pre-swollen conidia compared to 6 h pre-swollen conidia (Figure 6A and B). Trypacidin might be lost from the conidia upon swelling and this could decrease the uptake by amoeba and, in addition, a decrease in killing. Interestingly, 8 h pre-swollen conidia of the ATCC46645 and DTO303-F3 strains are actually more effective in killing amoebae. This suggests that trypacidin is most likely not involved but other killing mechanisms are functional in these two strains at this time-point, for example via different mycotoxins like gliotoxin or fumagilin (Guruceaga et al., 2018; Scharf et al., 2012). *A. fumigatus* strains lacking fumagillin cause less damage to epithelial cells when compared to the wild-type strain (Guruceaga et al., 2018). It should be mentioned that production of trypacidin is temperature dependent and is high when the fungus is grown at 25°C but very low at 37°C (Hagiwara et al., 2017), the latter being the temperature used in this study to culture the fungal strains. We expect that the amount of trypacidin is low but might vary between the five strains. Further research is required to assess this difference.

Gliotoxin has been shown to be an important determinant for virulence in the *G. melonella* infection model (Reeves et al., 2004). An increase in gliotoxin production by *A. fumigatus* led to a decrease in survival of *G. melonella* larvae. We showed that the virulence of Af293 conidia is lower in *G. melonella* larvae and zebrafish embryos, but also that this strain has less germlings after an 8 h incubation period in CZD medium. The amount of LDH released in the medium by A549 cells is also low after 12 h of co-incubation, which indicates that the slower germination of Af293 could be the reason for the decrease in virulence. Future research should determine whether gliotoxin production and virulence of the strains used in this study correlate as described by (Reeves et al., 2004). Another mycotoxin produced by *Aspergillus* species is fumitremorgin. Af293 contains a mutation in *ftmD* (Afu8g00200), which is part of the fumitremorgin gene cluster and essential for production of fumitremorgin C (Kato et al., 2013). A point mutation replacing arginine by a leucine at position 202 of the protein is responsible for the lack of fumitremorgin C production (Kato et al., 2013, Supplementary Table 6). The absence of this mycotoxin could explain why Af293 is less virulent compared to ATCC46645, CEA10, DTO271-B5 and DTO303-F3.

Previous research showed that Af293 is more sensitive to pH stress when compared to DTO271-B5 and DTO303-F3 when grown in medium at pH 8, but it is more resistant to copper stress as compared to these strains (Valdes et al., 2020). Notably, strains ATCC46645, CEA10, DTO271-B5 and DTO303-F3 have SNPs in the multicopper oxidase *abr1*, when compared to Af293 (Supplementary Table 2). Gene *abr1* is described to have a role in the DHN-melanin synthesis gene cluster (Tsai et al., 1999), but its role in copper stress is still unknown. With the SNPs observed in *abr1* and the observed copper resistance in Af293, this could indicate that *abr1* is important for the fungus to cope with copper stress.

There are no clusters of isolates originating from the environment, invasive or non-invasive infections in the phylogenetic tree that is based on SNPs within the

genome (Figure 10). It is also observed that Af293, CEA10, DTO271-B5 and DTO303-F3 are located in the same cluster, whereas ATCC46645 is located in a separate cluster. Based on the SNPs in the virulence-related genes, Af293 differs most from the other four strains (Figure 9). This shows that the subset of virulence-related genes used are not enough to determine genetic differences and to explain differences in virulence between the strains.

In conclusion, it is shown that differences observed in the four infection models especially depends on the genetic make-up of each *A. fumigatus* strain rather than being derived from an invasive or non-invasive infection. The most striking result is the reduced virulence of Af293 in the different infection systems, which, in part, is explained by reduced initiation of germination.

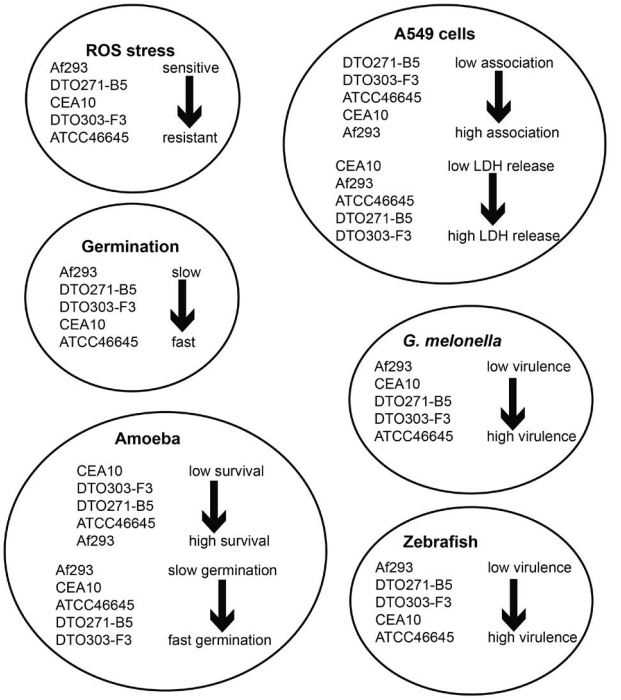

Figure 11. Summary of the main findings. A complete overview can be found in Supplementary Table 5.

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Supplementary data

Supplementary Table 1. Strains used for the assembly of the phylogenetic tree.

Strain ID	Reference
IFM 59355-1	(Hagiwara et al., 2014)
IFM 59355-1	(Hagiwara et al., 2014)
IFM 59355-2	(Hagiwara et al., 2014)
IFM 59355-2	(Hagiwara et al., 2014)
IFM 59356-1	(Hagiwara et al., 2014)
IFM 59356-1	(Hagiwara et al., 2014)
IFM 59356-2	(Hagiwara et al., 2014)
IFM 59356-2	(Hagiwara et al., 2014)
IFM 59356-3	(Hagiwara et al., 2014)
IFM 59356-3	(Hagiwara et al., 2014)
IFM 59361-1	(Hagiwara et al., 2014)
IFM 59361-2	(Hagiwara et al., 2014)
IFM 60237	(Hagiwara et al., 2014)
Af293	(Abdolrasouli et al., 2015)
Af65	(Abdolrasouli et al., 2015)
12-7505446	(Abdolrasouli et al., 2015)
12-7505220	(Abdolrasouli et al., 2015)
09-7500806	(Abdolrasouli et al., 2015)
12-7504652	(Abdolrasouli et al., 2015)
12-7504462	(Abdolrasouli et al., 2015)
12-7505054	(Abdolrasouli et al., 2015)
08-12-12-13	(Abdolrasouli et al., 2015)
08-36-03-25	(Abdolrasouli et al., 2015)
08-31-08-91	(Abdolrasouli et al., 2015)
08-19-02-61	(Abdolrasouli et al., 2015)
08-19-02-30	(Abdolrasouli et al., 2015)
10-01-02-27	(Abdolrasouli et al., 2015)
08-19-02-46	(Abdolrasouli et al., 2015)
08-19-02-10	(Abdolrasouli et al., 2015)
Afu_942/09	(Abdolrasouli et al., 2015)
Afu_1042/09	(Abdolrasouli et al., 2015)
Afu_343/P/11	(Abdolrasouli et al., 2015)
Afu_591/12	(Abdolrasouli et al., 2015)
Afu_124/E11	(Abdolrasouli et al., 2015)
Afu_166/E11	(Abdolrasouli et al., 2015)
Afu_257/E11	(Abdolrasouli et al., 2015)
Afu_218/E11	(Abdolrasouli et al., 2015)
A1163	(Fedorova et al., 2008)
LMB-35Aa	(Paul et al., 2017)
ISSF_21	(Singh et al., 2016)
1F1SW_F4	(Singh et al., 2016)
MO68507EXP	(Lind et al., 2017)
MO54056EXP	(Lind et al., 2017)
MO76959EXP	(Lind et al., 2017)
MO69250EXP	(Lind et al., 2017)
MO79587EXP	(Lind et al., 2017)
MO78722EXP	(Lind et al., 2017)
MO91298SB	(Lind et al., 2017)
MO89263LAB	(Lind et al., 2017)
V130-54	(Ballard et al., 2018)
V130-18	(Ballard et al., 2018)
V130-14	(Ballard et al., 2018)
V130-15	(Ballard et al., 2018)

V157-48	(Ballard et al., 2018)
V157-40	(Ballard et al., 2018)
V157-39	(Ballard et al., 2018)
V157-59	(Ballard et al., 2018)
V157-47	(Ballard et al., 2018)
V157-62	(Ballard et al., 2018)
V157-61	(Ballard et al., 2018)
V157-60	(Ballard et al., 2018)
V157-80	(Ballard et al., 2018)

Supplementary Table 2. Virulence-related genes as described by (Puértolas-Balint et al., 2019), with the type of SNP (low (L), moderate (M) or high (H) impact) indicated per strain.

Function	Gene ID	Gene Name	ATCC46645			CEA10			DTO271-B5			DTO303-F3		
			L	M	H	L	M	H	L	M	H	L	M	H
Thermotolerance	Afu1g03992	thtA	x			x	x		x	x		x		
	Afu3g06450	pmt1	x						x			x		
	Afu5g04170	hsp90												
	Afu8g02750	cgrA												
Resistance to immune response	Afu1g03200	mfsC	x	x		x	x		x	x		x	x	
	Afu1g10380	nrps1	x	x		x	x		x	x				
	Afu1g10390	abcB	x	x		x			x					
	Afu1g12690	mdr4												
	Afu1g13330	arp2												
	Afu1g14330	abcC												
	Afu1g14550	sod3				x								
	Afu1g15490	mfsB	x			x	x		x	x		x		
	Afu1g17250	rodB	x											
	Afu1g17440	abcA		x		x	x		x	x		x	x	
	Afu2g17530	abr2	x			x			x	x		x	x	
	Afu2g17550	ayg1	x	x		x	x		x	x			x	
	Afu2g17600	pksP	x	x			x		x			x	x	
	Afu3g02270	cat1				x			x					
	Afu3g03500	mdr3	x	x		x	x		x	x		x	x	
	Afu3g09690	catA		x			x		x	x				
	Afu3g10830	gstA	x			x			x			x		
	Afu3g12120	ppoC	x	x		x	x		x	x		x	x	
	Afu4g00180	ppoB	x			x			x			x		
	Afu4g10000	mdr2	x	x		x	x		x	x			x	
	Afu4g10770	ppoA	x	x			x			x			x	
	Afu4g11580	sod2												
	Afu4g13390	arpA												
	Afu4g14530	tpcF	x	x		x	x		x	x				
	Afu5g06070	mdr1		x		x	x		x	x		x	x	
	Afu5g09240	sod1												
	Afu5g09580	rodA	x	x		x	x							
	Afu6g03470	fmpD	x	x		x	x					x	x	
	Afu6g03890	catA	x			x			x	x		x		
	Afu6g04360	atrF	x	x		x	x		x	x		x	x	
	Afu6g07210	sod4	x									x		
	Afu6g09930	yap1	x	x		x	x		x					
	Afu6g12522	skn7					x							

	Afu7g00480	abcE	x	x		x	x		x	x		x	x	
	Afu7g05500	gstB				x			x			x	x	
	Afu8g01670	cat2	x			x			x			x		
	Afu8g05710	mfsA	x	x		x	x		x	x		x	x	
Cell wall	Afu1g01380	och4	x			x			x					
	Afu1g04260	ENGL1	x	x		x	x		x	x		x	x	
	Afu1g07690	afpmt2	x											
	Afu1g12600	chsD	x	x			x		x	x				
	Afu1g13280	pmi1												
	Afu1g15440	ags3	x	x		x	x		x			x		
	Afu2g01170	gel1	x	x			x			x			x	
	Afu2g01450	mnn9					x						x	
	Afu2g01870	chsA	x	x								x	x	
	Afu2g05150	mp2	x	x		x	x		x	x		x	x	
	Afu2g05340	gel4	x			x			x			x		
	Afu2g11270	ags2	x	x		x	x		x	x				
	Afu2g12850	gel3	x	x			x							
	Afu2g13440	chsE	x	x		x	x		x	x		x	x	
	Afu2g15910	anp1												
	Afu2g17560	arp2	x						x	x				
	Afu2g17580	arp1												
	Afu3g00910	ags1	x	x		x	x		x	x		x	x	
	Afu3g06690	rho3												
	Afu3g10340	rho2												
	Afu3g12690	glfA	x			x			x			x		
	Afu3g13200	gel6	x	x		x	x		x	x		x	x	
	Afu3g14420	chsG	x	x								x		
	Afu4g03240	mp1	x			x			x			x		
	Afu4g04180	chsB												
	Afu4g06820	ecm33	x				x						x	
	Afu5g00760	chsC		x			x			x			x	
	Afu5g02740	afmnt3	x			x			x			x		
	Afu5g08580	och1	x	x		x	x		x	x		x	x	
	Afu5g10760	mnt1				x								
	Afu5g12160	afmnt2												
	Afu5g14060	rho4	x			x			x			x		
	Afu6g06900	rho1												
	Afu6g11390	gel2	x											
	Afu6g12400	fks1	x	x		x	x		x	x		x	x	
	Afu6g12410	gel7		x			x			x			x	
	Afu6g14040	och2	x			x	x		x			x	x	
	Afu8g02040	och3	x							x				
	Afu8g02130	gel5		x			x							
	Afu8g04500	pmt4	x			x			x			x	x	
	Afu8g05630	chsF		x		x								
Toxins and secondary metabolites	Afu1g14660	laeA												
	Afu2g17540	abr1	x	x		x	x		x	x			x	
	Afu2g17970	fgaFS	x	x									x	
	Afu2g17980	easK	x	x					x					
	Afu2g18000	fgaDH											x	

	Afu2g18010	easM	x									x	x	
	Afu2g18020	fgaAT	x									x	x	
	Afu2g18030	fgaCat	x	x									x	
	Afu2g18040	dmaW		x					x	x			x	
	Afu2g18050	fgaOx1								x		x	x	
	Afu2g18060	fgaMT		x						x			x	
	Afu3g12900	hasB	x	x		x			x			x		
	Afu3g12940	hasF		x		x	x			x		x	x	
	Afu3g12950	hasG	x	x		x	x			x			x	
	Afu4g10460	hcsA	x											
	Afu4g14480	tpcL												
	Afu4g14490	tpcJ	x	x			x		x				x	
	Afu4g14500	tpcI	x	x		x			x			x		
	Afu4g14520	tpcG												
	Afu4g14540	tpcE												
	Afu4g14570	tpcB		x						x			x	
	Afu4g14580	tpcA	x			x			x			x	x	
	Afu4g14770	osc3	x	x		x			x			x		
	Afu4g14780	cyp5081A1	x	x						x				
	Afu4g14790	cyp5081B1	x	x		x	x		x	x		x	x	
	Afu4g14800	sdr1	x							x				
	Afu4g14820	null		x		x			x					
	Afu5g12710	null	x	x		x	x		x	x		x	x	
	Afu5g12720	null	x	x		x	x		x	x		x	x	
	Afu5g12750	null		x										
	Afu5g12760	null	x	x								x	x	
	Afu5g12770	null										x		
	Afu5g12780	null	x			x			x			x		
	Afu5g12790	null	x											
	Afu6g09630	gliZ		x		x			x			x	x	
	Afu6g09640	gliI		x		x	x		x	x		x	x	
	Afu6g09660	gliP	x	x		x	x		x	x		x	x	
	Afu6g09670	gliC	x			x			x					
	Afu6g09690	gliG												
	Afu6g09710	gliA							x	x				
	Afu6g09720	gliN	x						x					
	Afu6g09730	gliF	x				x		x					
	Afu6g09740	gliT												
	Afu8g00190	ftmC	x	x										
	Afu8g00200	ftmD	x	x		x	x		x	x		x	x	
	Afu8g00370	fma-PKS	x	x		x	x		x	x		x	x	
	Afu8g00380	fmaC				x			x					
	Afu8g00390	fmaD		x						x			x	
	Afu8g00400	null	x	x					x	x		x	x	
	Afu8g00410	metAP		x			x			x			x	
	Afu8g00420	fumR		x			x			x			x	
	Afu8g00430	null		x										
	Afu8g00440	psoF				x			x			x		
	Afu8g00460	fpaI	x			x						x		
	Afu8g00470	fmaE	x			x			x			x		

	Afu8g00490	Fma-KR	x	x		x	x					x	x	
	Afu8g00500	null	x	x		x	x		x	x		x	x	
	Afu8g00510	fmaG		x		x				x		x	x	
	Afu8g00540	nrps14	x	x		x	x		x	x		x	x	
	Afu8g00550	psoC		x										
	Afu8g00570	null				x						x		
	Afu8g00580	psoE		x			x						x	
	Afu8g00520	fma-TC	x			x						x		
Allergens	Afu1g05770	exg12	x	x			x			x			x	
	Afu1g06830	aspf26												
	Afu1g09470	aspfAT	x											
	Afu1g14560	msdS	x			x			x					
	Afu1g16190	aspf9		x			x			x			x	
	Afu2g00760	aspfPL	x	x		x	x		x	x		x	x	
	Afu2g03720	aspf11	x			x			x			x		
	Afu2g03830	aspf4	x	x		x	x		x	x		x	x	
	Afu2g10100	aspf8	x			x			x			x		
	Afu2g11260	luA	x			x	x		x					
	Afu2g11850	aspf23												
	Afu2g12630	aspf13												
	Afu2g15430	AspfSXR	x											
	Afu3g00590	aspHS	x	x								x	x	
	Afu3g07430	aspf27				x			x			x		
	Afu3g14680	aspfLPL3	x			x			x			x		
	Afu4g01290	csn	x	x		x			x			x		
	Afu4g06670	aspf7	x			x			x			x		
	Afu4g09580	aspf2												
	Afu5g02330	aspf1	x	x		x	x		x	x		x	x	
	Afu5g03520	aspfPUP		x		x			x					
	Afu5g11320	aspf29	x			x								
	Afu6g02280	aspf3				x			x					
	Afu6g03620	mreA	x	x		x	x		x	x		x		
	Afu6g04920	fdh	x	x		x	x		x	x		x	x	
	Afu6g06770	aspf22				x				x				
	Afu6g10300	aspf28												
	Afu7g05740	null												
Nutrient uptake	Afu1g01550	zrfA	x	x		x	x		x	x		x	x	
	Afu1g09280	ptcB					x							
	Afu1g10080	zafA					x			x				
	Afu1g16950	pig-a				x								
	Afu1g17200	sidC	x	x		x	x		x	x		x	x	
	Afu2g03860	zrfB	x			x			x			x		
	Afu2g04010	tpsB	x	x		x	x		x	x		x	x	
	Afu2g05730	mirC	x			x			x			x		
	Afu2g07680	sidA	x											
	Afu2g08360	pyrG												
	Afu2g09030	dppV		x			x			x			x	
	Afu3g03400	sidF												
	Afu3g03420	sidD	x	x		x	x		x	x		x	x	
	Afu3g03640	mirB	x			x			x			x		

	Afu3g03650	sidG	x			x			x	x		x		
	Afu3g05650	orlA	x	x		x			x			x		
	Afu3g09820	dvrA												
	Afu3g11400	pep2											x	
	Afu3g11970	pacC	x	x		x	x			x			x	
	Afu4g07040	ctsD												
	Afu4g08720	plb1				x								
	Afu4g09320	dppIV	x			x			x					
	Afu4g09560	zrfC	x			x			x			x		
	Afu4g11800	alp1												
	Afu4g12470	cpcA	x	x		x			x			x	x	
	Afu4g13750	mep20		x										
	Afu5g01340	plb2		x		x								
	Afu5g03790	fetC	x			x			x			x		
	Afu5g03800	ftrA												
	Afu5g05480	rhbA				x			x					
	Afu5g08570	pkaC2	x	x		x	x			x			x	
	Afu5g08890	lysF	x	x		x	x			x			x	
	Afu5g09210	alp2	x						x			x		
	Afu5g11260	sreA												
	Afu5g13300	pep1	x											
	Afu6g01970	areA	x	x			x			x				
	Afu6g03590	mcsA				x								
	Afu6g04820	pabA	x	x		x	x			x			x	
	Afu6g12950	tpsA	x			x								
	Afu7g04910	Null	x	x		x	x		x	x		x	x	
	Afu7g04930	pr1		x			x			x				
	Afu7g05930	mepB	x	x		x								
	Afu8g02760	amcA												
	Afu8g07080	mep	x	x		x			x	x			x	
Signalling and regulation	Afu1g05800	mkk2	x			x			x			x		
	Afu1g06900	crzA		x		x			x					
	Afu1g12930	gpaB				x								
	Afu1g12940	sakA												
	Afu1g13140	gpaA												
	Afu1g15950	pbs2	x			x			x	x		x		
	Afu2g00660	tcsB	x	x		x	x			x			x	
	Afu2g01260	srbA		x			x			x		x	x	
	Afu2g07770	rasB												
	Afu2g12200	pkaC	x				x							
	Afu2g12640	gprD	x	x			x		x	x				
	Afu2g13260	medA	x	x		x	x			x		x	x	
	Afu3g05900	ste7	x	x		x	x		x			x		
	Afu3g10000	pkaR				x			x	x		x		
	Afu3g11080	bck1	x	x		x	x		x	x		x	x	
	Afu3g11250	ace2	x	x		x	x		x	x		x	x	
	Afu4g13720	mpkA										x		
	Afu5g06420	steC/ste11	x			x			x				x	
	Afu5g08420	sho1	x											
	Afu5g09100	mpkC	x	x		x	x		x	x		x	x	

	Afu5g09360	calA				x							
	Afu5g11230	rasA											
	Afu5g12210	sfaD	x										
	Afu6g08520	acyA	x	x			x		x	x			
	Afu6g10240	fos-1	x	x		x	x			x		x	x
	Afu6g12820	mpkB	x			x			x	x		x	
	Afu7g04800	gprC	x										

*null = no gene name assigned.

Supplementary Table 3. Shared SNPs with the location and amino acid change in *fma-PKS* (Afu8g00370) in the ATCC46645, CEA10, DTO271-B5 and DTO303-F3 strains.

Reference (Af293)	Alternative	Location	Amino acid change
A	T	2335	Cysteine → Serine
T	C	2082	Asparagine → Serine
C	T	1624	Valine → Isoleucine
T	A	746	Tyrosine → Phenylalanine
A	G	641	Serine → Proline

Supplementary Table 4. Base pair (BP) change, location and amino acid (AA) substitution in the genes with SNPs shared between ATCC46645 and DTO303-F3. Shared SNP's are displayed in bold.

Gene ID	Gene name	Group	ATCC46645			DTO303-F3		
			BP Change	Location	AA change	BP Change	Location	AA change
Afu6g 07210	*sod4*	Resistance to immune response	**G → A**	**30**	**Arg → Arg**	**G → A**	**30**	**Arg → Arg**
						A → C	54	Thr → Pro
						T → C	95	Ile → Ile
						G → A	115	Leu → Leu
						T → G	128	Val → Gly
						A → C	146	Ser → Ser
						A → T	204	Thr → Ser
						C → A	242	Ala → Asp
						A → C	243	His → Pro
						A → C	249	Asn → His
						A → C	249	Asn → Thr
Afu2g 01870	*chsA*	Cell wall	**G → C**	**734**	**Leu → Phe**	A → C	906	Val → Val
			G → T	**645**	**Ala → Ala**	T → C	879	Thr → Thr
			G → A	**442**	**Ala → Ala**	G → T	857	Val → Val
			C → T	**331**	**Ala → Ala**	T → G	780	Lys → Asn
			A → G	**229**	**Phe → Phe**	**G → A**	**734**	**Leu → Phe**
			CATC → GACT	**116**	**Asp Asp → Ser His**	**G → C**	**645**	**Ala → Ala**
			T → C	**46**	**Val → Val**	**G → A**	**442**	**Ala → Ala**
			C → T	**33**	**Arg → Gln**	A → C	394	Leu → Leu
			C → A	**22**	**Gln → His**	**C → T**	**331**	**Ala → Ala**
						A → G	**229**	**Phe → Phe**
						G → A	192	Pro → Ser
						G → T	186	Leu → Met
						A → C	171	Trp → Gly
						T → G	156	Asp → Ala
						A → C	117	Asp → Glu
						CATC → GACT	**116**	**Asp Asp → Ser His**

						G → T	110	Pro → Thr
						T → G	109	Glu → Ala
						G → T	88	Pro → His
						T → G	55	Ser → Arg
						T → C	**46**	**Val → Val**
						C → T	**33**	**Arg → Gln**
						C → A	**22**	**Gln → His**
Afu2g 17970	*fgaFS*	Toxins and secondary metabolites	**G → A**	**6**	**Leu → Leu**	**G → A**	**6**	**Leu → Leu**
			G → C	**21**	**Asp → His**	**G → C**	**21**	**Asp → His**
			G → A	**152**	**Lys → Lys**	C → T	66	Asp → Asp
			T → C	**197**	**Gly → Gly**	T → A	68	Ile → Ile
						G → A	**152**	**Lys → Lys**
						T → C	**197**	**Gly → Gly**
						G → C	271	Ser → Thr
Afu2g 18010	*easM*	Toxins and secondary metabolites	**C → A**	**108**	**Arg → Arg**	**C → A**	**108**	**Arg → Arg**
			G → A	**467**	**Gln → Gln**	G → A	114	Ala → Ala
						G → T	136	Gly → Val
						T → C	161	Asp → Asp
						T → G	233	Ile → Arg
						A → C	237	Ile → Leu
						A → C	253	Ser → Arg
						G → T	260	Glu → Asp
						A → C	332	Asp → Ala
						T → G	334	Ile → Ser
						T → C	336	Ile → Thr
						G → A	**467**	**Gln → Gln**
						G → T	476	Val → Phe
						C → A	477	Ala → Glu
Afu2g 18020	*fgaAT*	Toxins and secondary metabolites	**C → G**	**364**	**Leu → Leu**	G → T	27	Ser → Ser
						C → T	51	Ser → Phe
						A → C	66	Ile → Leu
						A → T	69	Lys → Asn
						A → C	85	Ile → Leu
						A → C	184	Asn → Thr
						T → G	265	Leu → Val
						C → G	**364**	**Leu → Leu**
						G → T	377	Gly → Stop
						C → T	380	Ala → Ala
						A → G	458	Lys → Arg
Afu2g 18030	*fgaCAT*	Toxins and secondary metabolites	**T → C**	**97**	**Leu → Leu**	A → C	487	Tyr → Asp
			A → G	**66**	**Pro → Pro**	T → C	466	Lys → Arg
			T → G	**37**	**Glu → Ala**	A → C	432	Leu → Arg
						C → CCGT	391	Asp insertion
						T → G	363	Asp → Ala
						T → G	253	Asp → Ala
						T → G	251	Asp → Ala
						G → A	236	Arg → Cys
						A → C	213	Gly → Gly
						A → C	207	Ser → Ala
						A → C	199	Phe → Val
						T → G	174	Asn → Thr
						G → T	169	Arg → Arg

						TTT → ATA	131	Lys → Tyr
						C → A	129	Ala → Ser
						T → A	128	Met → Leu
						C → A	127	Gly → Trp
						C → A	126	Lys → Asn
						T → A	126	Lys → Stop
						C → A	125	Leu → Phe
						G → A	124	Asp → Asp
						C → A	124	Asp → Tyr
						C → A	123	Arg → Ile
						T → A	123	Arg → Stop
						C → T	122	Met → Ile
						T → A	122	Met → Leu
						C → A	103	Thr → Thr
						T → C	**97**	**Leu → Leu**
						A → G	**66**	**Pro → Pro**
						A → G	54	Val → Ala
						T → G	**37**	**Glu → Ala**
Afu5g 12760		Toxins and secondary metabolites	**T → G**	**278**	**His → Pro**	A → T	422	His → Gln
			C → T	**200**	**Arg → Arg**	G → A	410	Arg → Cys
			C → T	**9**	**Arg → Lys**	G → A	359	Ala → Val
						A → C	358	Cys → Trp
						T → G	292	Thr → Pro
						T → G	282	Tyr → Ser
						T → G	**278**	**His → Pro**
						A → C	250	Asn → Lys
						T → G	228	Lys → Asn
						C → T	**200**	**Arg → Arg**
						G → A	114	His → Tyr
						TA → AT	86	Met → Leu
						T → C	35	Asn → Asp
						T → C	33	Ser → Gly
						C → T	31	Ala → Thr
						C → T	**9**	**Arg → Lys**
Afu3g 00590	*aspHS*	Allergens	**A → G**	**51**	**Val → Val**	T → G	122	Asn → Thr
			G → T	**27**	**Gln → Lys**	A → C	116	Val → Gly
						A → G	**51**	**Val → Val**
						G → T	**27**	**Gln → Lys**

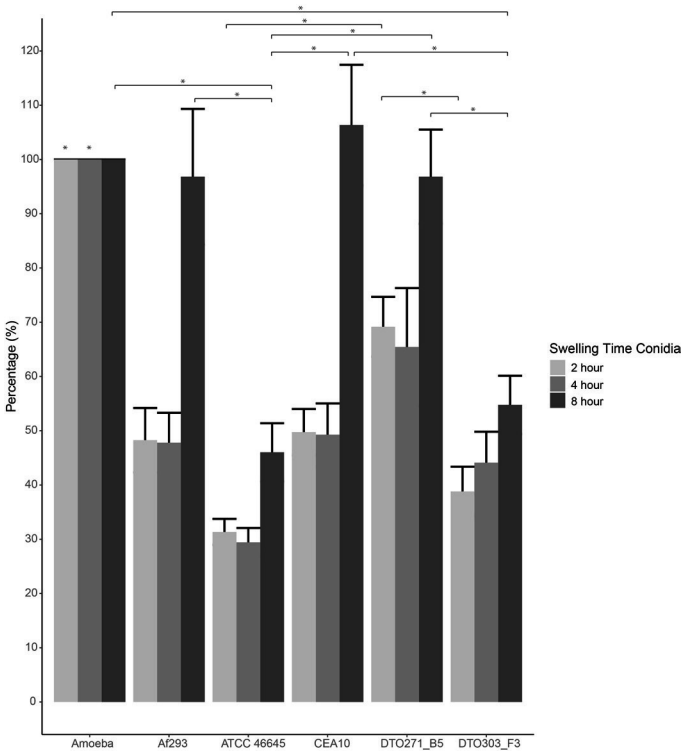
Supplementary Table 5. Summary of comparison of *A. fumigatus* strains

	Af293	**ATCC46645**	**CEA10**	**DTO271-B5**	**DTO303-F3**
Stress					
Peroxide (mm)	32	26	27	29	26
Menadione (mm)	37	27	29	29	28
A549	**Af293**	**ATCC46645**	**CEA10**	**DTO271-B5**	**DTO303-F3**
Association (conidia/cell)	0.19	0.14	0.17	0.11	0.12
Internalization (%)	82	77	77	77	80
Germination (%)	68	87	75	86	85
IL-8 release 12 h	8.2	18.2	6.6	7.8	8.8
LDH release 12 h	8.1	8.4	6.2	10.1	14.6
Apoptotic/necrotic cells 12 h	*Similar for all strains*				

Protostelium aurantium	**Af293**	**ATCC46645**	**CEA10**	**DTO271-B5**	**DTO303-F3**
Uptake (4.5h swollen) (%)	16	35	30	28	32
Fungal survival (%)					
4 h swollen	88	56	67	60	52
6 h swollen	62	47	28	36	28
8 h swollen	71	96	127	90	80
Germination with amoeba (% hyphae)					
4 h swollen	0	0.5	0	11	2
6 h swollen	1	16	2	47	29
8 h swollen	0.8	42	0	49	63
Germination no amoeba (% germlings)					
4 h swollen	0	0	0.2	0.4	0.8
6 h swollen	0.8	10	3	2	4
8 h swollen	3	54	39	20	25
Survival of amoeba (%)					
4 h swollen	48	31	50	69	39
6 h swollen	48	29	49	65	44
8 h swollen	97	46	106	97	55
Galleria melonella	**Af293**	**ATCC46645**	**CEA10**	**DTO271-B5**	**DTO303-F3**
Survival *Galleria melonella* (%)					
$1*10^5$ conidia mL^{-1}	87	73	73	53	33
$1*10^6$ conidia mL^{-1}	80	47	40	40	13
$1*10^7$ conidia mL^{-1}	60	20	53	33	27
Survival conidia	*Similar for all strains*				
Zebrafish	**Af293**	**ATCC46645**	**CEA10**	**DTO271-B5**	**DTO303-F3**
Survival zebrafish embryo's (%)	92	82	66	79	79

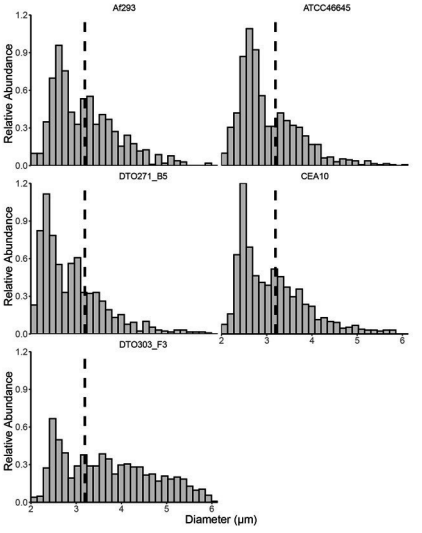
Supplementary Table 6. Base pair (BP) change, location and amino acid (AA) substitution in the *FtmD* (Afu8g00200) gene. The SNPs described by (Kato et al., 2013) is highlighted in bold.

BP change	**Location**	**AA change**	**Strain**			
			ATCC46645	**CEA10**	**DTO271-B5**	**DTO303-F3**
A → C	14	Ile → Leu			X	X
A → C	20	Asp → Ala			X	X
C → T	75	Ile → Ile			X	X
G → A	83	Ala → Thr			X	X
A → C	109	Ser → Arg			X	X
A → C	132	Thr → Pro			X	X
C → T	141	Pro → Leu			X	X
A → C	145	Lys → Thr			X	X
T → C	160	Arg → Arg	X	X	X	X
T → G	175	Val → Gly			X	X
T → G	**202**	**Leu → Arg**	**X**	**X**	**X**	**X**
T → C	233	Ile → Thr	X	X	X	X
C → T	249	Ala → Val			X	X
A → C	271	Met → Leu			X	X
T → G	299	Leu → Arg			X	X
C → T	311	Arg → Arg			X	X
T → G	314	Val → Gly			X	X



Supplementary figure 1. Amoeba survival after 18 hours incubation with 4 (light grey), 6 (grey) or 8 (dark grey) hour swollen conidia. Bar represent 3 individual experiments ± SE.

A



B

Strain	Diameter (µM)
Af293	2.67
ATCC46645	2.67
CEA10	2.53
DTO271_B5	2.4
DTO303_F3	2.53

Supplementary figure 2. Relative abundance of the diameter (µM) of the conidia measured with the coulter counter (A), dashed line represents the mean of all the measured particles. The diameter of the bin with the most measurements (highest frequency) per strain (B).

6

Summary and General Discussion

Esther M. Keizer

Introduction and scope

The *Aspergillus* genus currently consists of 446 species (Houbraken et al., 2020), which are common and abundant fungal species worldwide. The environmental growth conditions of *Aspergillus* species are diverse. For instance, growth is observed at a wide temperature (6 - 55 °C) and pH (pH 1.5 - 12) range as well as at relatively low humidity and oxygen level (Krijgsheld et al., 2013). *Aspergilli* are saprotrophic fungi that grow on organic debris (Latgé, 1999). Their asexual reproductive structures, known as conidia, germinate when exposed to proper environmental conditions (such as substrate, temperature and humidity). As a result, branching hyphae are formed that establish an interconnected network called mycelium (Adams et al., 1998). Hyphae in a mycelium will specialize, forming secretion, resistor and aerial hyphae (Champe & Simon, 1992; Tegelaar et al., 2020). Part of the aerial hyphae differentiate into conidiophores that form conidia. These conidia can form new mycelia after their dispersal (Yu et al., 2006). *Aspergillus* conidia are easily dispersed via air, water droplets and other vectors such as insects (Teertstra et al., 2017). On average we inhale several hundred *Aspergillus fumigatus* conidia per day (Mullins et al., 1984), as there are up to 80 and 4 10^7 of these conidia per m^3 of regular air or air close to composting facilities, respectively (Wéry, 2014).

Upon inhalation, *Aspergillus* conidia can cause allergic reactions and lung infections (Figure 1, **Chapter 1**). *A. fumigatus* and to a lesser extent *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans*, can cause invasive and non-invasive infections (Stevens et al., 2000). Hyper-sensitivity towards *A. fumigatus* allergens can occur in patients with asthma or cystic fibrosis (Agarwal, 2009). A more severe infection in immunocompetent patients is an aspergilloma (fungal ball). This non-invasive infection is caused by proliferation of the fungus in lung cavities and mostly occurs in patients with lung lesions, for example due to a previous pulmonary tuberculosis infection (Denning et al., 2016). A chronic pulmonary aspergillosis, typified by chronic inflammation and fibrosis, can also occur in immunocompetent patients (Latgé & Chamilos, 2019). The most severe *Aspergillus* infections occur in immunocompromised patients and are known as invasive pulmonary aspergillosis. This infection starts by germination of conidia in the lung. The resulting hyphae cause damage and inflammation, and eventually the fungus can disseminate into other organs (Zmeili & Soubani, 2007).

Lung epithelial cells represent the first line of the host defence against a fungal infection. It consists of basal cells, ciliated cells and cells that produce mucus and antimicrobial components, such as surfactant protein D, that are part of the innate defence at the lung lining together with phagocytic cells (Ordonez et al., 2019). Deeper in the lung is the alveolar epithelium. which consists of type I and type II cells. Type I cells are involved in gas exchange and cover 95 % of the epithelial surface. They are in close contact with type II cells that secrete surfactant. Conidia can be taken up by these epithelial cells and end up in acidic phagolysosomes, which kill the conidia upon germination (Wasylnka & Moore, 2002). Germination of the conidia outside the epithelial cells leads to the release of antimicrobial peptides and interleukin-8 (IL-8) (Balloy et al., 2008; Osherov, 2012). Other cells involved in the host defence against fungal infections are macrophages and neutrophils. Macrophages kill conidia upon germination in the acidic phagolysosomes, just as occurs in epithelial cells. In macrophages this is the result of the production of NADPH-oxidase dependent reactive oxygen species (ROS) (Philippe et al., 2003).

LC3-associated phagocytosis promotes fungal killing by macrophages upon germination (Akoumianaki et al., 2016). Neutrophils have several mechanisms to kill conidia, for example by triggering the apoptosis program in the conidia by ROS release (Shlezinger et al., 2017).

Fungal infections are treated with polyenes, azoles, flucytosine and echinocandins (Dudakova et al., 2017; Geißel et al., 2018; Gray et al., 2012; Vermes et al., 2000). The former two classes target ergosterol (synthesis) in the plasma membrane, while the latter class targets synthesis of β(1,3)-glucan in the fungal cell wall. This glucan is one of the main components of the cell wall of *A. fumigatus* together with α(1,3)-glucan, mannan, galactomannan, and chitin (Latgé et al., 2017) (Figure 2, **Chapter 1**). Conidia are covered with additional layers of the pigment 1,8-dihydroxynaphthalene (DHN)-melanin as well as the rodlet hydrophobins RodA and RodB and the stealth protein CcpA (Jahn et al., 1997; Paris et al., 2003a; Voltersen et al., 2018). These melanin and protein layers shield the immunogenic cell wall polysaccharides from immune recognition, which strongly reduces immune activating pathways (Aimanianda et al., 2009; Luther et al., 2007; Voltersen et al., 2018). Yet, these layers do not perfectly cover the cell wall polysaccharides because some patches of β-glucan can be observed at the surface of dormant spores (Steele et al., 2005) (Supplementary Figure 5, **Chapter 2**). Germination of the conidia leads to the breakdown of the outer pigment and protein layers disclosing more immunogenic structures, thereby enabling interactions with immune cells (Hohl et al., 2005; Luther et al., 2007; Voltersen et al., 2018). Both resting and germinating conidia can be internalized in phagocytic and epithelial cells. DHN-melanin was reported to reduce phagocytosis by macrophages and to block acidification of the phagolysosome, thus preventing killing of these spores (Amin et al., 2014; Escobar et al., 2016; Thywißen et al., 2011). In addition, DHN-melanin inhibits killing of conidia by macrophages via PI3-Akt pathway activation, inhibition of caspase activation, and by mitochondrial cytochrome c release (Volling et al., 2011). Together, DHN-melanin has an important role in survival of conidia when exposed to the immune system.

Knowledge of virulence mechanisms of *A. fumigatus* is increasing, but still a lot is unknown about the interaction of *A. fumigatus* with its host at the molecular level. In this Thesis I studied the interaction of *A. fumigatus* conidia with epithelial cells to identify molecules from the host and the fungus that play a role in the early steps of the infection process of *A. fumigatus*. In addition, I investigated if *Aspergillus* is able to produce immune evasive molecules that can play a role in infection. Furthermore, I addressed the heterogeneity of the pathogenic potential of *A. fumigatus* isolates in context of different fungal infection models.

Role of extracellular molecules in infection

Conidia that enter the lung will attach to and be internalized by the epithelial cells. **Chapter 2** describes the role of the EphA2 receptor in the association and internalization of conidia with type II A549 lung epithelial cells. Inhibition of the EphA2 receptor with an EphA2-specific antibody or the small molecule dasatinib did not alter the association of dormant conidia but did reduce the internalization by 50 %. This was in line with the role of this receptor in the pathogenic dimorphic fungus *Candida albicans* (Swidergall et al., 2018). EphA2 recognizes fungal β(1,3)-glucan (Swidergall et al., 2018). Yet, no correlation was found between internalization and the increased exposure of this glucan in swollen conidia. In line with these results,

deletion of the hydrophobin RodA, which increases exposure of β(1,3)-glucan (Valsecchi et al., 2019), also did not promote internalization (**Chapter 2**). This suggests that the patches of glucan that are exposed at the surface of the conidia are sufficient for recognition by EphA2. The C-type lectin receptor Dectin-1 also recognizes β(1,3)-glucan in the fungal cell wall and also has a role in the internalization of conidia of *A. fumigatus* (Brown & Gordon, 2001; Han et al., 2011). Dual inhibition of Dectin-1 and EphA2 showed an additive effect on reduced internalization of conidia but still did not abolish internalization completely. This implies that additional receptors are involved in internalization of conidia. Such receptors may recognize β(1,3)-glucan but may also interact with other cell wall components. MelLec may be involved in internalization by recognizing DHN-melanin (Stappers et al., 2018), while other receptors may recognize the rodlet proteins RodA and RodB or the stealth protein CcpA (Figure 2, **Chapter 1**). Since patches of β-glucan are observed at the surface of resting conidia (Steele et al., 2005), mannan and / or α(1,3)-glucan may also be exposed to some extent at the surface of resting conidia. Therefore, receptors may also recognize these molecules. Furthermore, 3 - 9 % of the wild-type *A. fumigatus* conidia expose glucosamine-containing components at the conidial surface (Bayry et al., 2014). It cannot be excluded that these components are recognized by receptors involved in internalization, but it should be noted that this low-level exposure of glucosamine-containing components on the surface of wild-type conidia was not sufficient to stimulate dendritic cells.

Interestingly, deletion of gene *pksP* that is essential for DHN-melanin production reduced internalization of conidia into type II A549 lung epithelial cells and shifted internalization to an EphA2-independent mechanism (**Chapter 2**). The role of DHN-melanin in EphA2-dependent internalization has not been described before. Upon germination, conidia increase in size and loose the DHN-melanin layer, which leads to recognition by the immune system of the host. The reduced internalization of conidia without DHN-melanin could promote immune recognition of germinating conidia because of the reduced uptake of these spores by for instance epithelial cells.

An EphA2-dependent upregulation of the immune response was observed for *C. albicans* evidenced by an IL-8 increase in an epithelial cell culture (Swidergall et al., 2018). **Chapter 2** shows that upon infection with *A. fumigatus* conidia no increase in IL-8 secretion is observed by type II A549 cells, and also no role for the EphA2 receptor in the immune response could be established. The only increase in IL-8 secretion by the A549 cells was observed after infection with conidia lacking DHN-melanin or the RodA protein with a high fungal load (10 conidia / cell). This supports the proposed downregulation of the immune response by *A. fumigatus* (Escobar et al., 2018). Yet, results in **Chapter 2** contrast other studies that do show upregulation of IL-8 upon infection with *A. fumigatus* (Chen et al., 2015; Oosthuizen et al., 2011). This difference could be explained by the removal of non-associated conidia after 2 hours of infection in **Chapter 2**, meaning that the increase in IL-8 secretion is mostly due to extracellular germination of conidia. Next to the absence of an increase in IL-8 secretion upon infection, no cell damage was detected by measuring by lactate dehydrogenease (LDH) release (**Chapter 2**), even though hyphae escaping the epithelium are observed (Escobar et al., 2018). This suggests that hyphae escape the epithelium via non-lytic exit (Seidel et al., 2020). It should be noted that I did measure LDH release under similar conditions in **Chapter 5**. The amount of LDH released in the culture medium is measured colorimetrically at 490

nm monitoring the reduction of NAD to NADH (Decker & Lohmann-Matthes, 1988). A drawback of this assay is the interference of fetal bovine serum (FBS), present in the culture medium, which results in high background readings (Aslantürk, 2018). This limits the assay to serum-free conditions, thereby limiting the culture time. The discrepancy in results of the LDH assay in **Chapter 2** and **5** shows that drawing conclusions from an LDH assay is difficult. Therefore, cell damage should be measured with different methods, such as dual acridine orange and ethidium bromide staining used in **Chapter 5**.

A role for DHN-melanin in conidial protection against peroxide has been described (Jahn et al., 1997; Sugareva et al., 2006). This peroxide-protective role is a well-established paradigm in the field, illustrated by the 240 and 66 references to these articles, respectively. This paradigm is based on the observation that an *A. fumigatus* UV mutant strain lacking DHN-melanin shows higher growth reduction in the presence of peroxide when compared to a wild-type strain. Notably, **Chapter 3** describes that this protection is not due to the absence of DHN-melanin since complementation of the defective *pksP* gene with a wild type version restored pigmentation, but did not restore the resistance towards hydrogen peroxide. Therefore, an additional mutation must be present in the *pksP* deletion strain. Genome sequencing of the wild-type, the UV-mutant and the *pksP* complementation strain revealed a mutation, changing a lysine to a glutamic acid, in the *cat1* gene encoding a mycelial catalase. Deletion of this gene reduces hydrogen peroxide resistance of mycelium but not of spores (Paris et al., 2003b). Together, these results indicate that the increase in peroxide sensitivity of the UV-mutant was due to a mutation in the *cat1* gene. To support this, I made a *pksP* deletion strain in a different *A. fumigatus* background. This CEA10 deletion strain lacked DHN-melanin, but as expected, did not show increased peroxide sensitivity. Furthermore, deletion of DHN-melanin in *Penicillium roqueforti* also did not alter sensitivity towards peroxide (**Chapter 3**). In addition, deletion of *abr2* of *A. fumigatus*, which is downstream in the DHN-melanin synthesis pathway when compared to *pksP*, did alter the colour of conidia, but did not affect peroxide sensitivity (Sugareva et al., 2006). All these results combined show that DHN-melanin does not protect conidia against hydrogen peroxide stress.

In general, melanins are described as molecules that protect against many stresses including oxidative compounds like peroxide (Cordero & Casadevall, 2017). This role must be refined based on my findings that DHN-melanin does not protect against peroxide and superoxides (**Chapter 3**). In contrast, deletion of genes involved in L-DOPA melanin synthesis in *Cryptococcus neoformans* and *A. nidulans* does increase peroxide sensitivity (Jahn et al., 2000; Wang & Casadevall, 1994). This difference in protection by the different types of melanin can be explained by their structure (Supplementary Figure 4, **Chapter 3**). In contrast to DHN-melanin, L-DOPA melanin contains a dihydroquinone and an indole group that are able to quench ROS radicals (Horstman et al., 2002; Jacobson, 2000). It was proposed that the reactive groups of L-DOPA melanin in fact quench radicals that result from the conversion of peroxide to hydroxyl radicals with transition metals via the Fenton reaction (Jacobson, 2000). It will be interesting to assess whether fungi protect themselves to hydroxyl radicals, for example with indole-containing secondary metabolites such as fumigaclavine A and fumitremorgin C (Tamiya et al., 2015). Their importance is suggested from the fact that all 71 tested *A. fumigatus* isolates produced these indole-containing secondary metabolites. In addition, strain Af293

which has an inactive fumitremorgin C gene (Kato et al., 2013) was more sensitive to peroxide and superoxides than 4 other strains that were tested (**Chapter 5**).

Chapter 4 describes secreted molecules of *A. niger* and *A. fumigatus* that compete for binding with antibodies to cell-surface human cellular receptors present on immune cells. The production of these molecules is inhibited by the global regulator of secondary metabolism LaeA since inactivation of its encoding gene induced their production. I thus propose that the molecules competing for binding are secondary metabolites. This is supported by the findings that these molecules are smaller than 3 kDa, are heat and proteinase K insensitive and that removal of proteins by acetone precipitation does not abolish binding activity of the supernatant (**Chapter 4**). The fact that ethyl acetate did not extract the activity from the culture supernatant indicates that the binding molecules have a hydrophilic nature.

Medium acidification affects the production of part of the molecules competing for binding. Deletion of *laeA* or the oxaloacetate hydrolase gene *oahA* resulted in absence of medium acidification and the production of molecule(s) competing for binding to CD181. The molecule(s) competing for binding to CD182 and CD88 are only produced in the *laeA* deletion strain, indicating that the secretion of these compounds is LaeA but not pH dependent (**Chapter 4**).

Secondary metabolites can be made via the mevalonate, shikimic acid, PKS and NRPS pathways. Gene deletions could reveal which pathways are involved in the production of the molecules that compete with receptor binding. For instance, one could delete the terpene cyclase gene that is essential for the production of volatile terpenes in *A. fumigatus* via the mevalonate pathway (Heddergott et al., 2014; Keller et al., 2005). One could also delete one of the seven genes needed for the production of aromatic secondary metabolites via the shikimic acid pathway (Choera et al., 2018) or gene *pptA* that is required for the synthesis of secondary metabolites via the PKS and NRPS pathway (Johns et al., 2017). Inactivation of the latter gene that encodes a 4'phosphopantetheinyl transferase (PPTase) in a *laeA* deletion background was done to show that the binding secondary metabolites are produced by one or both of these two pathways. However, the addition of siderophore medium that is needed for the *pptA* deletion strain to grow (Oberegger et al., 2003) abolished the production of the molecules competing for binding, even in the Δ*laeA* strain with the *pptA* gene still being present (**Chapter 4**). The siderophore medium is obtained by growing an *A. niger* wild-type strain on medium supplemented with glutamine and lacking iron and removing the *A. niger* mycelium before use by filtration (Márquez-Fernández et al., 2007). Possibly, deletion of *laeA* leads to iron limitation conditions that in turn induces the production of the molecules that compete with binding to receptor molecules. The addition of spent medium could reverse the iron limiting conditions and therefore inhibits the production of the molecules competing for receptor binding. As a next step, synthetic siderophores may be added to the medium instead of siderophores contained in spent medium of *A. niger*. It has already been shown that *E. coli* is able to recognize and use such synthetic siderophores (Gaspar et al., 1999). Alternatively, strains may be grown in the presence of citrate. It has been shown that the de-protonated form of this organic acid is also used by *A. niger* for iron uptake. Notably, the production of citric acid is lacking in *laeA* deletion strains (Niu et al., 2015; Odoni et al., 2017).

Heterogeneity in virulence between *A. fumigatus* isolates

Differences in virulence have been observed between *C. albicans* isolates. A widely used lab strain of this dimorphic fungus deviates in macrophage response and in cell wall composition when compared to clinical isolates (Gerwien et al., 2020). The frequently used isolates Af293 and CEA10 of *A. fumigatus* also show differences in virulence. Af293 has a lower low-oxygen fitness than CEA10 (Kowalski et al., 2016), which leads to reduced biofilm formation and development (Kowalski et al., 2020). It has also been described that CEA10 grows faster than Af293 in a zebrafish infection model and that this results in faster clearance by the host immune system (Rosowski et al., 2018), thereby making it less virulent. Another difference between CEA10 and Af293 is that conidia of CEA10 associate better to type II A549 epithelial cells but are internalized with a lower efficiency compared to conidia of Af293 (**Chapter 2**). **Chapter 5** compares the virulence of *A. fumigatus* isolates from invasive human infections (Af293, ATCC46645 and CEA10) and non-invasive canine sino-nasal infections (DTO271-B5 and DTO303-F3) in different infection models. Results show that Af293 has the lowest resistance to H_2O_2 and superoxides and has the lowest germination rate in medium and in co-incubation with amoebae. Af293 was also the least virulent strain in *G. melonella* and zebrafish, whereas ATCC46645 and CEA10 were the most virulent, respectively. No differences in virulence of the strains were observed in the A549 epithelial cells model. These results indicate that delayed germination of Af293 conidia causes it to be less virulent in the *G. melonella* and zebrafish models and leads to better survival after co-incubation with amoebae. Alternatively, the defect in the hypoxia response in Af293 (Kowalski et al., 2016) could be the reason why this strain is less virulent in the *G. melonella* and zebrafish models. It may also be that clinical isolates such as Af293, CEA10 and ATCC46645 each have accumulated mutations in the host, favouring the survival in the niche from which they have been isolated. Indeed, *A. fumigatus* strains derived from canine sino-nasal infections show mutations that favour the survival of the fungus in the sinus (Valdes et al., 2020). On the other hand, passing and culturing in the lab on rich medium may also have selected for mutations favouring growth in laboratory conditions.

A549 lung epithelial cells were used as a model for lung epithelial cells in **Chapter 2** and **5**. A549 cells are derived from a lung carcinoma and therefore represent an immortalized cell line (Lieber et al., 1976). It is known that A549 cells do not show all characteristics of type II epithelial cells, for example, they do not secrete certain proteins that are normally present in the surfactant released by type II cells (Mao et al., 2015). It has also been shown that the culture method of A549 cells affects the resemblance of A549 cells to type II cells found in the lung epithelium. Cells were grown in submerged cultures in **Chapter 2** and **5**, whereas culturing of A549 cells in an air-liquid interface model or long-term culturing increase the characteristics they share with fresh isolated type II cells (Cooper et al., 2016; Wu et al., 2017). A more advanced model which can be used to study host-pathogen

interaction are lung organoids. They represent the lung epithelium and contain basal cells, ciliated cells and mucus producing cells. They have already been used for viral and bacterial infections and can in the future be used for fungal infections. The possibility to produce organoids from cells of patients who are susceptible to fungal infections, makes it possible to obtain more insight in fungal infections in context of this disease (Sachs et al., 2019). Future research should reveal if the behaviour of *A. fumigatus* observed with A549 cells in a submerged culture is also observed in lung organoids or in the optimized culture conditions for A549 cells.

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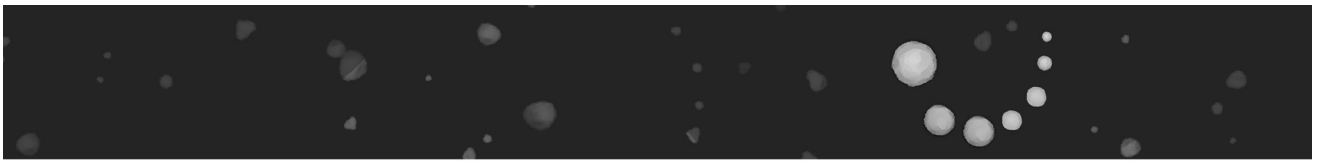

Appendix

Nederlandse Samenvatting
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Nederlandse Samenvatting

Het genus *Aspergillus* omvat een veelvoorkomende groep schimmels, waartoe 446 soorten behoren. *Aspergilli* groeien onder meer op organisch afval en hun eisen aan de omgevingscondities om te groeien zijn relatief gering. Bij gunstige omstandigheden kiemen de sporen van deze schimmels, conidia genoemd, en wordt een netwerk van schimmeldraden (hyfen) gevormd dat bekend staat als mycelium. Vanuit het mycelium ontwikkelen conidioforen zich in de lucht, waarmee de schimmel nieuwe conidia vormt die zich makkelijk verspreiden door de lucht. Zo ademen we enkele honderden *Aspergillus fumigatus* sporen per dag in die door mucociliaire klaring en met componenten van het aangeboren afweersysteem worden opgeruimd. Echter, in patiënten met een verzwakt immuunsysteem kan het inademen van conidia van aspergilli leiden tot levensbedreigende infecties. Doordat steeds meer patiënten medische behandelingen ondergaan die het immuunsysteem onderdrukken, zoals chemotherapie, orgaantransplantaties en gebruik van corticosteroïden, neemt het aantal infecties met deze opportunistische pathogenen toe.

Aspergilloses zijn infecties van de luchtwegen door *Aspergillus* soorten en worden ingedeeld in invasieve en niet-invasieve infecties. Deze infecties worden meestal veroorzaakt door *A. fumigatus* en in mindere mate door *A. flavus*, *A. niger*, *A. terreus* en *A. nidulans*. De meest levensbedreigende infecties zijn invasieve infecties in de long, ook wel invasieve pulmonaire aspergillose genoemd. Hierbij gaan de ingeademde conidia kiemen en groeien de hyfen in het omliggende longweefsel. In patiënten met astma, taaislijmziekte of tuberculose komen non-invasieve *A. fumigatus* infecties voor. Voorbeelden hiervan zijn een allergische reactie op de ingeademde schimmelcomponenten of de vorming van een aspergilloma (schimmelbal). *Aspergillus* infecties kunnen behandeld worden met polyenen, azolen en echinocandinen welke respectievelijk binden aan ergosterol in de plasmamembraan, de synthese van ergosterol remmen en de synthese van glucanen remmen. Voor met name azolen is er een sterke toename van resistentie bij *A. fumigatus* wat succesvolle behandeling in de wegstaat.

De celwand van conidia en hyfen van *A. fumigatus* bestaat uit een basis van chitine en α-glucanen, waarop zich een laag van β-glucanen bevindt. De volgende laag van de celwand bestaat uit een mengsel van mannan, galactomannan en α-glucanen. Bij de hyfen zit er in deze laag ook nog galactosaminogalactan welke een rol speelt bij aanhechting en immunologisch afscherming van het oppervlak van de hyfen. Dit is niet aanwezig bij conidia, die wel extra beschermende lagen bevatten bestaande uit het groene pigment DHN-melanine en de rodlet eiwitten en het eiwit CcpA. Gezamenlijk zorgen deze lagen ervoor dat de conidia niet herkend worden door het immuunsysteem. Als de conidia gaan kiemen breken de buitenste DHN-melanine en eiwit lagen af, wat zorgt voor herkenning en activatie van het immuunsysteem. Daarnaast zorgt DHN-melanine ervoor dat er geen verzuring op zal treden in interne compartimenten van cellen waar de conidia via fagocytose of endocytose in terecht komen, waardoor ze in deze cellen kunnen overleven. Tenslotte beschermt DHN-melanine tegen actieve zuurstofradicalen zoals peroxide.

Het doel van dit proefschrift was om nieuwe moleculen van de schimmel en de gastheer te identificeren die een rol spelen in de eerste stappen van een *A. fumigatus* infectie. Daarnaast heb ik gekeken naar moleculen die uitgescheiden

worden door *A. niger*, met een mogelijke immuun-ontwijkende functie, die een rol kunnen spelen in infecties. Als laatste heb ik gekeken naar de heterogeniteit in virulentie tussen verschillende *A. fumigatus* isolaten in verschillende infectie modellen.

Opname van conidia in epitheelcellen

In de longen kunnen conidia via endocytose worden opgenomen door epitheelcellen. Hiervoor is het actine skelet van de cellen belangrijk, maar ook de Dectin-1 receptor. De Dectin-1 receptor herkent β-glucanen in de celwand van conidia en hyfen. Opname van hyfen in epitheelcellen is over het algemeen lastiger, doordat deze te lang zijn geworden. **Hoofdstuk 2** laat zien dat inhibitie van Dectin-1 met een antilichaam de opname van conidia in type II A549 long epitheelcellen met 50 % verminderd. Inhibitie van de EphA2 receptor, die ook β-glucanen herkend, verminderd ook de opname van conidia. De EphA2 receptor kan geremd worden met een antilichaam of door de kinase inhibitor dasatinib, beide resulteren in een vermindering in opname van 50 %. Inhibitie van Dectin-1 en EphA2 op hetzelfde moment verminderde de opname van conidia tot 35 %, en liet dus zien dat er nog steeds opname mogelijk is. Dit impliceert dat ook andere receptoren bij de opname van conidia betrokken zijn.

Als conidia beginnen te kiemen, exposeren ze meer β-glucan, mede door de verwijdering van DHN-melanine en de beschermende eiwitlaag. Dit heeft echter geen effect op de opname van conidia (**Hoofdstuk 2**). Wel zien we dat de deletie van het gen *pksP,* waardoor synthese van DHN-melanine wordt uitgeschakeld, de binding van conidia aan de type II epitheelcellen verhoogd, maar dat de opname van conidia verminderd. Inhibitie van de EphA2 receptor had geen remmend effect op de opname van conidia die DHN-melanine miste. Dit laat zien dat DHN-melanine nodig is voor opname in epitheelcellen via de EphA2 receptor. Aangezien EphA2 β-glucan herkend en geen DHN-melanine is hier waarschijnlijk een co-receptor van EphA2 bij betrokken.

Voor de opportunistische pathogene gist *Candida albicans* is de EphA2 receptor zowel nodig voor opname in epitheelcellen als voor de initiatie van de immuun response van deze cellen. In type II long epitheelcellen is echter na infectie met wild-type *A. fumigatus* conidia geen initiatie van de immuun response (IL-8 productie). Alleen na infectie met grote hoeveelheden *A. fumigatus* conidia (10 conidia/cel) die DHN-melanine of een rodlet eiwit missen is een verhoging in IL-8 productie te zien (**Hoofdstuk 2**). Bij de wild-type en genetisch gemodificeerde conidia was er geen remming te zien in IL-8 productie bij inhibitie van de EphA2 receptor.

De rol van DHN-melanine in peroxide bescherming

Peroxide is een onderdeel van de reactieve zuurstofradicalen (ROS) die het immuunsysteem maakt om pathogenen, waaronder *A. fumigatus*, te doden. Een UV mutant van de ATCC46645 stam die geen DHN-melanine meer maakt is gevoeliger voor peroxide in een plaat assay dan de wild-type stam. **Hoofdstuk 3** laat zien dat complementatie van het defecte *pksP* gen in deze stam niet de resistentie tegen peroxide in de plaat assay hersteld, dit ondanks het feit dat DHN-melanine wel weer wordt gemaakt. Deletie van *pksP* in een CEA10 achtergrond leidde ook niet tot een hogere peroxide gevoeligheid in de plaat assay. Deze data laat zien dat DHN-melanine niet beschermt tegen peroxide en dat de gevoeligheid van de melanine

mutant in de ATCC46645 mutant waarschijnlijk komt door additionele mutaties ten gevolge van de UV-mutagenese. Genoomanalyse liet zien dat naast mutaties in het *pksP* gen er ook een mutatie zit in *cat1* (**Hoofdstuk 3**). Deletie van dit gen dat codeert voor een uitgescheiden catalase dat tot expressie komt in hyfen leidt niet tot een verhoogde gevoeligheid van conidia voor peroxide, maar wel van de filamenteus groeiende schimmel. Dit is overeenkomstig de resultaten van de ATCC46645 melanine mutant in **Hoofdstuk 3**, waarbij geen verhoogde gevoeligheid te zien was wanneer conidia werden blootgesteld aan peroxide, maar wel de groei van mycelium werd geremd. De hypothese is nu dat de toename in peroxide gevoeligheid in de ATCC46645 melanine mutant komt door de mutatie in het *cat1* gen waardoor er geen of onvoldoende actief catalase door hyfen wordt uitgescheiden om het peroxide in het groeimedium af te breken.

Immuun modulerende eiwitten uitgescheiden door *Aspergillus niger*

Micro-organismen zoals *Aspergillus* scheiden veel verschillende moleculen in hun omgeving uit, zoals eiwitten, koolhydraten en secundaire metabolieten. Secundaire metabolieten zijn kleine moleculen die niet nodig zijn voor groei, maar bijvoorbeeld ter verdediging tegen andere micro-organismes worden gemaakt zoals het antibioticum penicilline. Voor pathogene bacteriën is bekend dat uitgescheiden moleculen een rol kunnen spelen bij het ontwijken van de immuunrespons waardoor een infectie zich verder kan ontwikkelen. In **Hoofstuk 4** wordt het groeimedium van drie *Aspergillus* soorten (*A. fumigatus*, *A. tubingensis* en de *A. niger* mutant D15#26) vergeleken in een competitie voor binding met immuunreceptoren en fluorescerende antilichamen. Deze assay laat zien dat alle drie de schimmelsoorten moleculen uitscheiden die binden aan de CD162, CD181 en CD182 receptor, terwijl de *A. niger* D15#26 mutant ook moleculen maakt die kunnen binden aan de CD88 receptor.

De *A. niger* D15#26 mutant verzuurt het kweekmedium niet, in tegenstelling tot de *A. niger* wild-type stam. De moleculen die binden aan de CD181 receptor worden ook geproduceerd in andere *A. niger* stammen die het kweekmedium niet verzuren, bijvoorbeeld door een deletie in het gen dat betrokken is bij de vorming van oxaalzuur. De reden dat de *A. niger* D15#26 mutant het medium niet verzuurd is door de inactivatie van het gen dat codeert voor de regulator van secundaire metabolieten LaeA. Een verdere analyse van het kweekmedium in **Hoofdstuk 4** laat zien dat de moleculen die de competitie voor binding aan gaan met de CD88 en CD182 receptor kleiner zijn dan 3 kDa en bovendien ook hitte en protease resistent zijn en relatief hydrofiel. Deze eigenschappen en de regulatie door *laeA* wijzen erop dat de moleculen die de competitie voor binding aangaan secundaire metabolieten zijn.

Secundaire metabolieten worden geproduceerd via de mevalonaat en de shikimaat route, alsmede de polyketide synthese (PKS) of de non-ribosomale peptide synthese (NRPS) routes. De PKS en NRPS routes worden beide geactiveerd door een 4'fosfopantetheinyl transferase (PPTase). Deletie van het coderende gen *pptA* in *A. niger* remt de productie van secundaire metabolieten via beide routes. Een deletie van het *pptA* gen in een *A. niger* stam die ook een *laeA* deletie bevat verzuurt het kweekmedium niet (**Hoofdstuk 4**). De toevoeging van gebruikt medium, met daarin onder andere sideroforen die nodig zijn voor groei ten gevolge van de deletie van *pptA*, remde in alle condities de productie van moleculen die de competitie voor binding aan gaan met immuun receptoren. Of de moleculen

die binden aan de immuunreceptoren secundaire metabolieten zijn die geproduceerd worden door de PKS en / of NRPS routes is daarom nog niet duidelijk.

Heterogeniteit tussen *Aspergillus fumigatus* stammen in virulentie

Binnen de soort *A. fumigatus* worden de Af293 en CEA10 stammen het meest gebruikt voor experimenten om virulentie mechanismes te begrijpen. Eerder onderzoek heeft laten zien dat CEA10 minder virulenter is in een zebravis infectie model door zijn snellere groei. In **Hoofdstuk 5** zijn vijf verschillende *A. fumigatus* isolaten vergeleken ten aanzien van ROS tolerantie en virulentie in 4 modelsystemen. Van de stammen die gebruikt werden zijn er drie geïsoleerd uit een invasieve humane infectie (Af293, CEA10 en ATCC46645) en twee uit een niet-invasieve infectie uit de sinus van een hond (DTO271-B5 en DTO303-F3). Deze vergelijking liet zien dat Af293 het gevoeligst is voor ROS zoals peroxide, terwijl ATCC46645 de grootste resistentie heeft voor deze moleculen (**Hoofdstuk 5**). Type II A549 longepitheel cellen zijn gebruikt als model voor het longepitheel wat de conidia tegenkomen als ze ingeademd worden. Hier zien we geen grote verschillen tussen de stammen, maar wel dat Af293 het meest associeert aan de longcellen en DTO271-B5 het minst. Als we deze vijf stammen vergelijken ten aanzien van hun virulentie in de *Protostelium aurantium* amoebe, die als model dient voor fagocyten, zien we dat conidia van Af293 het minst kiemen en het beste overleven na een infectie van deze amoeben. Ook in medium zonder amoeben blijkt dat Af293 het minst snel kiemt in vergelijking met de andere vier stammen. Dit kan betekenen dat langzamer kiemen een strategie is van Af293 om te overleven in de amoeben en misschien ook in andere infectie modellen. Dit is inderdaad wat er wordt gezien als de vijf stammen worden vergeleken ten aanzien van virulentie in *Galleria melonella* larven en zebravis embryo's. In beide modellen is Af293 het minst virulent, in de *G. melonella* larven is ATCC46645 het meest virulent en DTO303-F3 in de zebravis embryo's.

Genoom analyse laat zien dat ATCC46645, CEA10, DTO271-B5 en DTO303-F3 meer verschillen van Af293 dan van elkaar in genen gerelateerd aan virulentie (**Hoofdstuk 5**). Wat daarnaast opvalt is dat deze vier stammen mutaties bevatten in het gen wat zorgt voor de productie van het secundaire metaboliet fumagillin. Het is mogelijk dat de verschillen in virulentie komen door mutaties in een van dergelijke virulentie gerelateerde genen. De fylogenetische boom in **Hoofdstuk 5** op basis van mutaties die voorkomen in het hele genoom, en niet alleen in de virulentie gerelateerde genen, laat zien dat ATCC46645 het verst verwijderd is van de andere vier stammen en niet Af293 zoals bij de virulentie gerelateerde genen. Wat we ook zien in de fylogenetische boom is dat er geen clusters zijn van stammen die geïsoleerd zijn uit de natuur, of klinische isolaten uit invasieve of niet-invasieve infecties. Er is alleen een cluster te zien van isolaten uit een niet-invasieve infectie, en dit zijn sequenties van isolaten uit dezelfde patiënt die op verschillende tijdstippen zijn geïsoleerd, wat verklaard waarom ze bij elkaar in een cluster te vinden zijn.

A

Dankwoord

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Curriculum Vitae

Esther Keizer was born August 13, 1992 in Swifterbant (the Netherlands). She followed her secondary education at the Almere College in Dronten and enrolled the Bachelor Biology at Leiden University September 2010. After her bachelor graduation, she was board member for a year of the Leiden student rowing association Asopos de Vliet and enrolled the master Molecular and Cellular Life Sciences at Utrecht University in September 2014.

As part of her master program, Esther completed two research internships. She performed her major internship (Microbiology, Department of Biology, Utrecht University) under supervision of Dr. Hans de Cock and Dr. Natalia Escobar. She studied the secretion of small molecules by *Aspergillus niger* that compete for binding with immune receptors. Esther performed her minor internship at Dupont Industrial Biosciences (Leiden, the Netherlands) on a high throughput transformation system of *Bacillus licheniformis*. Esther obtained her MSc degree August 2016 and started in October the same year her PhD project (Microbiology, Department of Biology, Utrecht University) under the supervision of Dr. Hans de Cock and Prof. Dr. Han Wösten. During her PhD she studied the interaction of *A. fumigatus* with epithelial cells on the molecular level and the heterogeneity of *A. fumigatus* strains in different infection models. Esther obtained an EMBO short term fellowship during her PhD project, which enabled her to study the interaction of *Aspergillus fumigatus* with amoebae for three months at the Leibniz Institute for Natural Product Research and Infection Biology - Hans-Knöll-Institut in Jena under the supervision of Dr. Falk Hillmann. The results of her research are described in this thesis.

List of Publications

Keizer E.M., Valdes I.D., Forn-Cuní G., Klijn E., Meijer A.H., Hillmann F., Wösten H.A.B., de Cock H. (2020) Comparison of virulence and genomic differences of five *Aspergillus fumigatus* isolates shows that Af293 is less virulent. PlosOne. *Under revision*.

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Keizer, E.M., Escobar N., Valdes I.D., Ordonez S.R., Ohm R.A., Wösten H.A.B., de Cock H. (2018) Differential gene expression of *Aspergillus fumigatus* and *Aspergillus niger* interacting with epithelial lung cells. Medical Mycology 56:S17. doi: 10.1093/mmy/myy036.

Keizer, E.M., Valdes I.D., van Baelen F., Schinkel M., Wösten H.A.B., de Cock H. (2018) Alternative mechanisms of protection of *Aspergillus fumigatus* conidia against reactive oxygen species. Medical Mycology 56:S156. doi: 10.1093/mmy/myy036.

Escobar N., Valdes I.D., **Keizer E.M.**, Ordonez S.R., Ohm R.A., Wösten H.A.B., de Cock H. (2018) Expression profile analysis reveals that *Aspergillus fumigatus* but not *Aspergillus niger* makes type II epithelial lung cells less immunological alert. BMC Genomics 19:534 doi: 10.1186/s12864-018-4895-3.