Studies on the release of neutrophil extracellular traps and IFN-γ as part of the innate immune response to *Aspergillus fumigatus* and on the fungal stress response via the hybrid sensor kinase TcsC

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1. Summary

Aspergillus fumigatus is a saprophytic mold that naturally inhabits the soil. Asexual reproduction yields hardy conidia that circulate in the air and are inhaled daily by humans. The fungus seems not to have evolved distinct mechanisms of pathogenicity, but is capable of responding to many stressful environmental cues present in its naturally harsh niche. The robust conidia present no problem to a fully functioning immune system, but if the innate immune system is compromised, the conidia can become activated and differentiate within the lung tissue to form invasive and disseminating hyphae. The resulting disease is called aspergillosis and is difficult to detect and to treat. To date, scientists have yet to find the factor(s) missing during immunosuppression that allow a healthy patient to easily dispose of *A. fumigatus*. We explored two possibilities: the production of neutrophil extracellular traps (NETs) and the release of IFN-γ by natural killer (NK) cells. We report here that NETs alone cannot kill the fungus, but do inhibit polar growth. Elongation of hyphal tips is abrogated due to zinc starvation, likely a consequence of the zinc-chelating, NETs-associated protein calprotectin. NK cells alone are also incapable of fungicidal activity, but their release of IFN-γ upon contact with *A. fumigatus* abrogates hyphal growth by a yet unknown mechanism. *In vitro* studies of the innate immune response, though helpful, are far from representative of the *in vivo* response. Neither NETs nor IFN-γ alone can manage *Aspergillus* infection, but in combination, these and other immune assaults certainly can. The difficulty lies in identifying the precise combination of immune cells and cytokine milieu that in a healthy individual prevent infection.

Additionally, we explored mechanisms by which the fungus responds to stress, namely the HOG MAPK pathway, historically involved in osmotic stress response. In filamentous fungi, certain stress signals are sensed by a cytoplasmic hybrid histidine kinase sensor and then passed through the HOG system via phosphorylation. We identified the putative hybrid sensor kinase in *A. fumigatus*, and generated a corresponding knockout mutant. The ΔtcsC mutant was indeed sensitive to osmotic stress, and resistant to the phenolpyrrole fungicide fludioxonil. In the wild type the addition of either osmotic stress or fludioxonil resulted in SakA phosphorylation and translocation to the nucleus. SakA, the Hog1 homolog in *A. fumigatus*, is located at the end of the HOG pathway, confirming the role of TcsC as the cytoplasmic sensor upstream of SakA. In hypoxia, on farnesol, and in high concentrations of divalent cations the ΔtcsC mutant exhibited a striking “fluffy” phenotype characterized by the production of tremendous aerial hyphae and little or no

differentiation, i.e., no conidiation. Though the ΔtcsC mutant showed no change in virulence compared to wild type, components of the TcsC signalling pathway remain promising targets for antifungal agents.

1.1 Zusammenfassung

Aspergillus fumigatus ist ein saprophytischer Schimmelpilz, der natürlicherweise im Boden vorkommt. Durch asexuelle Vermehrung entstehen Dauerformen, sogenannte Konidien, die über die Luft verteilt und vom Menschen täglich eingeatmet werden. Nach dem jetzigen Kenntnisstand besitzt *A. fumigatus* keine ausgefeilten Pathogenitätsmechanismen, ist aber in der Lage sich an Stresssituationen effizient anzupassen und überlebt so auch unter harten Bedingungen. Konidien sind zwar robust, stellen aber kein Problem für ein funktionstüchtiges Immunsystem dar. Ist die angeborene Immunität aber beeinträchtigt, so keimen die Sporen aus und es bilden sich Hyphen, die invasiv in das Gewebe eindringen und sich so ausbreiten. Die daraus resultierende Erkrankung, invasive Aspergillose genannt, läßt sich nur schwer nachweisen und behandeln. Die Mechansimen, die *A. fumigatus* in gesunden Personen effizient eliminieren, die aber im immungeschwächten Patienten fehlen, sind bis heute kaum verstanden. In dieser Arbeit haben wir zwei mögliche Elemente untersucht: die Produktion von *neutrophil extracellular traps* (NETs) und die Freisetzung von IFN-γ durch *natural killer* (NK) Zellen. Wir konnten zeigen, dass NETs den Pilz nicht abtöten können, aber immerhin sein Wachstum reduzieren. Das Spitzenwachstum der Hyphen wird dabei durch Zink-Mangel inhibiert, der vermutlich auf den NET-assoziierten Zink-Chelator Calprotectin zurückgeht. NK Zellen besitzen allein keine fungizide Aktivität, aber sie setzen nach Kontakt mit *A. fumigatus* IFN-γ frei, das wiederum das Hyphenwachstum durch einen bisher unbekannten Wirkmechanismus beeinträchtigen kann. *In vitro* Studien der angeborenen Immunantwort sind wichtig, spiegeln aber meist die Komplexität der *in vivo* Antwort nur unzulänglich wider. Für sich allein können weder NETs noch IFN-γ eine *Aspergillus* Infektion eliminieren, im Zusammenspiel miteinander und mit anderen Elementen der Immunantwort sind sie dazu aber offensichtlich in der Lage. Das wissenschaftliche Hauptproblem liegt in der Identifizierung der genauen Kombination von Immunzellen und Cytokin-Milieu, die eine Infektion in gesunden Personen unterbindet.

Als weiterer Punkt dieser Arbeit wurde ein Element des HOG MAPK Signalwegs untersucht, der in vielen Pilzen die Adaptation an hyperosmolaren Stress steuert. Filamentöse Pilze nehmen bestimmte Stresssituationen durch cytoplasmatische Hybrid-Histidin-Kinase Sensoren wahr, die dann das Signal als Phosphorylierung an Elemente des HOG Signalwegs weitergeben. In dieser Arbeit haben wir die putative Hybrid-Sensorkinase TcsC in *A. fumigatus* identifiziert und eine entsprechende

Deletionsmutante hergestellt. Die ΔtcsC Mutante erwies sich als sensitiv gegenüber osmotischem Stress und resistent gegenüber dem Phenolpyrrol-Fungizid Fludioxonil. Im Wildtyp führt osmotischer Stress und die Zugabe von Fludioxonil zu einer Phosphorylierung und anschließenden Translokation des SakA Proteins in den Zellkern. Das Hog1-homologe Protein SakA ist die terminale Komponente des HOG Signalwegs, und unsere Daten belegen, dass TcsC in *A. fumigatus* als cytoplasmischer Sensor oberhalb von SakA fungiert. Hypoxie, Farnesol oder hohe Konzentrationen divalenter Kationen induzieren in der ΔtcsC Mutante einen auffälligen Wachstumsphänotyp. Diese "fluffy" genannte Wuchsform zeichnet sich durch eine massive Zunahme der Lufthyphen und eine stark reduzierte Differenzierung und Konidienbildung aus. Obwohl für die ΔtcsC Mutante keine Attenuierung nachgewiesen werden konnte, so bietet der TcsC Signalweg dennoch interessante Ziele für neue anti-mykotische Wirkstoffe.

2. Introduction

Ascomycota is a phylum of the Fungi kingdom defined by the presence of an ascus, a sexual spore-bearing cell. The sexual process involves the production of haploid ascospores via meiosis of the diploid cell in the ascus. In the order Eurotiales, the asci develop within a closed, spherical structure, called the cleistothecium (see **Figure 1**). Within the cleistothecium, the asci are well protected, but can only be released by rupture of the cleistothecium, i.e., there is no specific mechanism for spore dispersal (Carlile *et al.*, 1994). Though the phylum is distinguished by the unique cells involved in sexual reproduction, the species *Aspergillus fumigatus* was thought to produce only asexually until quite recently. The name *Aspergillus,* in fact, comes from the Latin *aspergillum*,

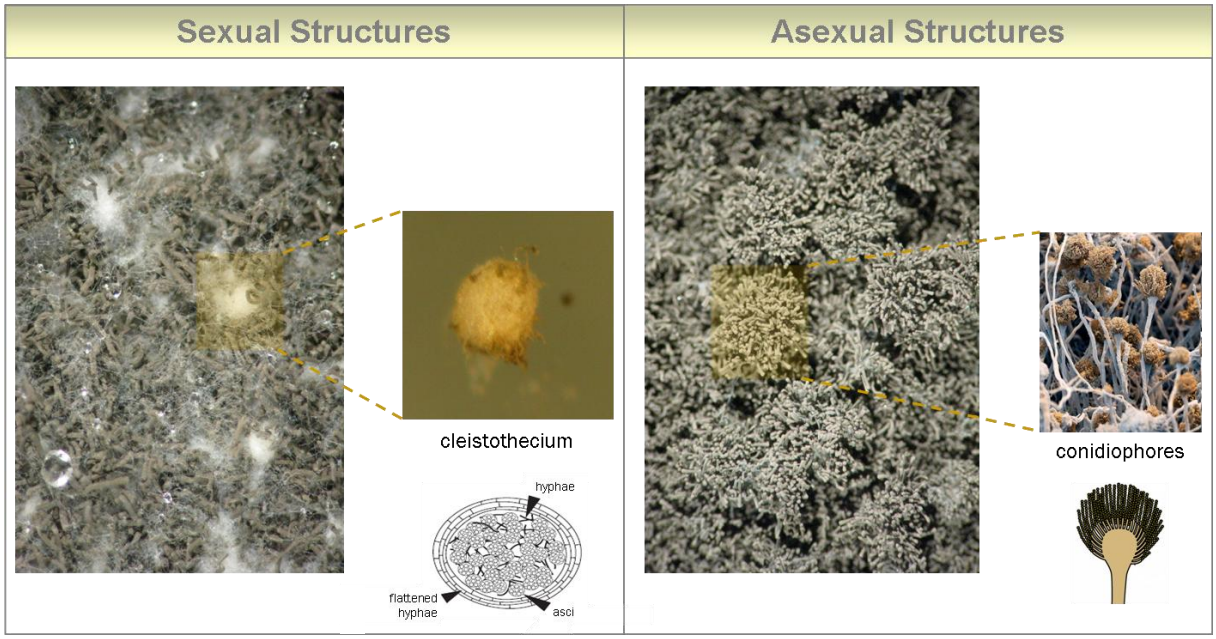

Figure 1. Morphology of the Aspergilli: sexual and asexual reproductive structures.

The name *Aspergillus* comes from the Latin *aspergillum*, a mop for distributing holy water, obviously in reference to the appearance of the conidiophore. The conidiophores produce and release large numbers of asexual spores called conidia, which are constantly dispersed into the air. It was very recently discovered that *Aspergillus fumigatus* can also reproduce sexually. Meiosis yields haploid asci from the diploid ascus. The asci develop within a closed structure called the cleistothecium. The lower magnification photos are of *A. fumigatus* strains AfS35 crossed with D141 on oatmeal agar after 4 months growth in the dark at 30°C. The cleistothecium was taken from the plate as indicated and photographed under higher magnification. Aforementioned photos taken in collaboration with Edita Szewczyk at the Research Center for Infectious Diseases, Julius-Maximilians-University Würzburg. Cartoon cleistothecium adapted from (Dyer and O'Gorman, 2012).

which is a mop for distributing holy water, and refers to an asexual structure called the conidiophore; the image is often used to depict the genus. Though the Aspergilli, of which there are approximately 250 species (Dyer and O'Gorman, 2012), all produce asexual

spores via the conidiophore, the members of this genus differ greatly from one another in other aspects. *A. glaucus* and *A. restrictus*, for example, are remarkable because of their tolerance of low water activities. *A. niger* is commonly used in fermentation processes; it produces citric acid used in soft drinks. *A. flavus* and *A. oryzae* are used in the production of Asian food and drinks (Carlile *et al.*, 1994). Researchers now have the genome sequences of nearly a dozen species at their disposal (available online at http://www.cadre-genomes.org.uk/index.html). Of the 250 species known, only a handful can act as pathogens, the most important of which (at least clinically) and indeed the topic of this study is *A. fumigatus*.

2.1 The innate immune response to Aspergillus fumigatus

The genus *Aspergillus* comprises saprophytic fungi that grow naturally in the soil as a mycelium, a mass of branching hyphae. Upon contact with air, the mycelia form conidiophores that produce and release large numbers of asexual spores called conidia. These conidia circulate in the air (1-100 conidia/m^3) and it is estimated that the average person inhales several hundred conidia per day. To a healthy immune system this presents no problem, but in an immunocompromised host *Aspergillus* can cause severe systemic infection, with a mortality rate of 80-90% (Latgé, 1999). Invasive aspergillosis, in most cases caused by *A. fumigatus* and to a lesser extent by *A. terreus* and *A. flavus* (Marr *et al.*, 2002), is currently the most important fungal disease (Latgé, 2001; McCormick *et al.*, 2010b), occurring in patients whose immune systems have been jeopardized, e.g., by leukemia, lymphoma, neutropenia, as well as stem cell or organ transplantation. As these medical practices become more common, the incidence of *Aspergillus* infection continues to rise as a consequence.

Aspergillosis is difficult to detect and therefore difficult to treat (McNeil *et al.*, 2001). Clinical symptoms alone are too vague to diagnose invasive aspergillosis with certainty; chest X-rays are too nonspecific and cannot be used for early diagnosis; histopathology cannot be used in neutropenic or thrombocytopenic patients and typically cannot distinguish *Aspergillus* from other filamentous fungi; and *Aspergillus* species are slow-growing, which means it may take several days to weeks for positive culture results. Galactomannan, a component of the *Aspergillus* cell wall, has been used in a double-sandwich enzyme immunoassay to diagnose invasive aspergillosis in Europe for over ten years. The assay is standardized, user-friendly, and both sensitivity and specificity are

generally high (Ostrosky-Zeichner, 2012; Walsh *et al.*, 2008). Optimal aspergillosis therapy is two-fold, requiring both the restoration of leukocyte counts and immune function coupled with effective antifungal treatment at the earliest stages of infection. Several antifungals are currently available (e.g., amphotericin B, voriconazole and other triazoles, and caspofungin), though use of these alone rarely yields complete remission (Traunmüller *et al.*, 2011). The pathogenicity of *Aspergillus* relies largely on its ability to grow as different morphotypes in changing environments within the human host—a characteristic that also makes treatment rather difficult, especially regarding response to antifungal therapy. *Aspergillus* conidia swell and germinate in the alveoli, where they are confronted by resident alveolar macrophages and epithelial cells (see **Figure 2**) (Herzog *et al.*, 2008). As infection persists, elongated hyphae penetrate blood vessels, spreading via the vascular route to other organs and the brain (Kradin and Mark, 2008). Depending on the time of treatment, the antifungal molecule must reach growing fungal cells within the alveoli, within a granuloma, embedded within the tissue, or travelling through the blood stream. The response rate to the antifungals currently available is therefore quite low (Lin *et al.*, 2001). The patient and scientific communities urgently need, not only a better understanding of the pathogen, but more importantly, insight into the pathogen-host interaction. Only then will better detection and therapeutic options become a possibility.

2.1.1 Resident alveolar macrophages versus conidia

The body's first line of defense against *Aspergillus* is the innate immune system. As shown in **Figure 2**, upon inhalation, conidia are confronted by resident alveolar macrophages, though no response is initiated until the resting conidia have begun to swell and shed their hydrophobic surface layer. Resting conidia are protected by a rodlet layer comprising RodA protein covalently bound to the cell wall, allowing this morphotype to avoid recognition by immune cells (Aimanianda *et al.*, 2009). As conidia grow (i.e., swell), this protein layer is shed, exposing carbohydrates of the cell wall and prompting the maturation of resident alveolar macrophages and subsequent cytokine release. Though alveolar macrophages can phagocytose conidia and kill them by producing reactive oxygen species (ROS) (Philippe *et al.*, 2003), Mircescu *et al*. have shown that neutrophils and not alveolar macrophages are essential for immune defense in the early stages of infection (Mircescu *et al.*, 2009). Recognition by both neutrophils and alveolar

macrophages is via the dectin-1 receptor, which binds β-glucan in the fungal cell wall, and has been shown to bind specifically to swollen conidia and hyphae but not resting conidia in *Aspergillus* (Hohl *et al.*, 2005). Dectin-1 is expressed on all monocytes, macrophages, dendritic cells, neutrophils, and eosinophils (Willment *et al.*, 2005). It was

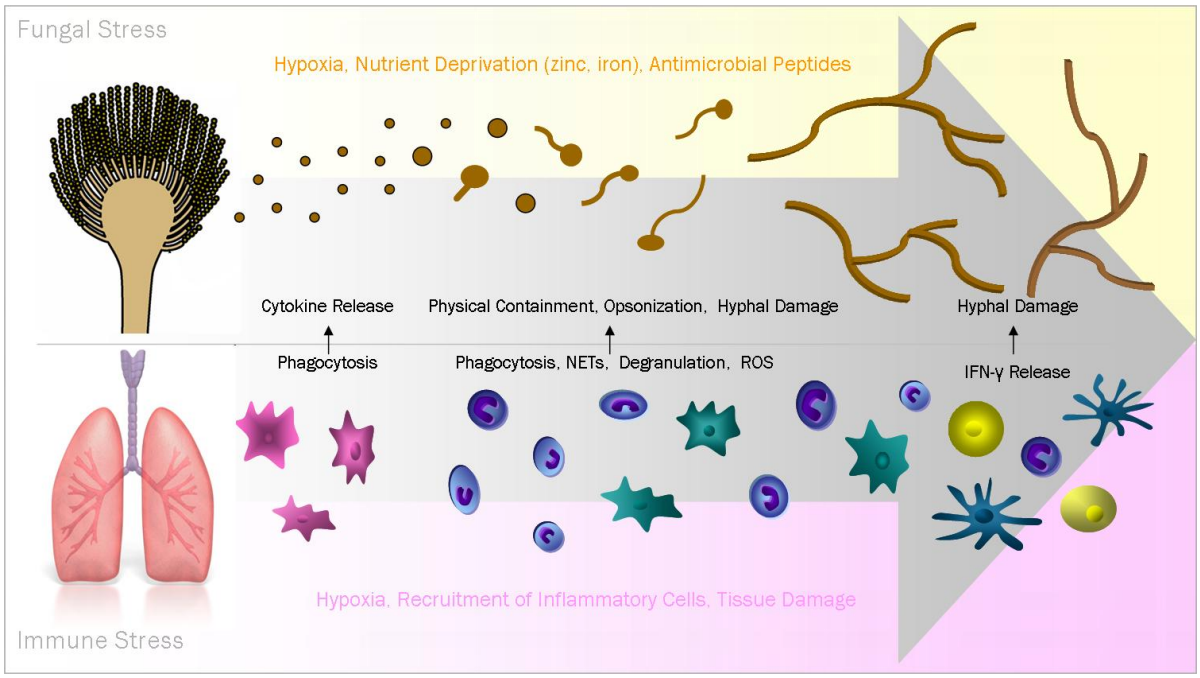

Figure 2. The innate immune response during *Aspergillus fumigatus* infection.

Upon inhalation, resting conidia are initially confronted by resident alveolar macrophages. Conidia that escape phagocytosis began to swell and germinate. At this stage they are attacked by recruited neutrophils and macrophages, both of which can phagocytose swollen conidia and small germlings. Larger germlings and hyphae are thought to be contained and killed directly by activated PMNs or indirectly by molecules released by PMNs and natural killer cells.

shown that during *Aspergillus* infection, dectin-1 is recruited *in vivo* to alveolar macrophage phagosomes that harbor conidia with exposed β-glucans (Hohl *et al.*, 2005), and that dectin-1-deficient mice are impaired in inflammatory response by alveolar macrophages and in recognition by recruited neutrophils (Willment *et al.*, 2005; Werner *et al.*, 2009). The Toll-like receptors (TLR) 2 and 4 also play a role in fungal recognition and subsequent inflammatory response (Mambula *et al.*, 2002; Meier *et al.*, 2003; Wang *et al.*, 2001; Netea *et al.*, 2006), though the *Aspergillus* binding partners are still unknown. It has been published that TLR2 and MyD88 (myeloid differentiation primary response gene [88]) signalling are required for efficient phagocytosis (Luther *et al.*,

2007), and that TLR2 dimerizes with TLR1 to activate NF-κB (Rubino *et al.*, 2012). As shown in **Figure 3**, binding of a pathogen associated molecular pattern (PAMP) to either dectin-1 or a TLR activates NF-κB, thereby inducing proinflammatory cytokine and chemokine production (Werner *et al.*, 2009). However, each pattern recognition receptor (PRR) has different adaptor protein partners that couple to different TNF receptor associated factor 6 (TRAF6) complexes (Dennehy and Brown, 2007). Clearly, the mechanism of *Aspergillus* recognition continues to elude researchers and it is thus vital for the future development of both diagnosis and treatment of *Aspergillus* infections that the details of recognition are elucidated.

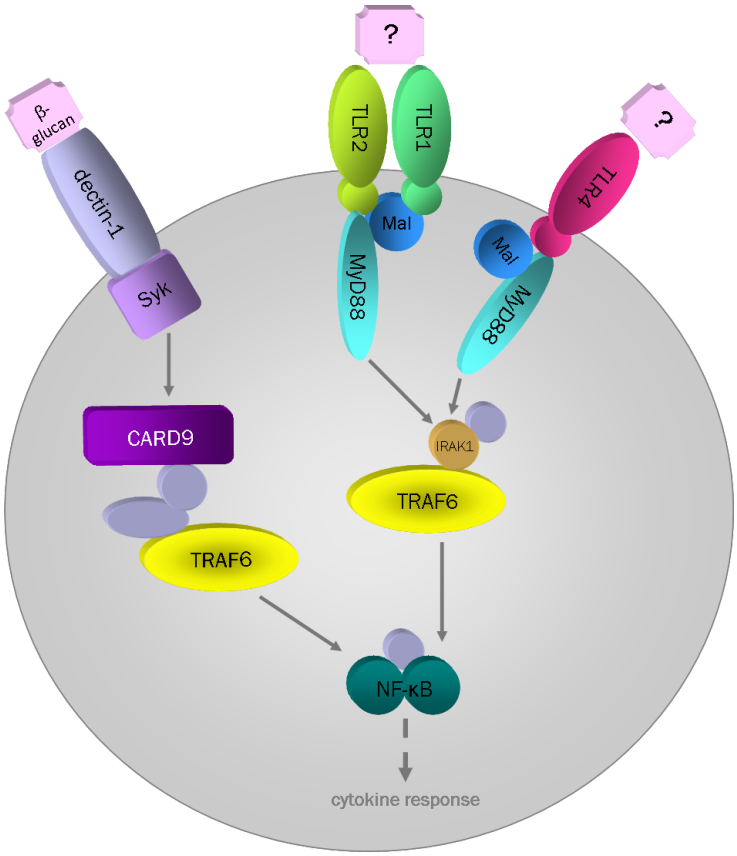

Figure 3. Dectin-1 and TLR signalling pathways in innate immune cells.

Both dectin-1 and TLR signalling lead to NF-κB activation and cytokine production. However, dectin-1 uses the Syk kinase and CARD9 adaptor protein to couple to the TRAF6 complex and activate NF- κB, while TLRs use the MyD88 adaptor protein and IRAK (IL-1 receptor-associated kinase). Dectin-1 binds β-glucan, a component of the *Aspergillus* cell wall. The binding partners for the TLR receptors involved in *Aspergillus* response are currently not known [adapted from (Dennehy and Brown, 2007; Netea *et al.*, 2006)].

2.1.2 Recruited neutrophils and macrophages versus germlings and hyphae

When polymorphonuclear neutrophils (PMN) are recruited to the site of infection, they can respond in three ways: phagocytosis, degranulation and production of ROS, or with the release of neutrophil extracellular traps (NETs). The conidia that manage to escape alveolar macrophages are phagocytosed by infiltrating PMNs (Behnsen *et al.*, 2007). Alternatively, they may germinate and are then attacked by PMNs that adhere to the hyphal surface, triggering a respiratory burst (Latgé, 2001), or become entangled in NETs—masses of decondensed chromatin decorated with antimicrobial proteins and opsonins (Papayannopoulos and Zychlinsky, 2009). NETs-associated proteins include neutrophil elastase, calprotectin, lactoferrin, and the long pentraxin 3 (Urban *et al.*, 2009a; Bottazzi *et al.*, 2009). NETs are believed to function by physically containing the microbe within the sticky mass of chromatin, thus forcing direct contact with or placing it in close proximity to the antimicrobial NETs-associated proteins. Neutrophil elastase activity is required for NET formation in PMNs (Papayannopoulos *et al.*, 2010), but is also capable of neutralizing the harmful effects of bacterial pathogens (Papayannopoulos and Zychlinsky, 2009). Calprotectin and lactoferrin are metal chelators and function by depleting the immediate environment of nutrients required for fungal growth, namely zinc and iron, respectively (Urban *et al.*, 2009c; Zarember *et al.*, 2007). Pentraxin 3 is a well documented opsonin (Bottazzi *et al.*, 2009) and is believed to bind directly to galactomannan in the *Aspergillus* cell wall (Garlanda *et al.*, 2002). The roles of each of these proteins, and indeed of NETs themselves, are still not well understood in the context of fungal infection. In fact, the mechanism of NETosis during fungal infections is not yet understood. The use of PMNs from patients with chronic granulomatous disease (CGD), in which the cells exhibit impaired nicotinamide adenine dinucleotide phosphate (NADPH) oxidase function, in the laboratory suggests a role for NADPH oxidase in the NETosis pathway, at least in response to *A. nidulans* (Bianchi *et al.*, 2011). Also, gene therapy was used in CGD patients to restore NETosis, thereby restoring neutrophil elimination of *A. nidulans* conidia and hyphae (Bianchi *et al.*, 2009). However, Henriet *et al*. showed that human leukocytes can kill *A. nidulans* by ROS-independent mechanisms and that CGD cells, even lacking a functioning NADPH oxidase, are able to damage *A. nidulans* hyphae (Henriet *et al.*, 2011). This suggests an alternative pathway to the respiratory burst and perhaps, therefore, to NETosis, known until now to depend on NADPH oxidase activity and ROS (Brinkmann and Zychlinsky, 2007; Fuchs *et al.*, 2007). Marcos *et al.* have recently shown that the G-protein coupled receptor CXCR2 mediates

NET formation independent of NADPH oxidase and involves Src family kinases (Marcos *et al.*, 2010). The current study explores not only the role of NETs in response to *A. fumigatus* infection, but the function of NETs-associated proteins involved in *A. fumigatus* attack, with hopes of elucidating the mechanism of NETosis in response to this very important fungal pathogen.

All three modes of PMN response lead to the recruitment of circulating monocytes to the site of infection, where maturation is induced (see **Figure 2**). Again, the mechanism of and reasons behind maturation are debated in the literature. Rosas *et al*. showed that β-glucans were insufficient for the induction of cytokine production by macrophages but were able to act in synergy with the TLR-mediated cytokine responses (Rosas *et al.*, 2008; Ferwerda *et al.*, 2008). It was originally believed that neutrophils, short-lived as they are, were first-response effector cells whose sole purpose was cell death to release toxic substrates. It has now become clear: while that remains true, neutrophils are also important recruiters and initiators of the body's defense against incoming pathogens (Ellis and Beaman, 2004). Their role in the innate immune response is just beginning to unfold and much work remains to fully understand the role of these altruistic cells.

2.1.3 Dendritic and natural killer cells versus germlings and hyphae

Dendritic cells act as the sentries of the immune system and are involved in both the innate and adaptive immune responses. In a murine model, the number of monocyte-derived dendritic cells correlates with survival of *Aspergillus*-infected neutropenic mice (Park *et al.*, 2010). It was recently shown that TNF-α (tumor necrosis factor alpha) produced by macrophages and mature dendritic cells in the lung acts as a molecular switch, modulating the activity of CD4 Tcells and promoting neutrophilic inflammation (Fei *et al.*, 2011). Maturation of resident dendritic cells is induced by contact with the pathogen and results in increased expression of costimulatory molecules and improved antigen presentation (Burns *et al.*, 2004). *Aspergillus* is recognized by dectin-1 on immature dendritic cells and binding of the PRR induces a proinflammatory cytokine response (Mezger *et al.*, 2008), which also seems to be inducible independent of MyD88 (Rogers *et al.*, 2005). Dendritic cells can also be activated by natural killer cells, a process requiring both cell contact and TNF-α production (at least *in vitro*). This interaction, however, is tightly regulated by the natural killer cell to dendritic cell ratio (Hamerman *et al.*, 2005; Piccioli *et al.*, 2002).

Not only do natural killer cells activate dendritic cells, but the reciprocal activation also occurs. Originally published in 1999, it is now well established that dendritic cells enhance the cytotoxicity of natural killer cells against microbes and induce the natural killer cell secretion of IFN-γ (interferon gamma) (Fernandez *et al.*, 1999; Hamerman *et al.*, 2005). Natural killer cell activation requires the concerted binding of cell surface receptors and the action of pro-inflammatory cytokines. Natural killer cells mediate pathogen death via two known mechanisms. The first is exocytosis of cytotoxic granule contents (perforin, granzymes, granulysin), which mediate a diverse range of cell death pathways. The second is via target cell apoptosis, which is mediated by the binding of Fas and TRAIL (TNF-related apoptosis-inducing) ligands on the surface of natural killer cells. The expression of Fas and TRAIL is regulated by IFN-γ (Smyth *et al.*, 2005). It is becoming increasingly evident that natural killer cells cannot only directly attack extracellular pathogens, including *Aspergillus* and other fungi, via granule exocytosis (Ma *et al.*, 2004; Schmidt *et al.*, 2011), but the secretion of IFN-γ alone protects the host from infection (Morrison *et al.*, 2003; Park *et al.*, 2009). The activity of natural killer cells in a virus-infected host has been studied in great detail, but the role of natural killer cells during fungal infection is still not well characterized, nor is the effect of IFN-γ secretion and the activity of this molecule against *A. fumigatus*, a denouement explained in part by the current study.

2.2 The Aspergillus fumigatus *stress response*

Aspergillus, though quite capable of causing invasive infection in a human host, does so accidentally (Tekaia and Latge, 2005). The reasons why only a handful of the hundreds of *Aspergillus* species are capable of causing infection are unclear, as is the mechanism enabling these *Aspergillus* species to be successful pathogens. The fungus has evolved mechanisms for surviving in decaying organic matter, its natural niche, but these mechanisms possibly aid the pathogen in avoiding immune clearance by any of the cells detailed in **Figure 2** and promote growth in hostile host tissues (Cramer *et al.*, 2011). The most obvious of these defenses is the complex fungal cell wall, comprising a number of polysaccharides that both activate and moderate the immune response, while protecting the intracellular compartments from exogenous stresses. These sugars include β-1,3/1,4,-glucan, chitin, and galactomannan (Latge, 2007). Intracellularly, *Aspergillus* depends on cytosolic sensors to recognize and respond to environmental stress. Soluble

histidine kinases and response regulators are components of a phosphorelay system required to both respond to environmental cues and for normal cell maintenance (Li *et al.*, 2010b). The system is part of a two-component signalling cascade present in prokaryotes and in eukaryotes, a prominent example in fungi is called the HOG (high osmolarity glycerol) pathway. The HOG pathway was originally identified in *Saccharomyces cerevisiae* as playing a crucial role in maintaining water balance in hyper- and hypoosmotic conditions (Bahn, 2008). In bacteria, virulence and antibiotic resistance are two outcomes of such signalling systems (Mascher, 2006). Since members of two-component phosphorelay systems have not been found in humans, and are valuable for fungal survival under stress, components of this pathway can be excellent drug targets.

2.2.1 The two-component HOG pathway

The HOG pathway is conserved from yeast to fungi and can be divided into two modules: the two-component system and the HOG MAPK (mitogen-activated protein kinase) module (see **Figure 4**). External stresses are sensed by a hybrid sensor kinase (HK), which then autophosphorylates a histidine residue in the histidine kinase domain. The phosphate is transferred through the system: to an aspartate residue in the response regulator receiver domain (REC) of the histidine kinase, then to a histidine residue of a histidine-containing phosphotransfer protein (HPt), and finally to an aspartate residue of the response regulator (RR). The response regulator can act as a transcription factor to trigger the expression of downstream target genes or directly activate the HOG MAPK module. Here again, a phosphorylation reaction runs through the module, now yielding a phosphorylated Hog1 MAPK, which is translocated to the nucleus, where it induces the expression of target genes necessary to defend the fungus from stress (Bahn, 2008). In pathogenic fungi, the HOG pathway governs the response to a wide array of stimuli, including osmotic shock, UV stress, oxidative and heavy metal stress, high temperatures, and osmotic stress (Bahn *et al.*, 2007). There is also evidence for activity of the HOG pathway (directly or indirectly) during growth, differentiation, and infection (Alonso-Monge *et al.*, 1999; Bahn *et al.*, 2006). These systems have been implicated in virulence in both plant and animal pathogens, and though the HOG pathway is highly conserved, little is known about its components and functions in *Aspergillus*, especially *A. fumigatus*.

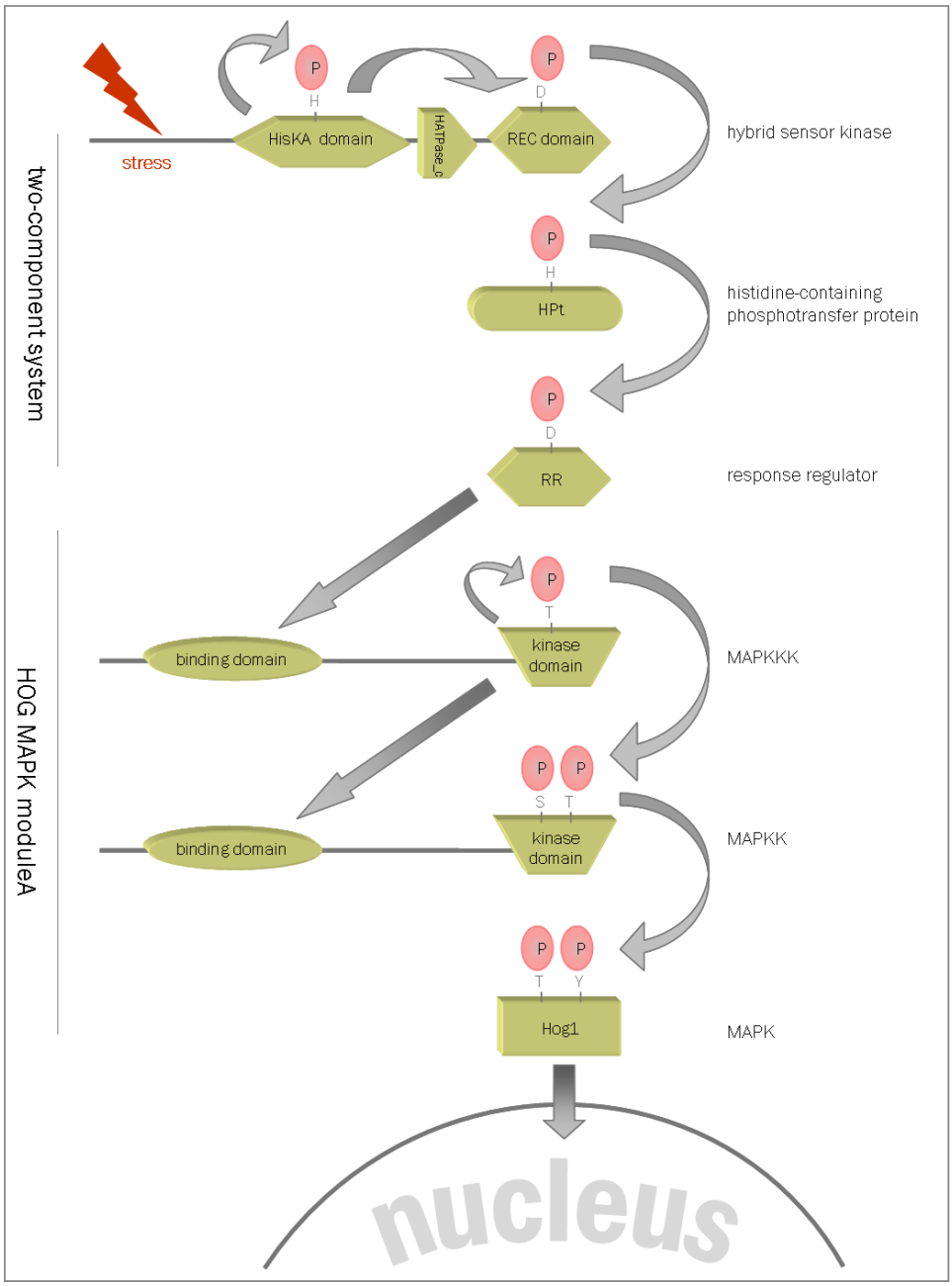

Figure 4. The two-component HOG pathway.

In bacteria and fungi a stress signal is sensed by an HK, which autophosphorylates and then transfers a phosphate through the two-component and HOG MAPK modules to the Hog1 MAPK. Phosphorylated Hog1 translocates to the nucleus where it activates the expression of stress-defensive genes [adapted from (Bahn, 2008)].

2.2.2 Hybrid sensor kinases

Initiating signal transduction through the two-component HOG pathway is a hybrid sensor kinase. Such proteins have been described in prokaryotes, slime molds, plants, and fungi, but not in animals. The fungal hybrid sensor kinases are quite diverse with regards to number in a single species, functions, and domain structures (Bahn, 2008), but among the diverse sensor kinases all those identified to date in fungi exist as hybrids, in which

the histidine kinase and response regulator domains are within a single polypeptide, as is an HATPase_c domain, required for ATP binding. Thirteen putative hybrid sensor kinases have been identified in *A. fumigatus* but only two have been studied functionally: the Group IV HK TcsA and the Group VI HK TcsB (Li *et al.*, 2010a). Deletion of *tcsA* or *tcsB* did not alter the growth or stress resistance of *A. fumigatus* (Du *et al.*, 2006; Pott *et al.*, 2000). It is now the phosphotransfer protein domain that is considered essential. In contrast to other filamentous ascomycetes, plants, and bacteria examined thus far, *A. fumigatus* contains only one histidine-containing phosphotransfer domain protein (Tekaia and Latge, 2005).

Group III hybrid sensor kinases are characterized by amino acid repeats at the N-terminus. These conserved sequences were denoted HAMP domains for the types of proteins in which they were initially discovered (histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins, phosphatases) (Buschart *et al.*, 2012; Li *et al.*, 2010a). Signal transduction occurs down a poly-HAMP chain whereby consecutive HAMP domains interconvert between two conformations (Airola *et al.*, 2010; Dunin-Horkawicz and Lupas, 2010). Group III hybrid sensor kinases have been implicated in virulence of both animal and plant pathogens, namely, the hybrid sensor kinase Nik1 in *Candida albicans* and the homologous BOS1 in *Botrytis cinerea* (Selitrennikoff *et al.*, 2001; Viaud *et al.*, 2006), respectively. In the present study, a single Group III HK in *A. fumigatus* was identified and named TcsC. Additionally, the role of the HOG pathway in *A. fumigatus* stress response and virulence by deletion of the *tcsC* gene was examined.

2.3 The fungus versus the host — Aims of the thesis

The study of any pathogen, or faux pathogen as may be the case with *Aspergillus*, requires not only an understanding of the pathogen itself but an understanding of the body's attack against this invader, and most importantly, an understanding of the interaction between the host and the microbe. The current study attempts this rather daunting task by starting with a simple premise: both the host and pathogen must combat a complex array of stresses as they meet one another. Their individual abilities to deal with these stresses, and in fact continue to thrive in such an environment, may reveal the key to *Aspergillus'* pathogenicity, but also the missing factor(s) in an immune system that is usually capable of dispelling this non-pathogenic, even ubiquitous, invader.

We identified stress signals and defense mechanisms of the innate immune system, namely NETosis and IFN-γ release by natural killer cells in response to *Aspergillus* infection. We simultaneously explored a pathway in *A. fumigatus* involved in defending the microbe from stresses normally encountered within its environmental niche, but also perhaps during infection of a human host.

3. Publication 1

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3.1 Contribution of co-authors

Review written by Frank Ebel and myself in collaboration with Jürgen Löffler.

Allison McCormick	Frank Ebel

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Microreview

Aspergillus fumigatus: contours of an opportunistic human pathogen

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Summary

***Aspergillus fumigatus* is currently the major airborne fungal pathogen. It is able to cause several forms of disease in humans of which invasive aspergillosis is the most severe. The high mortality rate of this disease prompts increased efforts to disclose the basic principles of *A. fumigatus* pathogenicity. According to our current knowledge, *A. fumigatus* lacks sophisticated virulence traits; it is nevertheless able to establish infection due to its robustness and ability to adapt to a wide range of environmental conditions. This review focuses on two crucial aspects of invasive aspergillosis: (i) properties of *A. fumigatus* that are relevant during infection and may distinguish it from non-pathogenic *Aspergillus* species and (ii) interactions of the pathogen with the innate and adaptive immune systems.**

It starts with the mould

Aspergilli are saprophytes that commonly grow on decaying plant material. They are able to utilize a wide range of organic substrates and adapt well to a broad range of environmental conditions. In contact with air the mycelium forms specialized structures, so-called conidiophores. These produce large numbers of conidia (asexual spores) that are efficiently dispersed through the air and inhaled by humans.

Aspergillus fumigatus is currently the most important airborne fungal pathogen causing different kinds of disease depending on the immune status of the host (e.g. invasive and non-invasive pulmonary infections or allergic bronchopulmonary aspergillosis). Most cases of invasive aspergillosis are associated with haematological malignancies, particularly haematopoietic stem cell transplantation, leukaemia or lymphoma. The risk of invasive *Aspergillus* infection is particularly high for patients with persistent neutropenia, graft-versus-host disease (especially with concomitant steroid therapy) and certain types of allogeneic transplantation (Segal *et al*., 2002; Camps, 2008). In all cases, recovery of granulocytes is pivotal for the survival of these patients.

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What makes the difference?

Fungal conidia of many species are inhaled by humans in substantial numbers, but invasive aspergillosis is caused predominantly by *A. fumigatus* and only to a much lesser extent by *Aspergillus terreus*, *A. flavus* and others (Marr *et al*., 2002; Morgan *et al*., 2005), while the other approximately 650 *Aspergillus* species are unable to provoke severe infections. This indicates a selective process that operates even in immunocompromised patients and eliminates most fungal invaders before or when they reach the lower respiratory tract (Fig. 1). Regrettably, our knowledge about this protective mechanism and the stage at which innocuous fungi are eliminated is still in its infancy.

Fungi are important pathogens for insects, amphibians and plants, and since most fungi grow best at ambient temperatures, it was speculated that vertebrate endothermy evolved primarily for protection against fungal infections (Casadevall, 2005). Invasive *Aspergillus* infections usually start in the non-inflamed lung, hence at normal body temperature. Under this condition many *Aspergillus* species are able to germinate and grow. *A. fumigatus* is a particularly thermotolerant organism: its temperature optimum ranges from 37°C to 42°C, but it can grow at up to 55°C and thereby approaches the upper temperature limit of eukaryotic organisms. This suggests that *A. fumigatus* evolved distinct mechanisms of stress resistance that might provide the basis of its virulence. Several mutants obtained by chemical mutagenesis were identified that grow at 42°C, but not at 48°C. Interestingly, none of them was attenuated in virulence (Chang *et al*.,

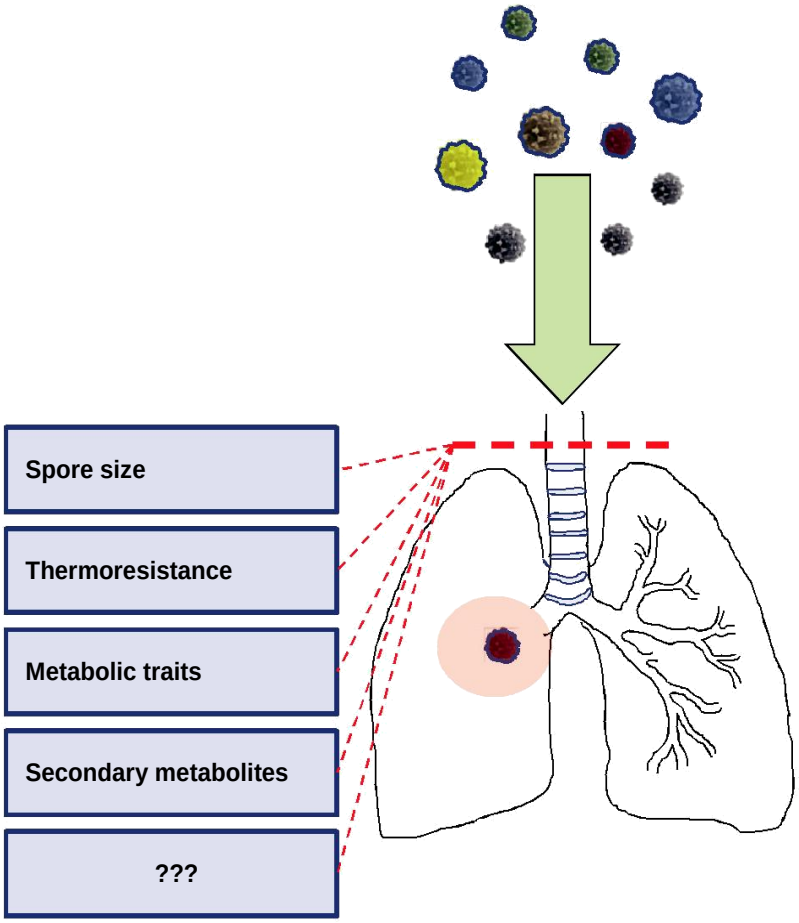

Fig. 1. What makes the difference? Conidia of numerous *Aspergillus* and other fungal species are constantly inhaled by humans, but *A. fumigatus* is responsible for the vast majority of infections. Potential criteria that may decide the success of infection are indicated.

2004). In contrast, disruption of the *cgrA* gene, which reduced growth at 37°C, but not at 25°C, led to an attenuation in virulence in a murine (37°C), but not in a *Drosophila melanogaster* (25°C) model of infection (Bhabhra *et al*., 2004). Further support for a correlation between thermotolerance and pathogenicity came from studies that compared different *A. fumigatus* isolates (Paisley *et al*., 2005) or *Aspergillus* species (Araujo and Rodrigues, 2004). Thus, certain genes that are required for thermotolerance seem to be also relevant for virulence. Thermotolerance may reflect a general hardiness that helps *A. fumigatus* to cope with different stress conditions. Although some traits have been implicated, it is still ambiguous whether they are distinct for *A. fumigatus*. It will be a challenge in the future to prove that human body temperature is the critical parameter that obviates infections by the numerous non-pathogenic *Aspergillus* species.

During infection *Aspergillus* must procure nutrition from the host. The finding that methylcitrate synthase is required for invasive *Aspergillus* infections (Ibrahim-Granet *et al*., 2008) indicates that the fungus feeds mainly on amino acids. This implies that the degradation of pro-teins is crucial during infection and *A. fumigatus* is well equipped with numerous proteases to make amino acids available (Monod *et al*., 2009). However, attempts to define relevant proteases by single mutations failed, as have recent studies in which PrtT, a regulator control-ling a subset of extracellular proteases, was deleted (Bergmann *et al*., 2009; Sharon *et al*., 2009).

According to our current knowledge *A. fumigatus* lacks sophisticated virulence factors that are solely dedicated to permit a pathogenic lifestyle. This distinguishes *A. fumi-gatus* from many bacterial pathogens and reflects its evo-lutionary background as a saprophytic soil dweller. Further research will have to uncover the secrets of its flexibility and robustness that distinguish this important and life-threatening opportunistic pathogen from its numerous non-pathogenic colleagues.

First encounter: host–pathogen interactions in the alveoli

Conidia of *A. fumigatus* are inhaled by humans and, due to their small size, travel deep into the respiratory system. Alveoli are the principal origin of systemic *Aspergillus* infections, although infection may also start from other

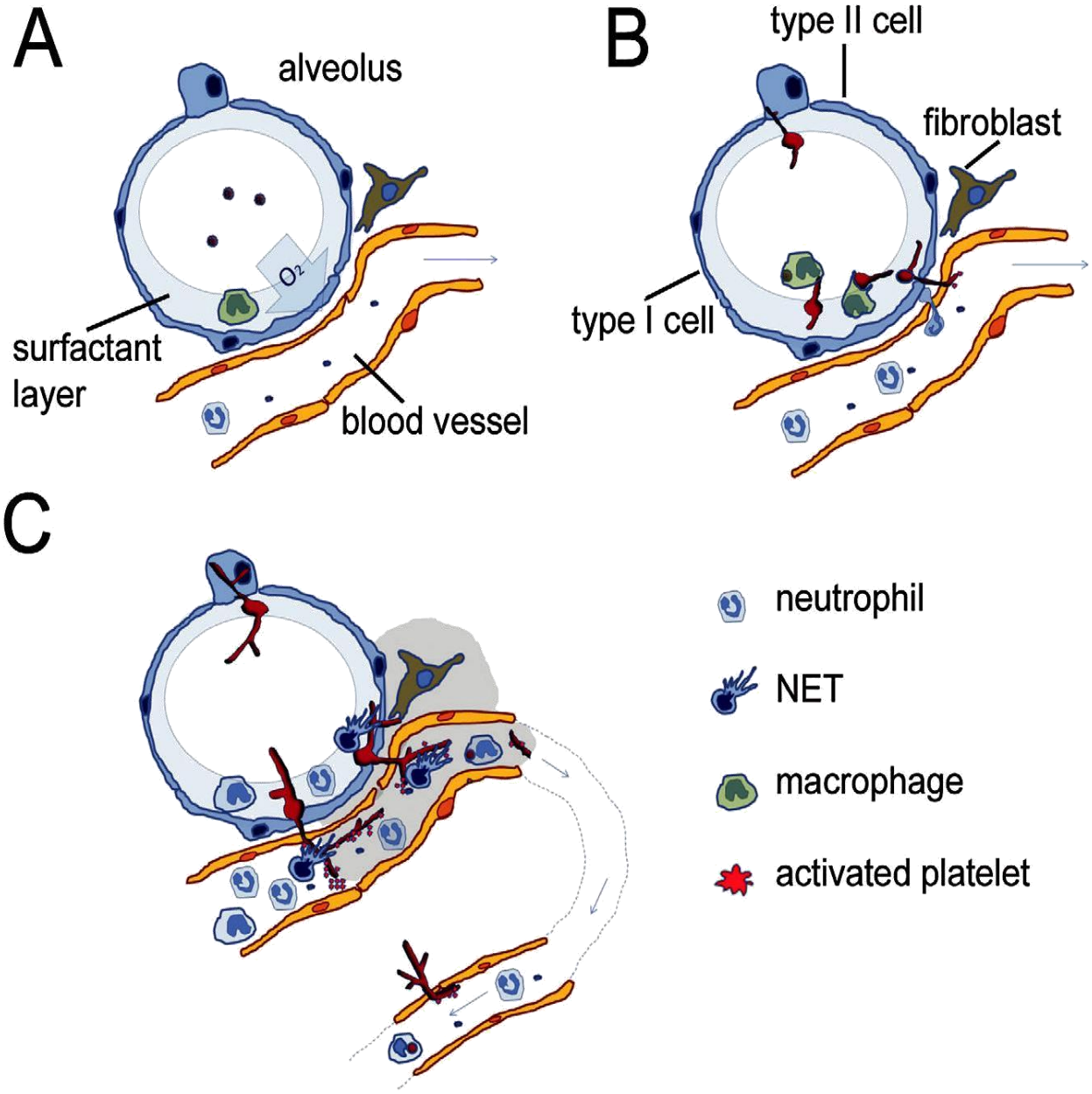

Fig. 2. Schematic representation of the innate immune response at an early stage of infection.
A. Resting conidia arrive in the alveolus.
B. Germination of the spores and initial interactions with alveolar macrophages and alveolar epithelial cells.
C. Later stage of infection characterized by hyphae that infiltrate blood vessels, activation of platelets, establishment of hypoxic conditions (indicated in grey) and vascular spread of infection.

anatomical sites, like the sinus. In the alveolus the fungus germinates in a highly specialized anatomical niche that consists of type I and type II epithelial cells, alveolar macrophages, interstitial fibroblasts and endothelial cells (Herzog *et al*., 2008) (Fig. 2A). The thin and flat alveolar type I cells cover 95% of the alveolar surface and mediate the gas exchange in collaboration with underlying endothelial cells. Fibroblasts produce extracellular matrix proteins and thereby build up the scaffold for the alveolus. Type II cells cover only 5% of the alveolar surface, but play an important role by keeping the alveolar space free of fluid.

Type II cells are additionally involved in the innate immune response. They release opsonins, such as complement and surfactant proteins, to the alveolar space and are able to respond to microbial infections with the production of cytokines (Herzog *et al*., 2008).

Conidia of different *Aspergillus* species were shown to activate the alternative complement cascade and asexual spores from clinical isolates induce a stronger response than non-pathogenic environmental isolates (Dumestre-Pérard *et al*., 2008). Thus, complement produced by alveolar cells might be an important player at this stage of infection.

In vitro, *A. fumigatus* conidia bind efficiently to the surface of A549 cells. These type II-like cells represent the standard model for studying interactions of *Aspergillus* with the alveolar epithelium. Conidia also bind to several matrix proteins, e.g. fibrinogen, laminin, and type I and type IV collagen (Bromley and Donaldson, 1996), and pre-incubation with fibrinogen or laminin impaired conidial binding to A549 cells (Bromley and Donaldson, 1996; Bouchara *et al*., 1997). Hence, receptors for matrix proteins may reside in the surface layer of resting conidia and mediate the primary adhesion to host tissue in the lung. However, the relevance of these findings for the virulence of *A. fumigatus* is still unclear.

Conidia are also internalized by A549 cells and travel to an acidic compartment comprising lysosomal markers. It has been reported that only 3% of these asexual spores survive, suggesting that A549 cells have the ability to kill conidia in a phagosomal compartment. The few conidia that survived in this hostile environment formed germ tubes, breached host membranes and escaped from the infected cell (Wasylnka and Moore, 2003). A549 cells infected with viable *A. fumigatus* conidia release IL-6 and IL-8 (Zhang *et al*., 2002), which underlines the role of type II cells in the innate immune response to fungi and in particular in the recruitment of neutrophils to the site of infection.

The innate immune response

Aspergillus conidia are able to withstand harsh conditions. This is due to a reduced water content, the accumulation of protective molecules in the cytoplasm, and a protective surface layer comprising two hydrophobin proteins (Thau *et al*., 1994; Paris *et al*., 2003) and a melanin layer (Langfelder *et al*., 1998; Tsai *et al*., 1998). Resting conidia shrouded in this hydrophobic mantle are immunologically inert particles (Aimanianda *et al*., 2009). Activation of resting conidia leads to an isotrophic growth that bursts open the rigid surface layer and thereby exposes the carbohydrates of the cell wall. Evidence is mounting to support the importance of fungal-specific glycostructures as target molecules for invariant, germ line-encoded pattern recognition receptors (PRRs) that are crucial for the innate immune response. Currently three main PRRs are believed to participate in the response to *A. fumigatus*: Dectin-1 and the Toll-like receptors TLR2 and TLR4. At least dectin-1 is of general importance for the recognition of fungal pathogens (Herre *et al*., 2004). Recognition of its ligand, β-1,3-glucan, by the innate immune system is evolutionarily old and can be traced back to ancient invertebrates, like the horse-shoe crab *Limulus polyphemus*. *Aspergillus* β-1,3-glucan triggers a strong inflammatory response and enhances phagocytosis by macrophages (Steele *et al*., 2005; Luther *et al*., 2007). Immunocompe tent mice are prone to *Aspergillus* infections if they lack dectin-1 (Werner *et al*., 2009), whereas TLR2 or TLR4 are only required after immunosuppression (Dubourdeau *et al*., 2006). The ligand of dectin-1, β-1,3-glucan, is hardly detectable on resting conidia, but prominent on swollen conidia and germ tubes. Interestingly, it is not traceable on hyphae (Hohl *et al*., 2005), a fact that has been discussed as a fungal stealth strategy. If this *in vitro* observation holds true during infection, the essential role of dectin-1 in the defence of *Aspergillus* is solely based on its importance in the combat of swollen conidia and germ tubes and therefore restricted to a very early stage of infection (Fig. 2B).

The *Aspergillus* molecules that are recognized by other PRRs are still under debate. Given their surface exposure and specificity for fungi, certain carbohydrates are excellent candidates for pathogen-associated molecular patterns (PAMPs) and the exemplary fungal pathogen *Candida albicans* was recently shown to be recognized by the concerted action of three PRRs that detect β-1,3-glucan (dectin-1), O-linked mannan (TLR4) and N-linked mannan (mannose receptor) (Netea *et al*., 2006).

In contrast to *Aspergillus*, *C. albicans* is a yeast and has a long record as a human pathogen. Thus, lessons learned from *Candida* may not necessarily apply to *Aspergillus*. Since purified carbohydrate ligands are usually not available, unambiguous proof for the rel-evance of certain *Aspergillus* glycostructures as PAMPs depends on appropriate mutants. Mutants in key enzymes of protein O-glycosylation and glycolipid syn-thesis have been analysed, but revealed no phenotype with respect to cytokine release in murine macrophages (Wagener *et al*., 2008; Kotz *et al*., 2010). This might be the consequence of a fundamental difference between yeasts and filamentous fungi: *C. albicans* produces highly mannosylated proteins and glycolipids, whereas comparatively smaller glycoconjugates are characteristic of *A. fumigatus*.

Resident alveolar macrophages engulf conidia and respond to this encounter by producing cytokines and chemokines. This triggers a massive recruitment of neutrophils, which is the hallmark that distinguishes a substantial inflammation from a daily skirmish. Neutrophils patrol through the bloodstream and have to be attracted to the site of infection. They are the executors of the acute inflammatory response and the particular susceptibility of granulopenic patients to severe *Aspergillus* infections underlines their relevance. Depletion experiments also assigned a critical importance to neutrophils, but not to alveolar macrophages (Mircescu *et al*., 2009). The ability of neutrophils to attack and kill *A. fumigatus* depends on TLR2, TLR4 and dectin-1 (Bellocchio *et al*., 2004; Werner *et al*., 2009). Elimination of conidia and small germ tubes is accomplished by phagocytosis, while the release of

microcidal molecules enables neutrophils to attack larger hyphal cells. Recently, the formation of neutrophil extracellular traps (NETs) triggered by *A. fumigatus* was demonstrated in the infected lung (Bruns *et al*., 2010). NETs represent an anti-microbial effector mechanism that mediates killing of a diverse range of bacterial pathogens as well as *C. albicans* (Papayannopoulos and Zychlinsky, 2009). NETs are unable to eliminate *A. fumigatus*, but reduce hyphal growth by depleting zinc ions (McCormick *et al*., 2010), a mechanism that might be valuable to confine infection.

A rapid influx of neutrophils into the lung can be observed in mice that inhaled larger numbers of conidia. After 2–3 h, samples obtained by bronchoalveolar lavage contained large aggregates of neutrophils and conidia, and germination was shown to be inhibited over a period of 24 h (Bonnett *et al*., 2006). In contrast, *gp91phox-/-*mice, which are deficient in phagocyte NADPH oxidation and therefore production of reactive oxygen species (ROS), are already susceptible to low doses of conidia (Bonnett *et al*., 2006). Alveolar macrophages from p47phox-/- mice, which are also deficient in ROS production, are impaired in killing of *A. fumigatus* (Philippe *et al*., 2003). These findings are in line with the fact that patients with Chronic Granulomatous Disease (CGD) who are deficient in ROS production are also more susceptible to *Aspergillus* infections. However, the concept that ROS are pivotal for killing of *Aspergillus* is still under debate. A *yap*1 mutant, although highly sensitive to ROS, behaved as wild type in confrontation experiments with human neutrophils and in a murine model of infection (Lessing *et al*., 2007), whereas a triple mutant lacking all three superoxide dismutase genes was more efficiently killed by macrophages, but not attenuated in virulence (Lambou *et al*., 2010). Mutations in the *tmpL* and the conidial catalase A gene are sensitive to oxidative stress *in vitro* and attenuated in virulence (Kim *et al*., 2009; Ben-Ami *et al*., 2010); however, killing assays with murine alveolar macrophages revealed no difference between the *catA* mutant and the wild type (Paris *et al*., 2003).

Recent data demonstrate that CGD patients have an impaired ability to form NETs. Restoration of ROS production by gene therapy was shown to reconstitute NET formation and to protect a CGD patient from a severe *Aspergillus nidulans* infection (Bianchi *et al*., 2009). The recent findings that NADPH oxidase restrains the innate immune response and limits inflammation provides another important tool to better understand the particular sensitivity of CGD patients to recurrent infections (Segal *et al*., 2010). Thus, apart from a potential direct action on microbes, ROS seem to play an important role in directing the innate immune response.

Natural killer (NK) cells represent a further facet of innate immunity. They are recruited early during *Aspergillus* infection and participate in the anti-fungal response (Morrison *et al*., 2003). At this stage, NK cells are the major source of IFN-γ (Park *et al*., 2009), a cytokine that is known to increase the microbicidal activity of phagocytes. Further studies are clearly required to define the role of NK cells in anti-*Aspergillus* immunity.

The large pentraxin PTX3 belongs to a family of acute phase proteins that represent the major humoral arm of innate immunity. PTX3 is produced by macrophages and epithelial cells in response to infection. Moreover, it is stored in larger quantities in the granules of neutrophils that release PTX3 during NETosis (Jaillon *et al*., 2007). PTX3 is an opsonin that mutually binds to the complement protein C1q and ficolin-2, a recognition molecule of the lectin complement pathway (Ma *et al*., 2009). Thus, PTX3, C1q and ficolin-2 might form complexes on the conidial surface and thereby amplify the innate immune response. Remarkably, PTX3 deficiency renders immuno-competent mice highly susceptible to *A. fumigatus* infection (Garlanda *et al*., 2002). Early administration of PTX3 enhances the conidiocidal activity of neutrophils and limits the inflammatory pathology (D'Angelo *et al*., 2009). The latter effect can be attributed to a faster elimination of PTX3-opsonized conidia (Garlanda *et al*., 2002) and a reduced neutrophil recruitment due to the binding of PTX3 to P-selectin (Deban *et al*., 2010). A fast elimination of PTX3-opsonized conidia and a concomitantly restrained inflammation provide a rationale for the fact that NADPH oxidase-deficient mice can be protected by the exogenous administration of PTX3 (D'Angelo *et al*., 2009).

Invasive pulmonary aspergillosis: tissue invasion and inflammation

After penetration of the epithelial layer of the alveoli, the fungus immediately comes in direct contact with the underlying blood vessels (Fig. 2C). Here, *A. fumigatus* requires no sophisticated adhesion and invasion mechanisms to breach epithelial or endothelial barriers. Instead it can rely on the robust architecture of its cell wall and the enormous driving force of the polarized hyphal growth. *Aspergillus* is a so-called angiotrophic fungus and infection of vessels is a characteristic histopathological feature of invasive *Aspergillus* infections (Kradin and Mark, 2008). As an organism that is used to growing in complex organic matter, *A. fumigatus* has a well-developed ability to follow gradients; during infection this will guide hyphae to blood vessels that transport oxygen and carbohydrates. Angioinvasion often results in infarction and consequently in reduced oxygen supply (Fig. 2C). Recruitment of neutrophils will furthermore disturb the integrity of the endothelial and epithelial barriers. Local obstruction of the airways may induce oedema, alveolar flooding and completely shut down the

oxygen supply. Consequently, the fungus has to adapt to a hypoxic environment. According to our current knowl-edge, *A. fumigatus* relies on its oxidative energy metabo-lism to do so. The putative transcription factor SrbA is essential for hypoxic adaptation and virulence (Willger *et al*., 2008). This important finding demonstrates that the adaptation to hypoxia is a prerequisite for the survival of *A. fumigatus* in the inflamed tissue and its ability to spread to different organs.

It has become evident only recently that hypoxia is also a strong signal to immune cells. Effector cells that are recruited from the bloodstream, like neutrophils and monocytes, travel along an oxygen gradient when enter-ing inflamed tissue. Hypoxia is deciphered by these cells as an activating signal and HIF-1, the central transcrip-tional activator of hypoxic adaptation in mammalian cells, activates the anti-microbial activities of phagocytes and has been discussed as a master regulator of the innate immune response (Nizet and Johnson, 2009). It will be a challenge, in future analyses of the innate immune response to *A. fumigatus*, to consider the hypoxic adaptation of both the pathogen and the host.

The adaptive immune response to *A. fumigatus*

The innate and the adaptive immune responses generally collaborate to defeat infections. T and B lymphocytes represent the two parts of the adaptive immune system. In contrast to the combat-ready innate defence, the adaptive response follows afterwards and reacts to signals originating from the innate immune response. The daily housekeeping work of eliminating inhaled fungal conidia relies solely on the innate immune system, whereas a concerted action of the innate and adaptive immune systems is required to fight established and potentially life-threatening infections.

Aspergillus-specific antibodies have been detected in immunocompromised patients suffering from invasive aspergillosis, but their functional importance is often con-sidered minor. However, administration of β-1,3-glucan-specific antibodies can be protective (Torosantucci *et al*., 2005) and the importance of antibodies in protection against aspergillosis clearly deserves more attention.

While the role of B cells is still under debate, it is generally accepted that T cells play an important role in the defeat of aspergillosis and fungal infections in general. The T cell response is in many ways linked to the innate immune response. Dendritic cells (DCs) infiltrate the infected region, differentiate in response to the pathogen and, when loaded with antigen, migrate to the draining lymph nodes to instruct T cells. T cells have the ability to either activate phagocytes or limit the immune response. The collateral tissue damage caused by an exaggerated inflammatory response contributes substantially to the morbidity of *Aspergillus* infections and the control of immune effector cells is therefore of prime importance. DCs are located at the cross-roads and direct the immune system either towards a balanced and protective Th1 or towards an excessive, inflammatory Th17 response. Production of IFN-γ by Th1 cells is fundamental to optimize the microbicidal activity of phagocytes. In contrast, stimulation of Th17 cells and the production of IL-23 by DCs promote a destructive inflammatory response and impair anti-fungal resistance (Zelante *et al*., 2007). Regulatory T cells (Tregs) limit the inflammatory response steered by Th1 cells and act in an antagonistic fashion to Th17 cells.

In conclusion, an efficient anti-*Aspergillus* immune response requires the coordinated actions of innate and adaptive immunity. Both arms are part of a highly inter-connected and interdependent network that must be finely tuned in order to find balance between protection and immunopathology. The adaptive immune system repre-sents the regulatory part and is crucial to activate, direct and finally limit the innate immune response, especially neutrophils which act as the major executors of aggressive anti-fungal measures.

As a tightly controlled innate immune response is pivotal to eliminate the pathogen, resolve inflammation and initiate tissue repair, attempts have been undertaken to develop new therapeutic concepts aimed at modulation of the adaptive immune response. The adoptive transfer of Th1 cells has already been successfully applied to treat human patients (Perruccio *et al*., 2005) and effective DC vaccination has been described in a murine model of infection (Bozza *et al*., 2003). More recently, an siRNA approach has been successfully applied in a similar infec-tion model to optimize the host response by dampening PI3K/Akt/mTOR inflammatory pathways (Bonifazi *et al*., 2010).

Systemic spread of infection

During invasive *Aspergillus* infection, hyphae commonly target blood vessels, as mentioned above. This often results in thrombosed vessels and the appearance of targetoid lesions (Kradin and Mark, 2008). Hyphae and conidia activate platelets *in vitro* and this host–pathogen interaction probably promotes thrombosis and contributes to inflammation *in vivo* (Rødland *et al*., 2010). The propensity to invade blood vessels is also a means for dissemination via the bloodstream. Viable fungal cells are rarely found in the peripheral blood, a fact that severely hampers diagnosis of disseminated *Aspergillus* infections. This is a consequence of the hyphal architecture that establishes tight cellular cohesion by a common cell wall and prevents the release of single cells or fragments. However, a detachment of short hyphal segments may

occasionally occur and drive the systemic spread of infection. Alternatively, phagocytes may ingest small fungal elements and displace them (Fig. 2C). Secondary blood vessel-borne infectious foci often grow with a characteristic sunburst appearance (Kradin and Mark, 2008). Thrombosis is a common feature of these lesions and instrumental to generate hypoxic conditions. *Aspergillus* furthermore inhibits angiogenesis through production of secondary metabolites, like gliotoxin, and thus enforces the formation of hypoxic conditions (Ben-Ami *et al*., 2009). The resulting disseminated abscesses are the hallmark of this late stage of infection and appear in different organs.

One of the major complications during *Aspergillus* infection is its dissemination into the central nervous system (CNS), surmounting the blood–brain barrier. CNS aspergillosis has been diagnosed with increasing frequency over the past decade; parenchymal abscesses represent the majority of these with true meningitis being rare. Although the blood–brain barrier consists of tight junctions between all endothelial cells in capillaries supplying brain cells, *A. fumigatus* is able to overcome this barrier and to penetrate into the cerebrospinal fluid (CSF). Infiltration of *A. fumigatus* into the CNS is often fatal because of reduced penetration capacities of most anti-fungal agents and impaired numbers of immune cells present in the CSF (Schwartz and Thiel, 2009).

Concluding remarks

Systemic infections by *A. fumigatus* are only found in patients with severely impaired immune defences. Clearly, such infections are rather a consequence of modern medicine and cannot have influenced the evolution of this opportunistic pathogen. So far, there is no evidence that *Aspergillus* acquired pathogenic traits in host–pathogen interactions with, for example, predatory protozoa. Also sequencing of the genome did not provide any hints for the presence of classical virulence factors. But in order to survive in the soil, *A. fumigatus* acquired a high level of stress tolerance and flexibility that could provide a basis for its pathogenicity. However, to distinguish itself from non-pathogenic moulds *A. fumigatus* appears to keep additional secrets that have yet to be disclosed.

Infections by filamentous fungi are a severe medical problem characterized by an increasing number of cases and limited therapeutic options. Hence, the identification of new therapeutic targets is an urgent need. Filamentous fungi rely on polarized hyphal growth to invade tissues and cross barriers. Therefore, studies on the hyphal organization and cell biology may uncover new Achilles heels of these pathogens.

During infection *Aspergillus* has to deal with changing conditions at different anatomical sites. For a deeper understanding of the interactions between the pathogen and the host, the experimental conditions have to be adapted to the reality of the infected tissue. Environmental parameters, such as the oxygen concentration, have to be considered and infection models have to mirror the complexity of the immune response. Travelling along this road will enable us to further shape the contours of this opportunistic pathogen.

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4. Publication 2

McCormick,A., Heesemann,L., Wagener,J., Marcos,V., Hartl,D., Loeffler,J., Heesemann,J., Ebel,F., 2010. NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. Microbes. Infect. 12, 928-936.

4.1 Contribution of co-authors

Heesemann, L. generated the α-galactomannan antibody (L10-13) used for immunofluorescence. Wagener, J. created the D141-mito-GFP strain used for the germ tube viability assay. Marcos, V. and Hartl, D. kindly provided us with neutrophils from CGD patients and aliquots of α-neutrophil elastase antibody used for immunofluorescence. Funding provided by the Sander Stiftung in collaboration with Loeffler, J. Frank Ebel and I wrote the manuscript and I performed all experiments.

Allison McCormick	Frank Ebel

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Original article

NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*

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Abstract

Neutrophil extracellular traps (NETs) represent a distinct mechanism to control and eliminate microbial infections. Our results show that conidia and germ tubes of the human pathogenic mold *Aspergillus fumigatus* are able to trigger the formation of NETs. Viable fungal cells are not essentially required for this host-pathogen interaction. Neutrophils engulf conidia and thereby inhibit their germination, a process that is independent of NETosis. In the experimental set-up used in this study neutrophils do not kill germ tubes, but reduce their polar growth and this inhibition depends on NETs as it can be overcome by the addition of DNase-1. The Zn^{2+} chelator calprotectin is associated with the *Aspergillus*-induced NETs and addition of Zn^{2+} abrogates the NET-mediated growth inhibition. In summary, our data provide evidence that NETs are not sufficient to kill *A. fumigatus*, but might be a valuable tool to confine infection.

Keywords: Aspergillus fumigatus; NET; Neutrophils; Innate immune response; Fungal infection

1. Introduction

Aspergillus fumigatus is the most prevalent airborne fungal pathogen causing life-threatening infections in immunocompromised patients. Although immunocompetent humans inhale several hundred *A. fumigatus* spores per day they commonly do not develop a detectable disease [1]. This protection relies essentially on cells of the innate immune system. Phagocytes are crucial for clearance of inhaled conidia and in particular neutrophils are thought to kill hyphae, which are too big to be phagocytosed [2]. Alveolar macrophages were originally thought to clear infection; however, this notion has been recently challenged by the finding that neutrophils, but not alveolar macrophages, are essential for clearance of *A. fumigatus* infections [3].

Neutrophils are in the first line of defense against microbial infections. They are short-lived, versatile cells that must be recruited to the site of infection [4]. Neutrophils can combat microbes by at least three distinct mechanisms: (1) phagocytosis, (2) release of anti-microbial molecules and (3) formation of neutrophil extracellular traps (NET). These NETs were described only recently [5]. They consist of a scaffold of DNA decorated by a distinct set of cytoplasmic and granular proteins, while other abundant cytoplasmic proteins do not associate with NETs [6].

The formation of NETs can be triggered by a variety of microbes or alternatively by interleukin 8 (IL-8) or phorbol myristate acetate (PMA). Several pathogens, such as *Staphylococcus aureus*, *Salmonella enterica* and *Candida albicans* induce NETs and when trapped within these structures are eliminated by NET-mediated killing [7]. Several proteins have been implicated in the anti-microbial activity of NETs, e.g., histones, elastase and calprotectin [5,6,8].

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Induction of NETs has been characterized in detail for *C. albicans* yeasts and hyphae [9] and first evidence that NET formation can be triggered by *A. fumigatus* conidia has been published [10]. In this study, we analyzed the ability of *A. fumigatus* to trigger NET formation as well as the impact of NETs on germination and growth of this fungal pathogen.

2. Materials and methods

2.1. Strains and materials

The *A. fumigatus* strain ATCC 46645 was used throughout this study. If required, fungal cells were killed by exposure to ultra violet (UV) light as follows. Resting conidia (RC) were incubated in 20 ml RPMI1640 plus 50 μg/ml gentamicin in a closed 50 ml Falcon tube overnight at room temperature to synchronize germination. Germ tubes (GT) were generated by incubating an additional 3-4 h at 37 °C with shaking. Morphotypes were verified microscopically. Cells were then centrifuged at 5000 g for 10 min at room temperature and resuspended in sterile deionized water. The fungal cells were subjected to UV-light in a sterile Petri dish for 4-6 h and then washed once in sterile deionized water. Killing was confirmed by plating the cells in RPMI1640 and confirming no growth occurred over a period of 1-2 days.

2.2. Isolation of neutrophils

Whole blood from healthy donors was diluted in an equal volume of PBS, layered over Ficoll solution (GE Health-care), and centrifuged at 300 g for 20 min at room temperature. The supernatant was removed; the remaining cells were washed once in PBS, and centrifuged at 200 g for 10 min at 4 °C. The supernatant was removed and the pellet resuspended in cold ammonium chloride lysis buffer. Lysis was carried out for 30 min at 4 °C followed by centrifugation at 200 g for 10 min at 4 °C. The cells were again resuspended in cold lysis buffer and incubated for 10 min at 4 °C. Neutrophils were then harvested by centrifugation and resuspended in RPMI1640.

2.3. Immunofluorescence

The monoclonal antibody (mab) L10-13 was raised by immunization of mice with culture supernatant of *A. fumigatus* grown in 2% collagen-water medium. This hybridoma was identified in a screening for antibodies that stained *A. fumigat*us hyphae in immunofluorescence. Primary antibodies to neutrophil elastase and calgranulin A were purchased from Abcam (ab21595) and Santa Cruz Biotechnology (sc-20174), respectively. The cover slips were first blocked in 1:100 goat serum in PBS at 37 °C for 30 min and washed once with PBS. Primary antibodies were used at 1:50 in PBS or as undiluted hybridoma supernatant at 37 °C for 1 h. The cover slips were then washed three times in PBS. Secondary antibodies, α-mouse IgM-Cy3 (Jackson ImmunoResearch) or α-rabbit-FITC (Abcam) were used at 1:1000 and 1:100, respectively, at 37 °C for 1 h. The cover slips were again washed three times with PBS and then mounted to glass slides in Vectashield containing DAPI (Vector Laboratories) and analyzed by fluorescence microscopy using a Leica SP-5 confocal laser scanning microscope (Leica Microsystems).

2.4. Germ tube preparation

RC were incubated overnight as described above. GT were generated by incubating at 37 °C for another 4 h with shaking. If necessary, the GT were stained with a final concentration of 0.3 mg/ml fluorescein-isothiocyanate (FITC, Sigma) in 0.1 M sodium carbonate buffer, pH 10.0. The staining was done at 37 °C for 30 min, with shaking, in the dark. Stained GT were washed twice with sterile deionized water and used immediately for infection experiments.

2.5. Resting conidia growth inhibition assay

Isolated neutrophils were seeded at 8×10^5 cells per well in 6-well plates with 3 ml RPMI1640 plus 50 μg/ml gentamicin and three poly-L-lysine-coated glass cover slips per well. Where appropriate, neutrophils were incubated with 25 nM PMA (Sigma) at 37 °C, 5% CO_2 for 30 min. The medium was then removed and fresh RPMI1640 added. PMA-treated neutrophils recovered an additional 20 min at 37 °C, 5% CO_2. If Cytochalasin D was used to inhibit phagocytosis, neutrophils were incubated in a final concen-tration of 10 μg/ml for 1 h prior to infection. If DNase-1 (Roche Applied Sciences, Mannheim, Germany) was used to degrade NETs, it was added at a final concentration of 20 U/ ml to neutrophil cultures prior to infection. 2.4×10^6 RC were added to each well and the infection carried out at 37 °C, 5% CO_2. At 6 h incubation, cells were fixed in 4% paraformaldehyde and stored in this solution at 4 °C until stained for immunofluorescence. Swollen conidia were distinguished from RC by immunofluorescence using the monoclonal antibody (mab) L10-13 and an appropriate Cy3-labeled secondary antibody. Samples were analyzed using a Leica SP-5 and the percentage of swollen conidia was determined using software provided with the microscope. At least 300 conidia were counted in total for each experimental parameter. Conidial growth in the absence of neutrophils was used as a control.

2.6. Germ tube viability assay

We generated an *A. fumigatus* mito-GFP strain to analyze viability of hyphae at a single cell level. Briefly, we inserted a phleomycin resistance cassette of pJW104 into the mito-GFP construct pRS54 [11]. The resulting plasmid pJW106 was transformed into D141 and positive clones were screened by fluorescence microscopy. Live cell microscopy studies were performed using a Leica SP-5 microscope (Leica Microsystems).

2.7. Germ tube growth inhibition assay

Neutrophils were seeded as above with the addition of PMA or DNase-1 in the same manner, as appropriate. 2.4 x 10^6 FITC-stained GT were added to each well and the infection carried out at 37 °C, 5% CO_2 for 3 h. Cells were fixed in 4% paraformaldehyde and stored in this solution at 4°C until stained for immunofluorescence. Hyphal growth during the experiment was determined after immunofluorescence staining using mab L10-13 and an appropriate Cy3-labeled secondary antibody. Samples were analyzed using a Leica SP-5. The length of the GT grown beyond the FITC-labelled portion was measured using software provided with the microscope. At least 100 randomly chosen GT were measured for each experimental parameter. Fungal growth in the absence of neutrophils was used as a control.

2.8. Neutrophil viability assays

To analyze neutrophil apoptosis during NET formation, neutrophils and fungal cells (GT or RC, without FITC-labeling) were co-incubated as above for 3 h. Cells were fixed in 4% paraformaldehyde and stored in this solution at 4 °C until stained for immunofluorescence. Fungal cells were labeled with the mab L10-13 and an appropriate Cy3-labeled secondary antibody. To analyze cell death, cells were stained with DAPI and apoptotic cells were labeled using an In Situ Cell Death Detection Kit (Roche Applied Sciences) according to the manufacturer's instructions. Briefly, a TUNEL reaction mixture, which incorporates fluorescein dUTP at DNA strand breaks, was used to detect apoptotic neutrophils. NETs were labeled with DAPI, present in the mounting medium.

To analyze neutrophil death during NET formation, an LDH Cytotoxicity Assay Kit (Cayman Chemical Company) was used according to the manufacturer's instructions. Briefly, lactate dehydrogenase (LDH) present in the cell culture supernatant was calculated by measuring the amount of formazan (which absorbs light strongly at 490 nm) produced. LDH released into the medium as a result of cell death is proportional to the amount of formazan produced.

2.9. Statistical analysis

The Student's t-test was used to determine the significance of differences. Results were plotted using Sigma Plot (Systat Software).

3. Results

Neutrophils are supposed to play a crucial role in the combat of *A. fumigatus* infections, but the precise mechanisms employed by this innate immune cell to kill the fungi are not well defined yet. We analyzed the interactions of human neutrophils and viable resting conidia (RC) of *A. fumigatus*. During co-incubation, the highly motile neutrophils engulfed many spores within the first hour (data not shown). After 3 h some neutrophils died and released large amounts of DNA.

Elastase, a characteristic component of NETs, was found in association with the extracellular DNA (**Fig. 1A** and **B**). Similar NET-like structures were observed when neutrophils were challenged with viable *A. fumigatus* germ tubes (GT) (**Fig. 1C**), whereas neutrophils cultured 3 h without fungal cells, appeared to be intact and elastase was only detectable after permeabilization of the membranes (**Fig. 1D**). We also challenged PMNs with UV-killed *A. fumigatus* conidia and GT, both were able to trigger NET formation as their viable counterparts (**Fig. 1E** and data not shown).

We quantified the release of lactate dehydrogenase (LDH) as a measure of cell death. PMA treatment of PMNs led to high LDH activity in the supernatant (approx. 80,000 µU/ml). Relative to this value, the release of LDH by non-treated PMNs and neutrophils challenged with either viable RC or GT reached approximately 10, 40 and 50%, respectively. Similar rates of cell death were obtained if cells were counted individually after DAPI staining (**Fig. 2B**). A parallel TUNEL staining revealed that only very few untreated PMNs showed clear signs of apoptosis, whereas the majority of staurosporine treated cells became apoptotic. Incubation with GT or RC did not increase the number of apoptotic cells compared to the untreated control (**Fig. 2B**). Breaks of the DNA strands, which are visualized by the TUNEL assay, were not detected in the NETs (**Fig. 2C** and **D**). In conclusion, these data suggest that the apoptotic program of neutrophils is not activated by *A. fumigatus* RC and GT.

NET-mediated killing of *C. albicans* has been analyzed using a plating assay to determine the number of surviving fungal cells [6]. Swollen conidia and GT of *A. fumigatus* have a strong tendency to form aggregates, a fact that severely hampers analysis based on plating assays. We therefore studied the impact of NETs on *A. fumigatus* at the single cell level. For this purpose we used mab L10-13 that recognizes swollen conidia and GT, but not RC (**Fig. 3A**). The reactivity of L10-13 mab was not influenced by proteinase K treatment, but was abrogated after perjodate and mild acid treatment (data not shown). These results and the fact that L10-13 belongs to the IgM isotype strongly suggest that L10-13 recognizes a surface exposed carbohydrate of the *A. fumigatus* cell wall that comprises galactofuranose (data not shown).

After co-incubation of viable RC and neutrophils samples were stained with L10-13 and DAPI and the percentage of swollen, L10-13 positive conidia was determined. The presence of neutrophils clearly reduced the percentage of swollen conidia after 6 h (**Fig. 3B**). We analyzed whether PMA as an additional trigger of NET formation [5] enhances the inhibitory effect on conidial germination, which was not the case (data not shown). We then analyzed germination separately for three conidial subpopulations: (1) spores that were associated with NETs, (2) spores that were associated with intact and apparently viable PMNs and (3) extracellular conidia that were not associated with PMNs or NETs. The percentage of swollen conidia for these three subpopulations is given in **Fig. 3C**. Germination of PMN-associated spores was clearly reduced compared to extracellular spores. Strikingly, germination of NET-associated conidia was not inhibited.

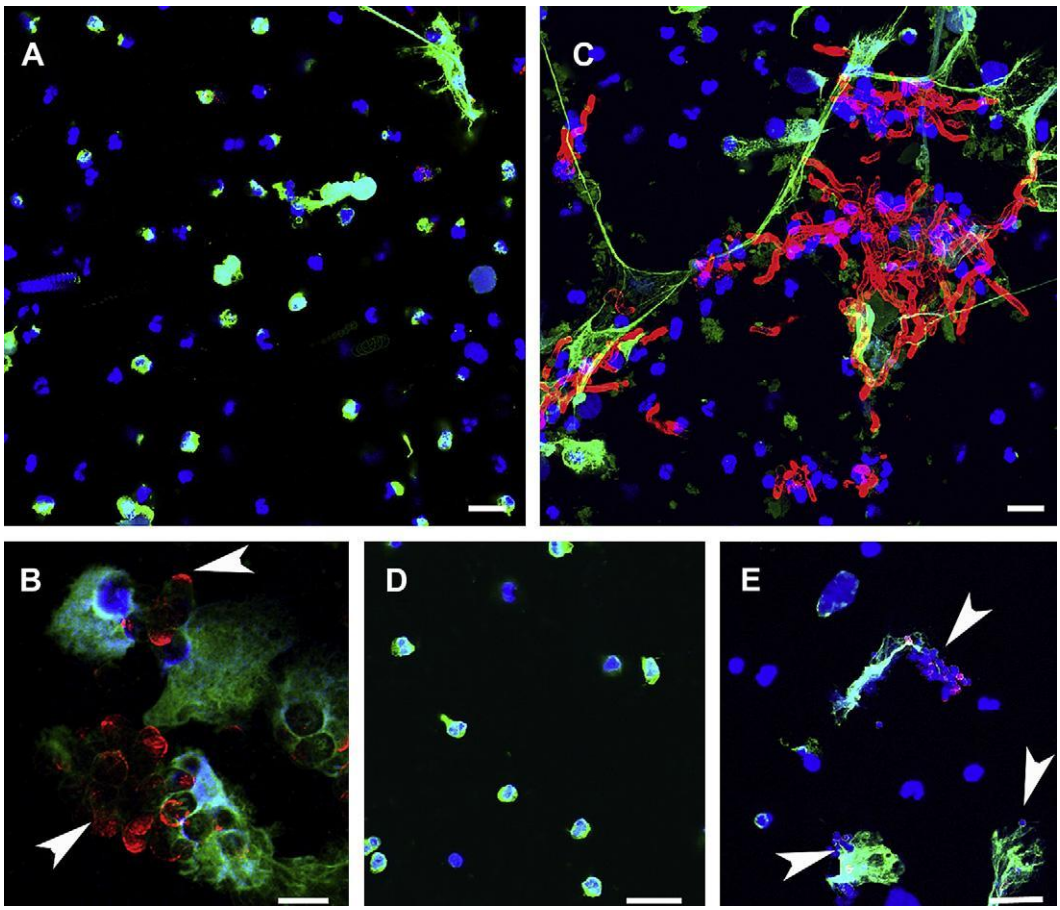

Fig. 1. *A. fumigatus* induces the formation of NETs by human neutrophils. Isolated neutrophils were incubated for 3 h in the presence of viable RC (panels A and B), viable GT (panel C), UV-killed conidia (panel E) and as a control without fungal cells (panel D). DNA was visualized by DAPI, shown in blue, fungal cells were stained in red using mab L10-13 and neutrophil elastase is depicted in green. Neutrophils in panel D were permeabilized to detect intracellular elastase. No permeabilization was performed in A, B, C and E. Clusters of conidia are indicated by arrowheads in B and E. Bars indicate 30 μm in A and C, 5 μm in B and 25 μm in D and E (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To analyze whether phagocytosis was responsible for the reduced germination of cell-associated spores, we treated PMNs with cytochalasin D. Under this condition, neutrophils were viable (**Fig. 2B**), but the conidia remained largely extracellular and the vast majority germinated after 6 h (**Fig. 3C**). We then induced NETosis by pre-treatment of neutrophils with PMA and incubated conidia with the resulting NETs in the presence of cytochalasin D. Although NETs were formed (data not shown) no inhibition of germination was detected (**Fig. 3C**). DNase-1 treatment of PMNs, which is known to degrade NETs and supplementation of the medium with Zn^{2+} ions had no impact on the level of germination (data not shown). Taken together, these data show that NETs are induced by *A. fumigatus* conidia, but are not able to inhibit germination.

To analyze the impact of NETs on the viability of *A. fumigatus* hyphae we used an *A. fumigatus* strain with GFP-tagged mitochondria. Fungal mitochondria are organized as a dynamic tubular network that continually changes its shape and moves throughout the cell [12]. The dynamic reorganization of these organelles reflects the cellular vitality. Co-incubation of this strain with NETs for several hours had no impact on the dynamics of the mitochondrial network (**supplementary file S1**) indicating that NETs were not able to kill hyphae.

We then determined the growth of GT in the presence of neutrophils. For this purpose we adapted a method described recently by Wozniok et al. [13]. GT were labeled with FITC and then co-incubated with neutrophils. At the indicated time points samples were fixed and stained with L10-13. Polar growth of hyphae resulted in terminal FITC-negative segments that were stained with L10-13. A representative picture of such a staining is shown in **Fig. 4A**. We determined the length of the FITC-negative segments as a measure for growth of the GT. The impact of PMNs on GT growth is shown in **Fig. 4B**. As before, we observed no evidence for killing of hyphae by NETs. However, we measured a reduced hyphal growth in the presence of NETs. The median values for the control hyphae and those co-incubated with neutrophils were 12.04 μm and 7.80 μm, respectively. This difference was highly significant (p value <0.0001) and reproducible in several experiments. In contrast to RC, no difference in hyphal growth was evident in the presence of DNase-1 (**Fig. 4C**) indicating that the formation of NETs is a prerequisite for the observed growth inhibition.

Calprotectin has recently been described as an important constituent of NETs and evidence has been provided that this

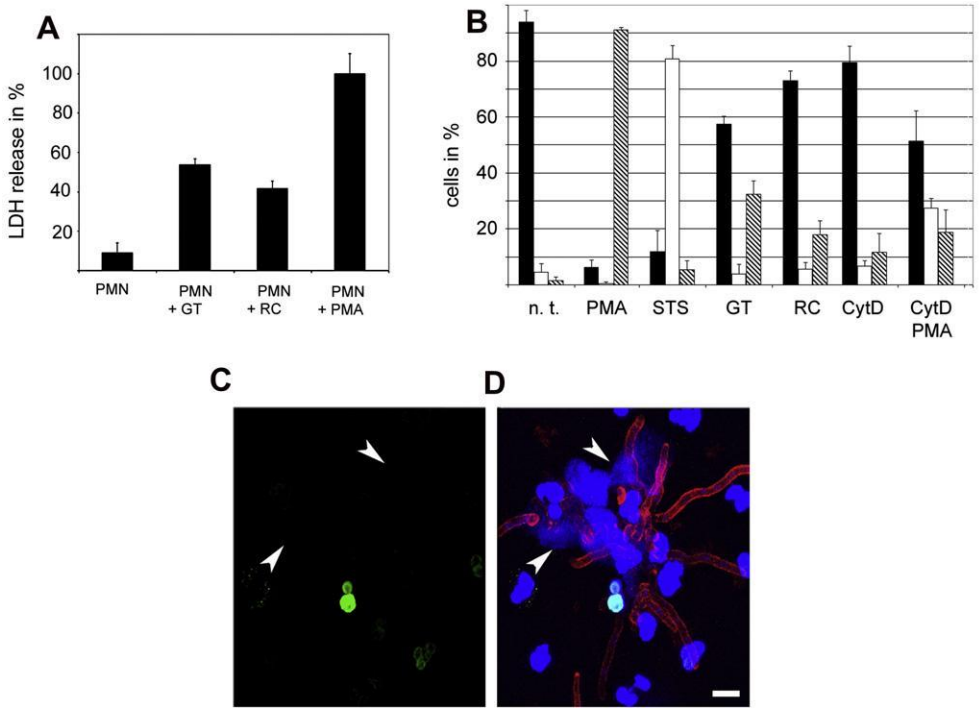

Fig. 2. Quantification of cell death and apoptosis in response to *A. fumigatus*. Panel A: Release of lactate dehydrogenase (LDH) was measured after 3 h to determine cell death. PMN treated with PMA formed many NETs and most of the cells died. The corresponding LDH release was set as 100%. Relative values are given for PMNs treated with RC or GT and for non-treated controls. Standard deviations were calculated from three independent values each. Panel B: PMNs were treated with different stimuli for 3 h and the percentage of intact cells (black bars), TUNEL positive apoptotic cells (open bars) and cells that underwent NETosis (hatched bars) is given. Standard deviations were calculated from three independent values each. The data are representative of two completely independent experiments. Three sets each comprising at least 100 PMNs were analyzed. Abbreviations: GT = germ tubes; RC = resting conidia; PMA = phorbol 12-myristate 13-acetate; STS = staurosporine; CytD = cytochalasin D; n.t. = not treated. Panel C and D: TUNEL staining of PMNs incubated with GT for 3 h. Green: TUNEL; red: L10-13; blue: DAPI. NETs are indicated by arrowheads. The bar in panel D represents 25 μm and is also valid for panel C (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein plays a crucial role in the NET-mediated killing of *C. albicans* [6]. We stained *A. fumigatus*-induced NETs for calgranulin A, a constituent of the calprotectin heterodimer. Calprotectin was detectable in large patch-like structures, but also on the hyphal surface (**Fig. 5A** and **B**, arrows and arrowheads, respectively). Calprotectin is known to bind metal ions and addition of Zn^{2+} to the medium restored the survival of NET-associated *C. albicans* [6]. Supplementation with Zn^{2+} also rescued the hyphal growth of *A. fumigatus* (**Fig. 6A**), but in the absence of PMNs addition of Zn^{2+} had no positive influence on hyphal growth (**Fig. 6B**). Addition of iron (ferritin at 1.0 μg/ ml) was not able to restore growth of GT in the presence of NETs (data not shown). Taken together, our findings demonstrate that the growth inhibition of *A. fumigatus* hyphae by NETs is at least in part mediated by the chelation of Zn^{2+} ions.

4. Discussion

The formation of NETs by neutrophils has recently been identified as a novel mechanism to combat microbes. Reports of NET-mediated killing have been published for bacteria, fungi and parasites [5,6,8]. The particular compromise of neutropenic patients underlines the importance of neutrophils for control of *A. fumigatus*. Apart from neutropenic patients, chronic granulomatous disease (CGD) patients also have a higher risk to develop systemic *A. fumigatus* infections [14]. Since CGD patients lack functional NADPH oxidase, reactive oxygen species (ROS) were for a long time believed tomediate killing of *A. fumigatus*. However, an *A. fumigatus* mutant that is highly sensitive to ROS turned out to be as virulent as the wild type [15]. Resistance of a CGD patient to a systemic *Aspergillus* infection was recently restored by NADP oxidase gene therapy and NETosis was discussed as a crucial element in the resistance to systemic *Aspergillus* infections [16]. An *Aspergillus nidulans* strain was isolated from this patient by bronchoalveolar lavage and PMA-induced NETs were shown to inhibit its germination and growth [16]. Although *A. nidulans* is generally regarded as non- or low-pathogenic fungus, infections of humans and in particular CGD patients have been reported [17]. However, infections by *A. nidulans* are rare and *A. fumigatus* is by far the dominating species causing systemic *Aspergillus* mycoses [1]. During revision of this work Bruns and co-workers described the formation of NETs by *A. fumigatus* infected human neutrophils and in vivo in a murine model of infection [18].

In this study, we show that *A. fumigatus* conidia and GT are able to induce the formation of NETs by human neutrophils. The fungal cells seem to play no active part in this interaction, as UV-killed conidia and GT are also able to trigger NET formation. NETs are the most recently described anti-

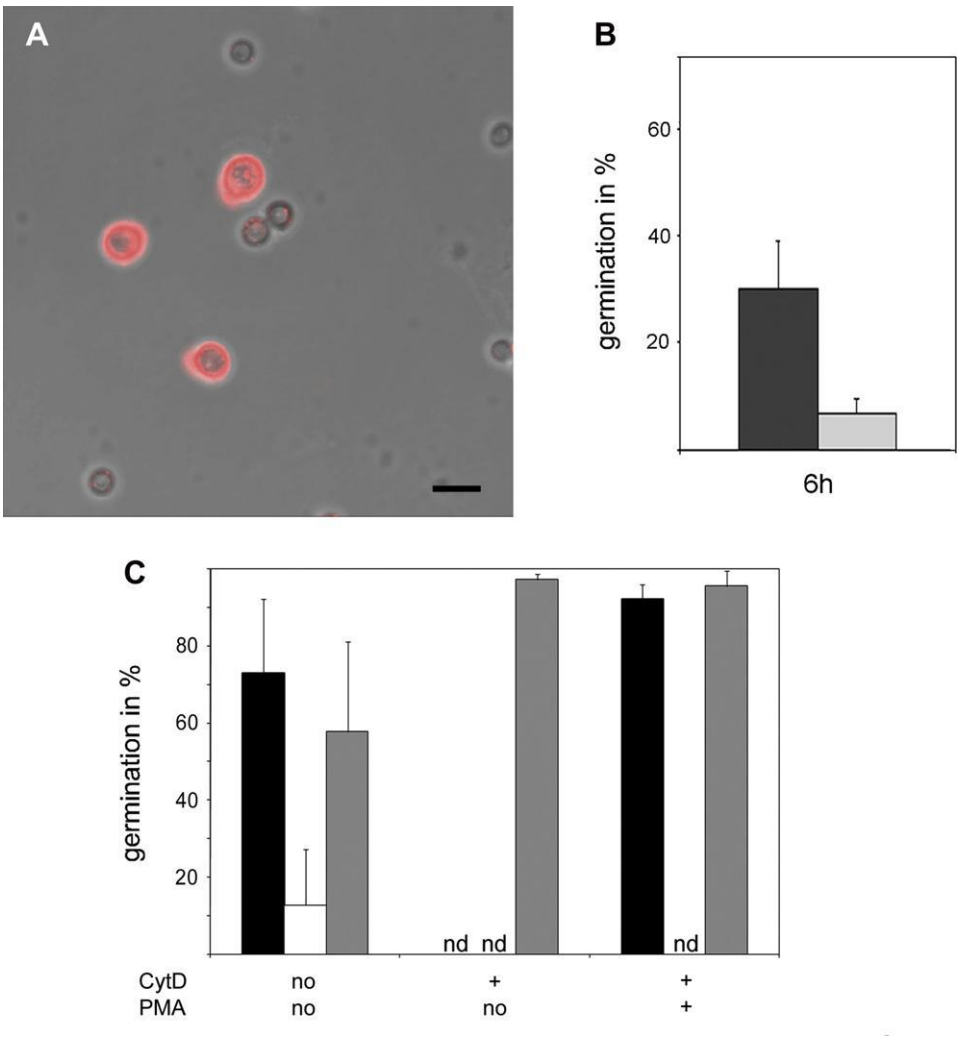

Fig. 3. PMN inhibit germination of conidia by phagocytic internalization. Panel A: Differential binding of the L10-1 monoclonal antibody to resting and swollen conidia. Bound antibody is shown in red. The bar represents 5 μm. Panel B: Resting conidia were incubated for 6 h with and without neutrophils. Activation of spores was determined microscopically after staining with mab L10-13. The percentage of germinated conidia is shown in the absence (black bar) and presence (gray bar) of neutrophils. Three sets each comprising at least 100 conidia were analyzed. Panel C: Germination of RC after co-incubation for 6 h with PMNs pre-treated or not with PMA and/or cytochalasin D to induce NETosis and block the actin cytoskeleton, respectively. The percentage of swollen conidia is shown for spores that were extracellular (gray bars), PMN-associated (white bars) or NET-associated (black bars). Standard deviations are shown for three groups of at least 100 cells each. The result is representative of two independent experiments. nd = not detectable.

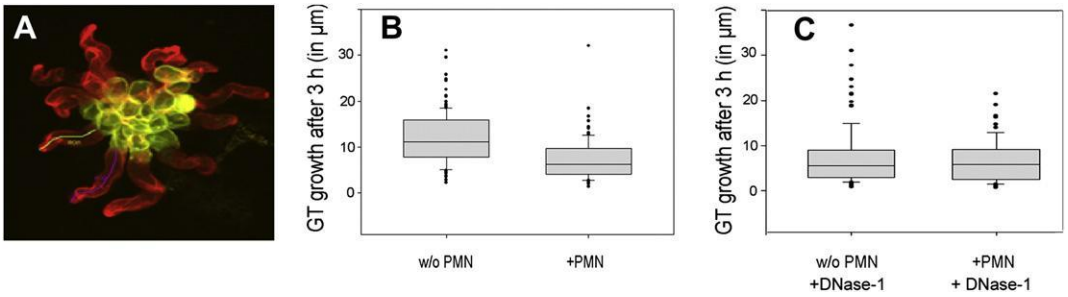

Fig. 4. NETs inhibit growth of *A. fumigatus* hyphae. Panel A: Small GT were labeled with FITC and grown for 3 h in cell culture medium. Fixed samples were stained with mAb L10-1 to visualize the entire length of the hyphae. Panel B: Labeled GT were grown for 3 h with and without neutrophils. Samples were analyzed as described above. The length of the apical FITC-negative segments was determined and depicted in a box diagram. The difference between the two samples was significant by Student's t-test ($p < 0.0001$). The data presented are representative for a set of three independent experiments. Panel C: Labeled GT were grown for 3 h with and without neutrophils in the presence of DNase-1. The length of the apical FITC-negative segments was determined and depicted in a box diagram. The presence of DNase-1 abolished the reduction in hyphal growth due to the presence of neutrophils. The data presented are representative of a set of two independent experiments.

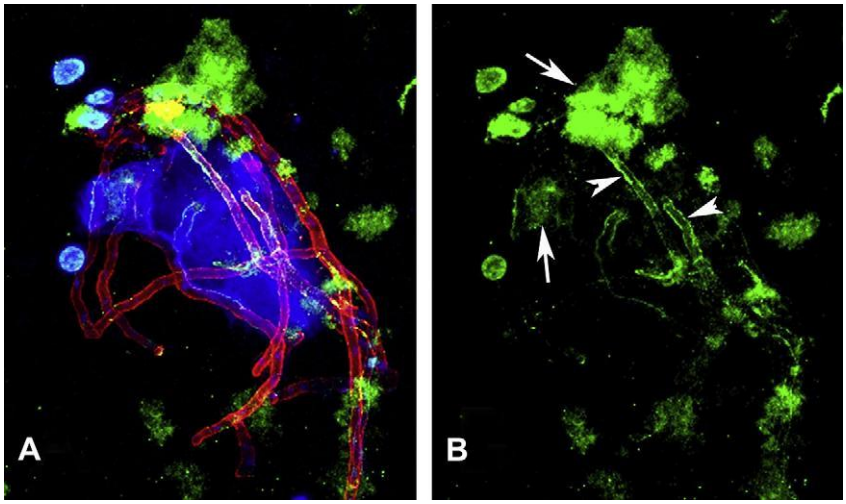

Fig. 5. Localization of calprotectin with NETs triggered by *A. fumigatus*. GT were co-incubated with neutrophils for 3 h and analyzed by immunofluorescence. Calprotectin is shown in green, GT are stained in red using mab L10-13 and DNA is visualized in blue by DAPI. Localization of calprotectin in patches and on the hyphal surface is indicated by arrows and arrowheads, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microbial mechanism employed by neutrophils. NET formation was shown to be triggered by *C. albicans* and NET-mediated killing has been described for *C. albicans* and *Cryptococcus neoformans* [6,9]. Incubation of PMNs with *A. fumigatus* led to the formation of NETs, but did not result in an enhanced frequency of apoptotic cell death. Single strand breaks, which are characteristic for apoptotic cells were not detectable in NETs. Our results show that approximately 40% of the neutrophils produced NETs after 6 h at an MOI of 1:3.

We found that co-incubation with neutrophils for 6 h reduced the germination of *A. fumigatus* conidia by approx. 60%. Phagocytosis of conidia occurs before NET formation and damage of internalized conidia within intact neutrophils may contribute to the observed inhibition of germination. To determine the impact of phagocytosis, we analyzed neutrophils that were treated with cytochalasin D, a drug that strongly inhibits actin dynamics. We found that such neutrophils were immotile and unable to phagocytose conidia. Consequently, conidia remained extracellular and germinated normally. This finding indicates that the phagocytic uptake by neutrophils is crucial for the observed inhibition of germination. Accordingly, degradation of NETs by DNase-1 or supplementation of the medium with Zn^{2+} ions had no impact on germination. We therefore assume that internalized conidia remain in a resting state due to the hostile conditions in the phagosome.

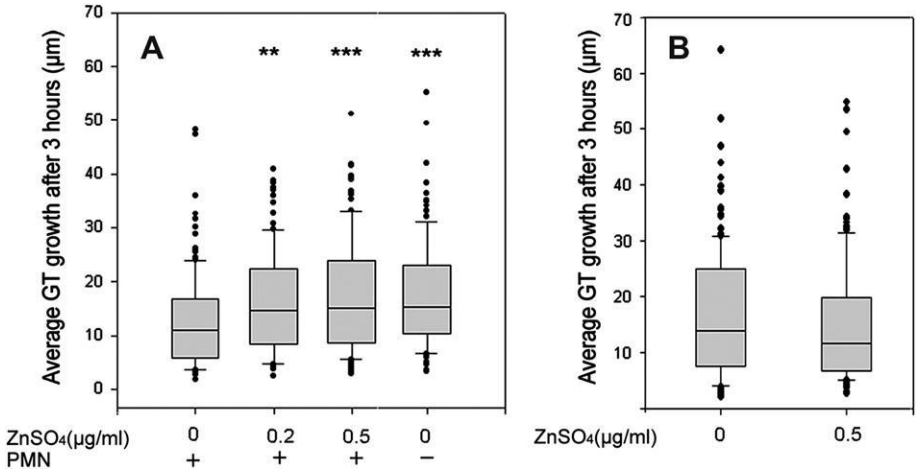

Fig. 6. Addition of Zn^{2+} ions rescues the growth inhibition by NETs. Panels A: FITC-labeled germ tubes were incubated for 3 h with and without neutrophils and in the presence of the indicated concentrations of extracellular Zn^{2+}. Samples were fixed and stained with mab L10-13. The length of the apical FITC-negative segments was determined and depicted in a box diagram. Addition of Zn^{2+} significantly increased hyphal growth in the presence of PMNs. Student's t-test: $^{**}p < 0.01$; $^{***}p < 0.001$. The data presented are representative for a set of three independent experiments. Panel B: FITC-labeled germ tubes were grown for 3 h in cell culture medium supplemented with and without 0.5 µg/ml Zn^{2+}. Samples were fixed and stained with mab L10-13. The length of the apical FITC-negative segments was determined and depicted in a box diagram. Addition of Zn^{2+} slightly reduced the hyphal growth, but this difference was not significant according to the Student's t-test. The data presented are representative of a set of two independent experiments.

In some experiments neutrophils that were pre-treated with PMA blocked germination as efficiently as non-treated cells. However, an additional treatment with cytochalasin D completely abrogated this effect. We observed in many experiments that a certain subset of neutrophils was especially motile and actively internalizing *Aspergillus* spores. Whether this subpopulation is more resistant to NETosis triggered by PMA remains to be analyzed. In conclusion, our results provide evidence that although conidia trigger NET formation, their germination is not impaired by NETs.

Neutrophils are short-lived cells that have to be recruited to the site of infection. Little is known about the role of neutrophils in non-infected, healthy tissue. Inhaled *A. fumigatus* conidia reach the alveoli where they encounter alveolar macrophages as the major resident phagocytic cell type. The impact of neutrophils on the elimination of inhaled conidia remains to be determined, while their importance in the combat of hyphae is generally accepted. We therefore addressed the question whether NETs induced by *A. fumigatus* GT are able to kill hyphae. GT of a strain in which mitochondria are tagged by targeted GFP expression showed a normal mitochondrial morphology and dynamics after co-incubation with neutrophils for several hours. This demonstrates that NETs *per se* are unable to kill hyphae. We then analyzed the fungal growth of single GT during co-incubation with neutrophils. Again we observed no evidence for killing of GT, but we detected a significant inhibition of the fungal growth. It is conceivable that the reduced hyphal growth was a consequence of NETosis, (1) since GT are too large to be taken-up by phagocytosis and (2) since DNase-1 treatment abrogated the observed growth inhibition. This is furthermore in line with very recent results showing that the oxygen consumption of *A. fumigatus* is reduced in the presence of NETs [18].

Calprotectin was recently identified as a major component of NETs that is important for killing of *C. albicans* [6]. Neutrophil-derived calprotectin was found inside *S. aureus* abscesses and shown to inhibit growth of this bacterium by chelation of Zn^{2+} and Mn^{2+} [19]. In this study, we detected calprotectin in association with NETs induced by *A. fumigatus* and in smaller amounts also directly on the fungal surface. Addition of Zn^{2+} rescued the growth inhibition observed for NETs, suggesting that chelation of Zn^{2+} by calprotectin is an important mechanism in this interaction. The iron chelator lactoferrin is another characteristic component of NETs [6] and was shown to inhibit growth of *A. fumigatus* in vitro [20]. However, in our experiments supplementation with ferritin was not sufficient to rescue hyphal growth in the presence of NETs, suggesting that the major mechanism operating here is the depletion of zinc by calprotectin.

In summary, our data show that *A. fumigatus* conidia and GT are able to induce the formation of NETs by human neutrophils. Neutrophils inhibit germination of RC, but this effect seems to be largely due to phagocytosis of the spores. More importantly, we observed a reduced polar growth of GT and hyphae due to the chelation of zinc. This effect was clearly dependent on NETosis. NETs *per se* seem to be unable to kill *A. fumigatus* and to clear infection. However, trapping the fungus in NETs and reducing its growth might be an important means to confine infection.

Acknowledgement

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micinf.2010.06.009.

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5. Publication 3

Bouzani,M., Ok,M., McCormick,A., Ebel,F., Kurzai,O., Morton,C.O., Einsele,H., Loeffler,J., 2011. Human NK cells display important antifungal activity against *Aspergillus fumigatus*, which is directly mediated by IFN-gamma release. J Immunol. 187, 1369-1376.

5.1 Contribution of co-authors

Bouzani,M., Ok,M., Kurzai,O., Einsele,H., Loeffler,J.: Primary author and supporting group, respectively, at the Medizinische Klinik und Poliklinik II, Universitätsklinikum Würzburg. Maria Bouzani performed the majority of the experiments and wrote the manuscript. I performed the experiment for Figure 6C and, together with Frank Ebel, proof-read the manuscript.

Allison McCormick	Frank Ebel

The Journal of Immunology

Human NK Cells Display Important Antifungal Activity against *Aspergillus fumigatus*, Which Is Directly Mediated by IFN-γ Release

Maria Bouzani,[*] Michael Ok,[*] Allison McCormick,[†] Frank Ebel,[†] Oliver Kurzai,[‡,§] C. Oliver Morton,[¶] Hermann Einsele,[*] and Juergen Loeffler[*]

Despite the strong interest in the NK cell-mediated immunity toward malignant cells and viruses, there is a relative lack of data on the interplay between NK cells and filamentous fungi, especially *Aspergillus fumigatus*, which is the major cause of invasive aspergillosis. By studying the in vitro interaction between human NK cells and *A. fumigatus*, we found only germinated morphologies to be highly immunogenic, able to induce a Th1-like response, and capable of upregulating cytokines such as IFN-γ and TNF-α. Moreover, priming NK cells with human rIL-2 and stimulating NK cells by direct NK cell–pathogen contact were essential to induce damage against *A. fumigatus*. However, the most interesting finding was that NK cells did not mediate anti-*Aspergillus* cytotoxicity through degranulation of their cytotoxic proteins (perforin, granzymes, granulysine), but via an alternative mechanism involving soluble factor(s). To our knowledge, our study is the first to demonstrate that IFN-γ, released by NK cells, directly damages *A. fumigatus*, attributing new properties to both human NK cells and IFN-γ and suggesting them as possible therapeutic tools against IA. *The Journal of Immunology*, 2011, 187: 1369–1376.

Natural killer cells are $CD56^+CD3^-$ lymphocytes that are cytotoxic against neoplastic and virus-infected host cells (1). They possess a sophisticated repertoire of activating and inhibitory receptors through which they perceive their environment. In response to stimuli, they produce and release cytokines and chemokines, such as IFN-γ and TNF-α, which shape the host's immunity (2, 3). Moreover, they exert a strong, cytolytic effect via the perforin–granzyme, Fas ligand (FasL), and TRAIL pathways (1). Increasing data provide evidence of direct NK cell action against extracellular pathogens, such as bacteria (4), parasites (5), and yeast (6). A role for NK cells and IFN against *Aspergillus fumigatus* in mice has been suggested (7). This was recently confirmed by Morrison et al. (8), who showed that, in neutropenic mice with invasive aspergillosis (IA), the recruitment of NK cells was a critical host defense mechanism. In a similar animal model, it was shown that NK cell-derived IFN-γ was the protective factor against IA (8, 9). Clinical data confirmed an antifungal and, more specifically, the anti-*Aspergillus* activity of IFN-γ (7, 10–14). These studies attributed the beneficial effect of IFN-γ to its immunoregulatory role with phagocytes of the innate immune system, which are conventionally involved in the host defense against *A. fumigatus* (11, 15).

In our study, we investigated the largely unknown interplay between human NK cells and *A. fumigatus*. We showed that human NK cells interact only with germinated morphotypes of the fungus, and direct physical contact is necessary to induce an NK cell response. This response involves expression of Th1-like cytokines and release of a soluble factor that has direct antifungal properties, which we identified as IFN-γ. These data provide new insights into NK cell biology demonstrating that *A. fumigatus*, an extra-cellular pathogen, is a direct target of these cells. Moreover, to our knowledge our results identify, for the first time, unrecognized properties of a key IL and designate IFN-γ as the mediator of a novel NK cell cytotoxic mechanism.

Materials and Methods

Cells

Institutional approval was obtained to isolate cells from the buffy coat of peripheral blood from healthy volunteer donors. After layering over a Ficoll standard density gradient (Biochrom AG), untouched NK ($CD56^+CD3^-$) cells were isolated by MACS negative selection procedure, using the NK cell isolation kit (Miltenyi Biotec), according to the manufacturer's protocol, and were resuspended at a concentration of 1×10^6 cells/ml in culture media (RPMI 1640 with 2 mM L-glutamine [Invitrogen] supplemented with 10% heat-inactivated FCS [Sigma-Aldrich] and 100 mg/ml gentamicin [Refobacin; Merck]). The human erythroleukemia cell line K562 (provided by R. Seggewiss, Medizinische Klinik II, Würzburg, Germany) was cultured in the same culture medium and at the same density as NK cells. In all experiments, except those performed with resting NK cells, NK cells were pulsed with 500 U/ml recombinant human (rh)IL-2 (Proleukin; Novartis) for 24 h before being used.

Fungal strains

A. fumigatus resting conidia (ATCC 46645) were cultivated for 3 d on beer mash plates at 28°C. Conidia were detached from the plate using endotoxin-free sterile water and were filtered through a cell strainer, 40-μm nylon mesh pore membrane, to obtain a single-fungal cell suspension. Swelling and synchronization of fungal growth were achieved by cultivating conidia in culture medium at room temperature and under continuous shaking at 200 rpm overnight. Germlings were obtained after an

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Abbreviations used in this article: FasL, Fas ligand; IA, invasive aspergillosis; rh, recombinant human; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]2H-tetrazolium-5-carboxanilide.

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additional 5-h incubation at 37˚C in the shaker. Fresh swollen conidia and germlings were used directly in cocultures with NK cells.

Infection experiments

After priming, NK cells were applied to 24-well plates at a density of 1 x 10^6 cells/ml, 1 ml/well, and were cocultured with swollen conidia or germlings at an E:T ratio of 1, at 37˚C and 5% CO_2 humidified air. Cocultures with the K562 cell line were established as positive controls. Evaluation of cell viability using trypan blue dye preceded all experiments. To estimate the effect of NK cell–fungal cell contact, cocultures were performed in the presence of transwell permeable membranes (Corning) with pores (0.4 μm) small enough to prohibit the contact of cells placed on opposite sides of the insert, but large enough to enable the free diffusion of molecules between the basal and apical compartments of the well. Cultures were harvested at 3, 6, and 12 h and were centrifuged at 5400 rpm for 5 min. Culture supernatants were stored at -80˚C. RNA was extracted from the cell pellets with the RNeasy mini kit and QiaShredder spin columns, according to Qiagen's protocol. QuantiTect reverse transcription kit (Qiagen) was used for the reverse transcription of 500 ng total RNA into cDNA following the manufacturer's protocol. To evaluate the importance of exocytosis of cytotoxic lysosomal proteins in fungal damage, NK cells were treated for 12 h with 5 mM EGTA (IDRANAL VI; Sigma-Aldrich). EGTA is a calcium (Ca^{2+}) chelating agent, inhibiting the Ca^{2+} flux required for degranulation. NK cells were also treated with 25 mM $SrCl_2$ (Sigma-Aldrich) for 24 h, to induce degranulation and thereby to eliminate cytotoxic granules (6). The cells were then washed three times in culture media before being used in the experiments. NK cell viability was not affected by such treatments (trypan blue exclusion).

Real-time quantitative PCR assays

Reactions were performed with a LightCycler 1.5 (Roche). Real-time PCR master mix was made with 10 μl Qiagen Quantifast system supplemented with 0.75 ml primers (5 μM; Tib-molbiol), 1 μl probes (3 μM; Tib-molbiol), and 4.5 μl RNase-free water per reaction, and 2 μl cDNA added as template. We investigated the expression of two genes, as follows: IFN-γ (primers, 5'-GCATCCAAAAGAGTGTGGAG-39, 59-GCAG-GCAGGACAACCATTAC-3'; probes, 5'-LC640-TCCAAGTGATGGCTG-AACTGTCG-PH-3', 5'-TCCAACGCAAAGCAATACATGAACTC-FL-3'), TNF-α (primers, 5'-CTCTGGCCCAGGCAGTCAGA-3', 5'-GGCGTTT-GGGAAGGTTGGAT-3'; probes, 5'-GCATTGGCCCGGCGGTTC-FL-3', 5'-LC640-CCACTGGAGCTGCCCCTCAGCT-PH-3'). Results were normalized against the housekeeping gene 5-aminolevulinate synthase expression (primers, 5'-CAGCTCCCGCTCTAAGTCCA-3', 5'-AATGAGTCGCCA-CCCACG-3'; probes, 5'-CCTGCCCCAGCACCATGTTGTTTC-FL-3', 5'-LC640GTGTCCATAACTGCCCCACACACC-PH-3') of the unstimulated control at each time point.

ELISA

IFN-γ and TNF-α release were quantified in culture supernatants, according to the manufacturer's instructions (BioSource International).

FACS analysis

The purity of NK cells was assessed by FACS analysis, consistently showing .95% $CD56^+CD3^-$ NK cells. The average proportion of $CD56^+CD3^-$ cells, evaluated immediately after isolation from PBMCs, was 96.53%. Twenty-four hours later, after priming with rhIL-2, the $CD56^+CD3^-$ cells accounted for 97.9%, and these cells were used in subsequent experiments. In detail, the contaminating WBC populations were: $CD3^+$ T cells, 1.8%; $CD20^+$ B cells, 0.19%; $CD14^+$ monocytes, 0.15%; $CD1c^+CD19^-CD14^-$ myeloid dendritic cells, 0.36% (CD56-PE, CD3-FITC, CD20-allophyco-cyanin, CD14-allophycocyanin, CD1c-PE, CD19-FITC [BD Biosciences]). The release of cytotoxic molecules was assessed by the detection of de-granulation markers CD107a,b on $CD56^+CD3^-$ NK cells (CD107a-FITC, CD107b-FITC, CD56-allophycocyanin, CD3-PerCP [BD Biosciences]).

Plating assays

Plating assays were performed by coculturing 4 x 10^5 NK cells with different morphologies of *A. fumigatus* at an E:T ratio of 1. The assay was performed for 3 and 6 h in 600 μl medium, at 37˚C and 5% CO_2 humidified air. In similar experiments, 4 x 10^5 germlings were incubated for 3 h in 600 μl culture supernatant. Supernatants were obtained after 6-h coculture of a high number of NK cells (8 x 10^6/ml) with germlings in an E:T ratio of 1. The mean concentration of IFN-γ, determined by ELISA, in these supernatants was 18 pg/ml. For plating assays, NK cells were lysed at each time point with 1.5 ml cold water, and, after serial dilutions, fungal cells were plated on Sabouraud agar. After overnight incubation at 37˚C, the CFU was counted. The results were normalized against a growth control, in which *A. fumigatus* was incubated on culture medium. Fungal growth in the different chambers of transwell plates and in the presence of Abs was also measured using a plating assay. When appropriate, NK cells were treated with 50 mg/ml human IgG (Talecris), 1.6 mg/ml blocking Ab against TRAIL, and 10 ng/ml FasL Ab (R&D Systems).

Analysis of fungal damage

A total of 2 x 10^5 fungal germlings was cultured in 150 μl NK cell supernatant (prepared as above), IFN-γ partially depleted supernatant, or culture medium supplemented with rhIFN-γ (Invitrogen) and at 37˚C. IFN-γ depletion was performed magnetically, using the IFN-γ secretion assay cell enrichment and detection kit (Miltenyi Biotec). IFN-γ was depleted by 85%. The mean concentration of IFN-γ, in IFN-γ partially depleted supernatants, was 2.5 pg/ml (as determined by ELISA). To mimic fungal damage caused by IFN-γ, we used rhIFN-γ. rhIFN-γ was diluted in culture medium to a final concentration equal to that in the undepleted supernatant of each experiment (as determined by ELISA). To evaluate the dose response, rhIFN-γ was diluted in RPMI 1640 without any supplements. This medium was inoculated with germlings and conidia, as described above. After a 3- to 6-h incubation period, the cultures were washed three times with cold water. A total of 400 μl PBS with 0.5 mg/ml 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]2H-tetrazolium-5-carboxanilide (XTT) (Sigma-Aldrich) and 50 μg/ml coenzyme Qo (Sigma-Aldrich) was added to fungus, which was then incubated for 1.5 h at 37˚C (16). Duplicate 100 μl samples of supernatant were transferred to an ELISA plate, and the OD was measured at 450 nm with 655 nm reference filter. Antifungal effect was calculated according to the following formula: percentage of damage = (1 - x/y) x 100, where x is the OD of the different culture conditions and y is the OD of the negative control (fungus alone).

Germlings growth inhibition assay

Germlings of the *A. fumigatus* strain ATCC 46645 (generated as described before) were resuspended in 0.1 M sodium carbonate buffer (pH 10.0) and 0.3 mg/ml FITC (Sigma-Aldrich) and incubated for 30 min at 37˚C in the dark with shaking. Germlings were washed twice in sterile deionized water and resuspended in fresh medium at 4 x 10^6 germlings/ml. Germling solution (100 μl) was used to inoculate each well of a 24-well plate containing 300 μl/well NK supernatant, rhIFN-γ–enriched medium, or supplemented medium and a glass coverslip. The mean concentration of IFN-γ was 5.5 pg/ml. The plate was incubated at 37˚C, 5% CO_2, and, after 6-h incubation, the cells were fixed in 3.7% formaldehyde for 5 min at room temperature. Germling growth beyond the FITC labeling was visualized by immunofluorescence using hybridoma supernatant containing the mAb L10-13 (against galactomannan) and a Cy3-conjugated secondary Ab. Coverslips were mounted to glass slides in Fluoroprep, and germling elongation was measured using the software package provided with the Leica SP-5 confocal laser scanning microscope (Leica Microsystems). At least 100 germlings were measured for each growth condition, and a Student t test was used to calculate significance.

Statistics

Estimation of p values was performed with the unpaired, two-sided Student t test; p . 0.1 NS, *p , 0.1, **p , 0.05, ***p , 0.001. Bars show arithmetic means of the values of the independent experiments - SEM.

Results

Human NK cells express Th1-like cytokines during interaction with A. fumigatus *germlings*

Both animal and clinical studies have demonstrated the protective effect of Th1 immunity against IA, with IFN-γ and TNF-α being the major mediators of this response (9, 17–22). To evaluate whether human NK cells express these cytokines upon incubation with *A. fumigatus*, we performed in vitro infection experiments with resting (conidia) and germinated (germlings) fungal morphologies. We observed that NK cells, challenged by germlings, upregulated the genes IFN-γ and TNF-α, in a time-dependent pattern, reaching a maximum between 6 and 12 h after stimulation (Fig. 1*A*, 1*B*). A similar temporal profile was also observed for the release of these cytokines, with the maximum being detected after 12 h of stimulation (Fig. 1*C*, 1*D*). Interestingly, resting conidia

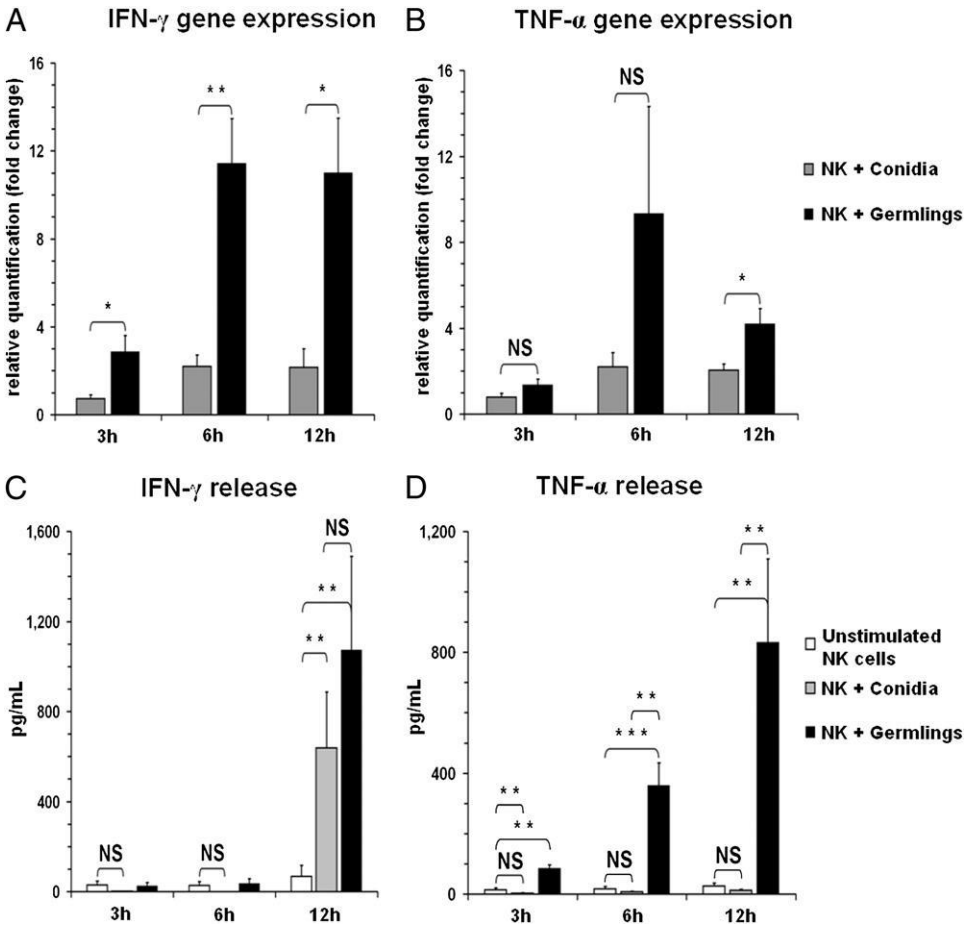

FIGURE 1. Human NK cells mediate a time-dependent Th1-like immune response following stimulation by *A. fumigatus*-germinated morphotypes. Measurement of the cytokine response of NK cells during in vitro interaction with *A. fumigatus* conidia and germlings at an E:T ratio of 1 over time by real-time quantitative PCR and ELISA. The time-dependent induction of IFN-γ (A, C) and TNF-α (B, D) was observed. This effect was especially prominent between 6 and 12 h of NK cell–fungus coincubation (A–D). Conidia were significantly less stimulatory than germlings (A, B). Germination of conidia caused cytokine release from NK cells in a time-dependent pattern that peaked after 12 h (C). Data from four independent experiments with three donors each. *p , 0.1, **p , 0.05, ***p , 0.001.

induced no effect on NK cells; however, cytokine release occurred following germination (Fig. 1*C*). Focusing fur-ther on the mechanism of this recognition, NF-kB transcription factor translocation to the nucleus was demonstrated in the pres-ence of germlings, but not by conidia, suggesting the involvement of pattern distinct recognition receptors in the interaction of NK cells with *A. fumigatus* (data not shown).

Human NK cells primed with rhIL-2 exhibit cytotoxicity against A. fumigatus *germlings*

To investigate whether NK cells interfere with *A. fumigatus* growth, we performed plating assays. When NK cells were co-incubated with conidia, there was no fungal damage observed, at all time points (Fig. 2*A*). However, when they were confronted for 3 h with germlings, there was a significant reduction of 40– 60% in the number of fungal colonies (Fig. 2*B*). To evaluate the influence of priming with rhIL-2 on the NK cell cytotoxicity, we repeated the previous experiments cocultivating germlings with either rhIL-2–primed NK or unprimed $NK_{w/o\ rhIL\text{-}2}$ cells, for 3 h. Resting $NK_{w/o\ rhIL\text{-}2}$ cells were unable to damage *A. fumigatus*, highlighting the importance of priming in the induction of cyto-toxicity (Fig. 2*C*).

NK cell stimulation by A. fumigatus *is contact dependent, but antifungal activity is not*

To investigate whether the interaction of NK cells with *Aspergillus* was contact dependent or mediated by soluble factors, the previous infection experiments were repeated using permeable transwell inserts (///) (Fig. 3*A*, 3*B*). These allowed the free circulation of soluble molecules, but prohibited direct contact between cells placed in opposite compartments. The compartments were inoculated as follows: NK /// germlings and NK + germlings /// germlings. NK + germlings and germlings alone served as positive and negative controls, respectively (Fig. 3*B*). Cytokine release was determined as before, and plating assays were per-formed after 3-h incubation. In the presence of the transwell membrane, without direct fungal contact (NK /// Germlings), cytokine release was abolished as compared with contact-stimulated NK cells (Fig. 3*A*, NK + Germlings). NK cells showed no antifungal activity across the membrane (NK /// Germlings), permitting unhindered growth of *A. fumigatus* (Fig. 3*B*). However, when NK cells were coincubated with the fungus at one side of the insert and germlings alone were at the other side (NK + Germlings /// Germlings), the CFU of germlings cultured alone was equivalent to the CFU of the positive control, where the germlings were cultured in contact with NK cells (Fig. 3*B*, NK + Germlings). These data show that direct contact is required as a first step in the stimulation of NK cells by *A. fumigatus*. NK cells then release soluble factors with antifungal properties. Thus, NK cell signaling induced by *A. fumigatus* is contact dependent, but the resultant antifungal effect is not.

*Soluble factors mediate the anti-*Aspergillus *effect of NK cells*

To confirm the effectiveness of the released soluble factors against the fungus, we performed plating assays where germlings were incubated, for 3 h, in culture supernatants. Incubation in supernatant caused a reduction in CFU development to 60% of that observed in culture medium alone (Fig. 4*A*). The antifungal activity of the supernatant was also assessed by the XTT assay. Interestingly, 40% fungal damage was observed after exposing germlings to culture supernatant for 3 h (Fig. 4B). These findings suggest that NK cells exert cytotoxicity against *A. fumigatus*,

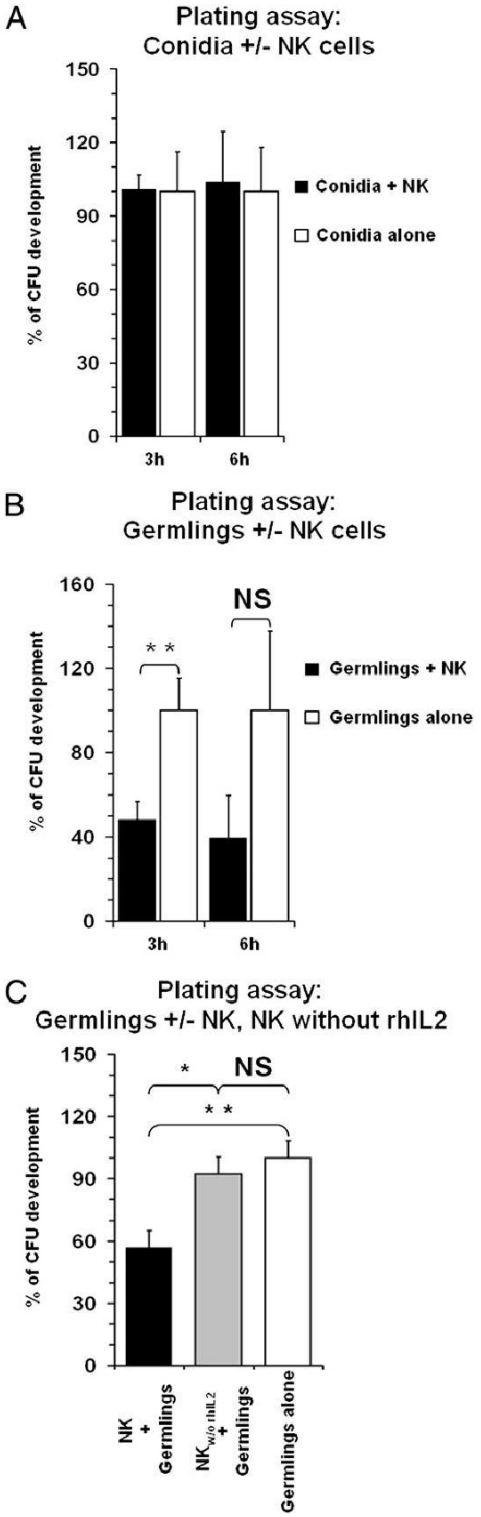

FIGURE 2. RhIL-2–primed NK cells exert direct cytotoxicity against *A. fumigatus* germlings. Plating assays were performed with NK cells primed with rhIL-2 and challenged with *A. fumigatus* conidia or germlings for the indicated times. Coincubation of resting conidia with NK cells for 3 and 6 h had no impact on conidial growth, compared with controls (A). In contrast, growth of germlings was clearly reduced after coincubation with NK cells (B). Without rhIL-2 priming, $NK_{w/o\ rhIL\text{-}2}$ cells failed to damage germlings (C), demonstrating that a pretreatment with rhIL-2 is essential to render NK cells capable of exerting cytotoxicity toward *A. fumigatus*. Experiments were performed independently, on different days, with two to four donors each time. Data from four experiments studying the NK cell effect on conidia and eight experiments studying the impact on germlings.

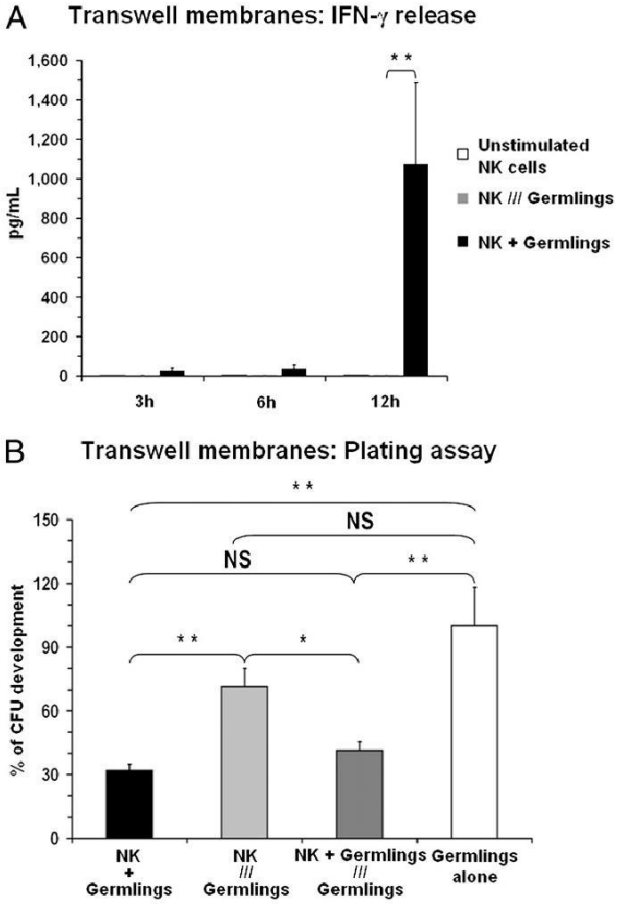

FIGURE 3. NK cell stimulation by *A. fumigatus* requires direct cell-to-cell contact. NK cells and germlings were placed in the two compartments of transwell chambers (NK /// Germlings). The insert prohibited cell contact between the two populations, but allowed free circulation of molecules between the compartments. Without direct contact to the fungus, NK cells showed no enhanced cytokine release, as quantified by ELISA (A), as well as no cytotoxicity, measured by plating assays (B). To determine whether soluble factors released by NK cells upon stimulation by the fungus are sufficient to trigger the observed cytotoxic effect, germlings were coincubated with NK cells in one compartment and germlings were placed in the other compartment (NK + Germlings /// Germlings). The antifungal effect across the membrane was normalized against a control in which germlings were cultured alone (B). Data from five independent experiments with one donor each. *p , 0.1, **p , 0.05.

through soluble molecules released by NK cells after direct contact with the fungus. *A. fumigatus* might be damaged by these factors without being in physical contact with the NK cells.

*NK cell anti-*Aspergillus *cytotoxicity is independent of NK cell degranulation*

Cytotoxic protein degranulation represents one of the two major killing mechanisms of NK cells against malignant and virus-infected cells (2). To determine whether this mechanism was responsible for *A. fumigatus* damage, we evaluated the expression of the lysosomal associated membrane proteins LAMP-1 and LAMP-2 (CD107a,b), which appear on the cell surface when degranulation occurs (23). CD107a,b–FITC Abs were incubated

Three experiments compared the cytotoxicity of primed and resting NK cells. The cultures were performed in duplicates. Each duplicate was plated twice on Sabouraud agar plates. *p , 0.1, **p , 0.05.

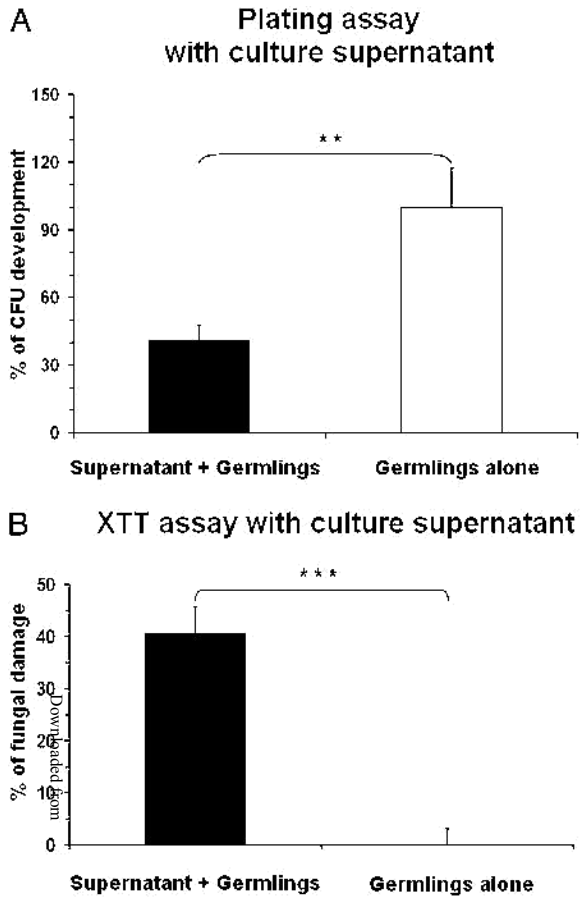

FIGURE 4. The anti-*Aspergillus* effect of NK cells depends on soluble factors. After 3-h incubation in NK cell culture supernatant, fungal germlings were plated on Sabouraud agar. The next day, the CFU was determined and normalized against the growth control. Data from five independent experiments with one donor each (A). In parallel experiments, damage of germlings was quantified after 3-h incubation in culture supernatant using an XTT assay. Antifungal activity was calculated in relation to the negative control (germlings incubated in culture medium). Data from six independent experiments, with two donors each (B). ***p* , 0.05, ****p* , 0.001.

with unstimulated NK cells (negative control), fungal germlings–NK cell cocultures, or K562 cell–NK cell cocultures (positive control) for 4 h and measured by flow cytometry. Surprisingly, *A. fumigatus* did not induce the surface expression of CD107a, b on NK cells, suggesting that exocytosis of cytotoxic proteins (perforin, granzymes, granulysine) is not the mechanism mediating *A. fumigatus* damage (Fig. 5A).

It has previously been shown that mobilization and increase of the intracellular Ca^{2+} are essential for exocytosis of the lytic granules (24). Moreover, the Ca^{2+} flux correlates with the surface expression of CD107a (25). To determine whether exocytosis is required for the observed antifungal activity, NK cells were treated with EGTA, a Ca^{2+} chelating agent (6), and thereafter challenged with *A. fumigatus* germlings or K562 cells (control) for 3 h. The antifungal activity of NK cells was not influenced by EGTA pretreatment (Fig. 5*B*), whereas the same treatment abolished their ability to kill K562 cells (trypan blue staining) (Fig. 5C). Depletion of cytotoxic proteins from NK cells mediated by $SrCl_2$ that triggers degranulation (6) did not affect the antifungal activity of NK cells (Fig. 5*B*). However, the same treatment abolished the cytotoxicity of NK cells against the K562 cells (Fig. 5*C*).

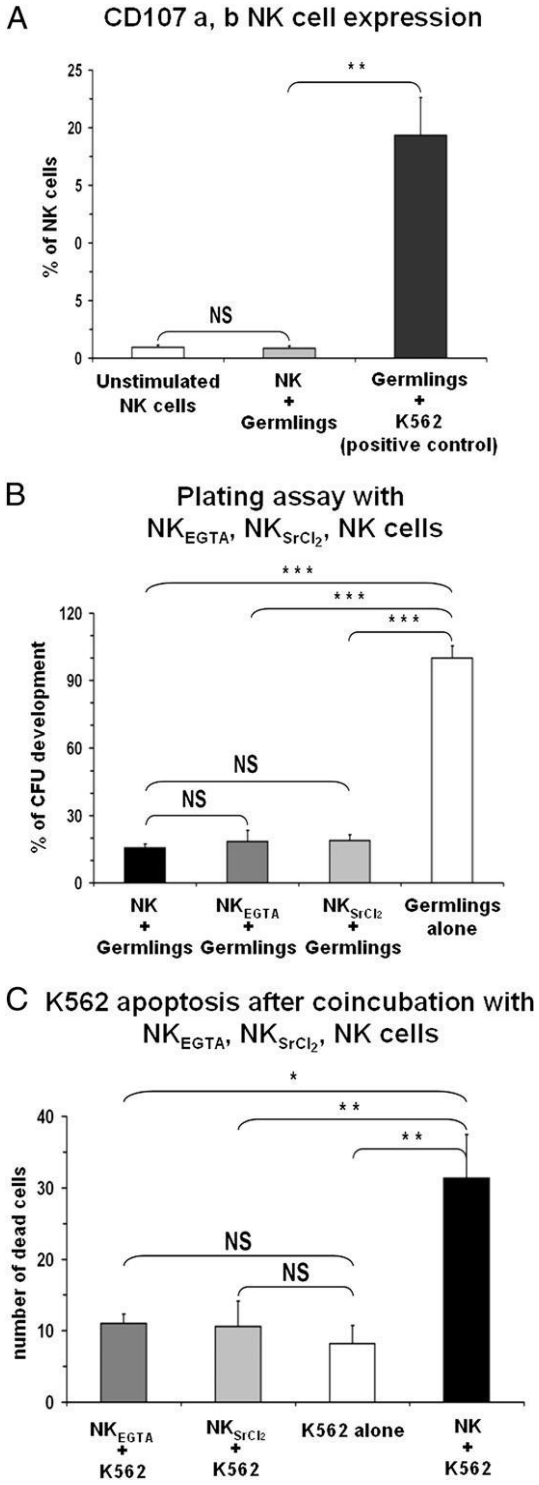

FIGURE 5. Anti-*A. fumigatus* activity is degranulation process independent. NK cells were coincubated for 4 h with FITC-labeled Abs directed against the degranulation markers CD107a,b, which are exposed on the NK cell surface upon release of cytotoxic proteins. In the presence of germlings, the expression of CD107a,b was equivalent to the unstimulated control (negative control). However, NK cells exposed these markers upon confrontation with K562 cells (positive control) (A). Plating assays were performed with NK cells previously treated with EGTA or $SrCl_2$. After 3-h coculture with germlings, the antifungal activity of EGTA- and $SrCl_2$-treated NK cells was equivalent to untreated controls (B). However, the same treatments abolished NK cell cytotoxicity toward K562 cells (trypan blue detection) (C). Data from three independent experiments with two donors each. **p* , 0.1, ***p* , 0.05, ****p* , 0.001.

The second major cytotoxic pathway engaged by NK cells involves the interaction of FasL and TRAIL expressed on NK cells, with their cognate receptors on target cells (1). We repeated our plating assays after treating NK cells with blocking Abs against FasL and TRAIL. Treated NK cells were equally as effective as untreated cells against *A. fumigatus*. This finding suggests that FasL and TRAIL do not mediate an antifungal response (data not shown). Collectively, these data showed that the two major cytotoxic mechanisms of NK cells are not involved in the anti-*Aspergillus* activity.

*IFN-γ demonstrates direct anti-*A. fumigatus *activity*

To date, IFN-γ has been recognized as a factor exhibiting indirect anti-*A. fumigatus* properties, via an increase in the antifungal activity of innate immune effector cells (26, 27). In our study, we investigated whether IFN- γ also acts directly against *A. fumigatus*, as a soluble factor released by NK cells to damage fungal germlings. XTT assays were performed after 3-h incubation of germ-lings in NK cell supernatants, partially depleted of IFN-γ (mean concentration of IFN-γ, 2.5 pg/ml) and undepleted supernatant (mean concentration of IFN-γ, 18 pg/ml) (Fig. 6*B*). This dem-onstrated that the depletion of IFN-γ decreased the anti-*Aspergillus* effect of the supernatant (Fig. 6A). To confirm the direct antifungal properties of IFN- γ, XTT assays were repeated after incubation of the fungus in culture medium supplemented with rhIFN-γ at concentrations equal to those detected in the complete supernatants by ELISA (Fig. 6*B*). In fact, damage in the presence of rhIFN-γ was equivalent to damage in undepleted supernatant, confirming the anti-*Aspergillus* properties of this cytokine (Fig. 6*A*). To quantify this observation and to investigate the presence of a potential, alternative anti-*Aspergillus* molecule, the hyphal length was additionally measured after 6-h incubation of germ-lings with low IFN-γ concentration NK supernatants and rhIFN-γ –enriched medium (mean concentration of IFN-γ, 5.5 pg/ml). Both NK supernatants and rhIFN-γ medium inhibited the hyphal elongation similarly. However, a trend of a higher inhibition caused by the NK supernatants was observed (Fig. 6*C*). It is worth noting the morphological alterations of the fungus caused by NK supernatant (Supplemental Fig. 1, Image 3) and rhIFN-γ medium (Supplemental Fig. 1, Image 2). Both culture conditions provoked similar structural distortions of the germlings, which acquired an uncommon flattened and twisted conformation. To provide further evidence for a direct role of IFN-γ against *A. fumigatus* and to exclude any synergistic effect with the supplements of the culture medium, we measured by XTT assay the effect of different concentrations of rhIFN-γ in unconditioned RPMI 1640 medium. A dose-dependent toxicity was observed toward germlings (Fig. 6*D*)..

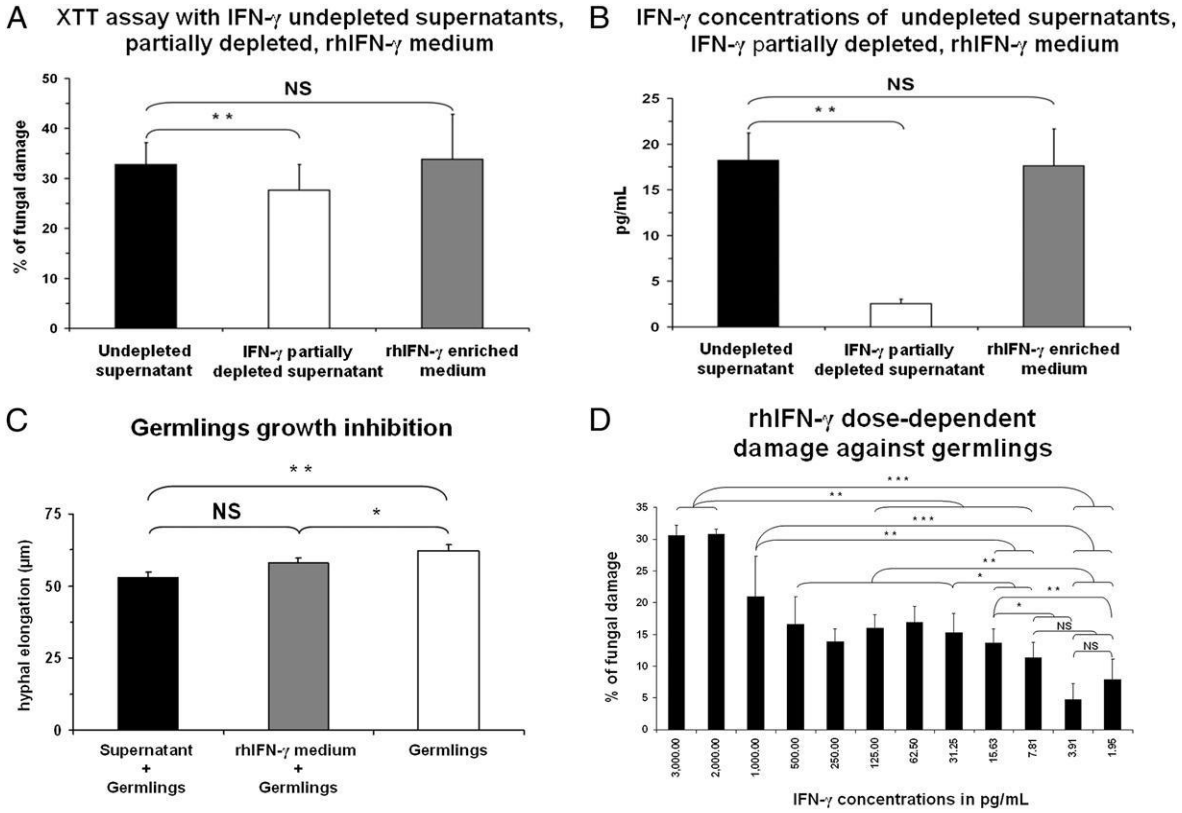

FIGURE 6. IFN-γ directly mediates NK cell damage against *A. fumigatus*. We compared the antifungal effect of undepleted and partially IFN-γ –depleted supernatants on germlings, after 3-h incubation using an XTT assay. Data from 10 independent experiments with one donor each. The bar on the right shows the fungal damage caused by the culture medium supplemented with rhIFN-γ at concentrations equal to those detected in NK cell supernatants (mean concentration, 17.63 pg/ml). This inhibition resembled that obtained with undepleted supernatants (left bar), whereas partial depletion of IFN-γ reduced the fungal growth inhibition (middle bar). Data from four independent experiments (A). The concentrations of IFN-γ of the experimental conditions of A are shown here (B). We confirmed the antifungal effect of IFN-γ, even at low concentrations, by measuring the hyphal elongation under the effect of NK supernatants and rhIFN-γ–supplemented medium with same amounts (mean, 5.5 pg/ml) of the cytokine, respectively. After 6-h incubation, both conditions caused an equivalent reduction of hyphal elongation, which was statistically significant compared with the growth control. Data from three independent experiments (C). A dose-damage relationship was established between the different concentrations of rhIFN-γ in unconditioned RPMI 1640 medium toward germlings. Data from eight independent experiments (D). **p* , 0.1, ***p* , 0.05, ****p* , 0.001.

In contrast, RPMI 1640 supplemented with rhIFN-γ demonstrated no effect against conidia (data not shown).

These results indicate the direct anti-*A. fumigatus* properties of IFN-γ, and show that IFN-γ is a factor mediating NK cell cytotoxicity toward the fungus.

Discussion

Since their discovery, NK cells have proven to be a useful tool in the fight against neoplasia and viral infections. NK cells are both potent effectors of innate immunity, exerting direct cytotoxicity without prior sensitization (1), and immunoregulatory mediators, shaping the response of other immune cells, such as dendritic cells and T cells (28–30). Interestingly, their role toward fungal pathogens, especially *A. fumigatus*, has been poorly elucidated. Our work aimed to study the direct interaction of human NK cells with *A. fumigatus*.

Studies in both humans and animals have documented the favorable effect of the Th1 immune response against IA. Proinflammatory cytokines such as IFN-γ and TNF-α are associated with increased antifungal activity of phagocytes, higher clearance of *A. fumigatus*, and better response to antifungal treatment (9, 17–22). Our results reveal that human NK cells challenged with *A. fumigatus* mediate a Th1-like response secreting IFN-γ and TNF-α. Interestingly, the release of these cytokines is morphotype dependent and is induced only by germlings and not by resting conidia. Moreover, in accordance with data in animals showing the importance of mouse NK cells for the clearance of *A. fumigatus* from the lungs (8), we demonstrated that human NK cells exert direct cytotoxicity against germlings; however, this is not the case with conidia. Both cytokine induction and antifungal activity suggest that NK cells perceive only germinated morphologies of *A. fumigatus*, recognizing that the fungus has escaped the first line of host defense, provided by alveolar macrophages and neutrophils (31–33). Priming of NK cells with rhIL-2 seems to be a prerequisite for NK cell–fungus interaction, suggesting an important role for cytokine-producing immune cells in the interplay of NK cells with *A. fumigatus*. We next questioned whether physical contact or soluble molecules mediated NK cell signaling and cytotoxicity. Separating the immune cells from the fungus, using transwell membranes, we observed a complete abrogation of both NK cell stimulation and fungal damage. However, when NK cells were cocultured with *A. fumigatus* on one side of the membrane, a condition allowing NK cell signaling, the antifungal effect on the other side was equivalent to the effect observed when NK cells and *A. fumigatus* were cultured in direct contact. These results indicate a two-step mechanism of cytotoxicity. First, germlings stimulate NK cells when in direct contact. In response, NK cells release soluble molecules able to harm the fungus not in physical contact with the immune cells.

The two major killing pathways of NK cells have been the object of extensive studies during the last two decades. Contact-dependent exocytosis of highly cytotoxic proteins and the activation of death receptors on target cells by TNF and FasL are the main means of attack by NK cells against tumor- and virus-transformed cells (1). Our results revealed that neither of these mechanisms is specifically involved in the anti-*Aspergillus* activity of NK cells. Instead, we demonstrate that IFN-γ released by NK cells in the presence of *A. fumigatus* germlings causes the observed antifungal effect. Moreover, we revealed that the anti-*Aspergillus* activity of IFN-γ is concentration dependent. This surprising finding is consistent with the previous report that NK-derived IFN-γ is essential to the host defense in a murine model of IA (9). It was shown that NK cell depletion and IFN-γ deficiency augmented similarly the severity of IA, suggesting that the contribution of NK cells to the anti-*A. fumigatus* effect is confined to IFN-γ production. More-over, depletion of NK cells from IFN-γ knockout mice had no adverse effect on the outcome of infection. This argues against an involvement of other effectors, apart from IFN-γ (e.g., cytotoxic proteins, perforin, granzymes, granulysine) in the NK cell-mediated anti-*A. fumigatus* activity (9).

Recent data on the human NK cell–*A. fumigatus* interaction recognized an activity of NK cells against germlings. Although we found that IFN-γ can directly inhibit the fungal growth, Schmidt et al. (34) implicated perforin as a major effector molecule of NK cell-mediated anti-*Aspergillus* activity. We believe that the discrepancies between their data and ours can be explained by the different study designs. Our study does not rule out that perforin, granzymes, granulysine, or other proteins of the NK cell granules have antifungal activity. However, our data demonstrate that the NK cell–*A. fumigatus* interaction does not involve substantial degranulation, which argues against a functional importance of the above mentioned proteins in the antifungal activity of NK cells (34). It is worth noting that our data cannot exclude the involvement of an additional factor, induced by the fungus and secreted by an unknown process. This might explain the minor, although significant decrease of the antifungal effect caused by the partially IFN-γ –depleted supernatant (Fig. 6*A*) and the trend toward an increased growth inhibition induced by the NK cell supernatant (Fig. 6*C*). Moreover, lower levels of hyphal damage caused by rhIFN-γ in unconditioned medium (Fig. 6*D*) compared with FCS and refobacin-supplemented medium (Fig. 6*A*) might reflect effects of the supplements.

Experimental infections in mice and clinical trials in humans have recognized the ability of IFN-γ to increase the phagocytic activity of innate immune effector cells against different species of fungi and especially *Aspergillus* (9–11, 13, 14, 17, 35). Our data suggest that in addition to acting as an immunoregulatory molecule, IFN-γ acts directly against *Aspergillus*, further explaining the antifungal effect observed in the previous studies. The concept of human proteins and peptides capable of eradicating pathogens is not new. Indeed, there is increasing evidence regarding the antibacterial and antifungal activity of chemokines, defensins, cathelicidins, and histatins (36–39). It is worth noting that, in our experiments, low concentrations of IFN-γ proved to be sufficient for anti-*Aspergillus* activity. Moreover, the finding that rhIFN-γ alone damages *A. fumigatus* underlines that the fungus is directly targeted by this major cytokine. If another factor is involved, it should originate from fungal germlings. Interestingly, Hu et al. (40) reported a synergistic cytotoxicity against hepatoma cell lines, resulting from the interaction of IFN-γ with a RNase. IFN-γ might cooperate with fungal ribotoxins, secreted by *A. fumigatus* (41), transforming them into suicide molecules for fungus.

In conclusion, despite the progress in antifungal diagnostics and treatment, IA remains a leading cause of morbidity and mortality in immunosuppressed patients. Our study sheds new light on the host defense against the most common cause of IA, *A. fumigatus*, and provides a rational framework for the use of NK cells and IFN-γ as therapeutic tools against IA.

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Disclosures

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6. Publication 4

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6.1 Contribution of co-authors

Jacobsen, ID. performed the mouse and egg infection experiments. Broniszewska, M. and Beck, J. assisted with phenotype testing and cloning, respectively. I performed all other experiments and wrote the manuscript together with Frank Ebel.

Allison McCormick	Frank Ebel

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The Two-Component Sensor Kinase TcsC and Its Role in Stress Resistance of the Human-Pathogenic Mold *Aspergillus fumigatus*

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Abstract

Two-component signaling systems are widespread in bacteria, but also found in fungi. In this study, we have characterized TcsC, the only Group III two-component sensor kinase of *Aspergillus fumigatus*. TcsC is required for growth under hyperosmotic stress, but dispensable for normal growth, sporulation and conidial viability. A characteristic feature of the ΔtcsC mutant is its resistance to certain fungicides, like fludioxonil. Both hyperosmotic stress and treatment with fludioxonil result in a TcsC-dependent phosphorylation of SakA, the final MAP kinase in the high osmolarity glycerol (HOG) pathway, confirming a role for TcsC in this signaling pathway. In wild type cells fludioxonil induces a TcsC-dependent swelling and a complete, but reversible block of growth and cytokinesis. Several types of stress, such as hypoxia, exposure to farnesol or elevated concentrations of certain divalent cations, trigger a differentiation in *A. fumigatus* toward a ''fluffy'' growth phenotype resulting in white, dome-shaped colonies. The ΔtcsC mutant is clearly more susceptible to these morphogenetic changes suggesting that TcsC normally antagonizes this process. Although TcsC plays a role in the adaptation of *A. fumigatus* to hypoxia, it seems to be dispensable for virulence.

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Introduction

Aspergillus fumigatus is a mold causing severe and systemic infections in immunocompromised patients [1]. The high mortality of these infections is largely due to the limited therapeutic options. Since *A. fumigatus* seems to lack sophisticated virulence factors, alternative therapeutic targets must be considered. The ability to respond to a plethora of environmental changes and to cope with different stress situations is vital for growth and survival of all microorganisms. This applies in particular to microbial pathogens that have to adapt to changing environments and a hostile immune response during colonization and invasion of the host. In fungi, sensing and responding to environmental stress is mediated by a set of receptors that are linked to a network of down-stream signaling pathways [2]. Interference with these signal transduction cascades can impede the fungal adaptation to stress and is considered a promising option to identify novel therapeutic targets. However, this approach is hampered by the conservation of many central signaling molecules in fungi and humans.

In bacteria sensing and processing of stress signals relies largely on two-component systems (TCS) that consist of a sensor histidine kinase and a response regulator. In fungi and other eukaryotes, hybrid histidine kinases (HHK) integrate both functions in a single protein. Fungal TCS are multistep phosphorelays composed of a sensor kinase (HHK), a histidine-containing phosphotransfer protein (HPt) and one or two response regulators. HHK are conserved within the fungal kingdom and depending on the species they govern the response to various stress signals, including osmotic stress, oxidative stress, hypoxia, resistance to anti-fungals and sexual development [3,4]. In contrast to other signaling molecules, TCS are attractive candidates for new therapeutic targets since they contribute to the virulence of fungal pathogens and are not found in vertebrates [3,5].

In fungi, eleven families of HHK have been described according to their protein sequence and domain organization [6]. Of several potential HHK present in the genome of *A. fumigatus* only two have been studied so far. Deletion of the Group VI HHK gene tcsB (AFUA_2G00660) had no severe impact on growth and stress resistance of *A. fumigatus*, but led to a slightly increased sensitivity to SDS [7]. A mutant in the Group IV HHK tcsA/fos1 (AFU6G10240) showed normal growth, no increased sensitivity to osmotic stress, but resistance to dicarboximide fungicides, like iprodione, and enzymatic cell wall degradation [8]. This is remarkable, since dicarboximide fungicides commonly target Group III HHK [9]. Several lines of evidence link Group III HHK to the high osmolarity glycerol (HOG) pathway that was initially

described as a signaling module enabling yeasts to adapt to high external osmotic pressure [10]. However, recent evidence suggests that in pathogenic fungi the HOG pathway is furthermore involved in the response to diverse kinds of stress [4].

In this study, we have analyzed TcsC, the sole representative of the Group III HHK in *A. fumigatus*. Group III HHK are found in bacteria, plants and fungi. They contain a characteristic cluster of HAMP domains that mediate signaling in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and certain phosphatases. Conformational changes in the spatial organization of the amphipathic helices in HAMP domains allow two conformations that either activate or inactivate the kinase activity of the output domain [11]. Single HAMP domains of membrane-bound HHK are found in close proximity to the membrane-spanning segment and transduce signals from the external input to the internal output domain. Group III HHK contain clusters of 4-6 HAMP domains, that according to a model developed recently for the osmo-tolerant yeast Debaryomyces hansenii, form a functional unit that is able to sense external signals. Changes in external osmolarity are supposed to alter the pattern of HAMP domain interactions and thereby modulate the inherent kinase activity of the protein [12]. The facts that Group III HHK are exclusively found in fungi and that certain fungicides can activate these sensor kinases in an uncontrolled and harmful manner makes them a potential Achilles heel of fungal pathogens that merits further investigations.

Results

The Group III HHK TcsC of *A. fumigatus*

The genome of *A. fumigatus* contains only one putative Group III HHK (AFU2G03560). The corresponding protein comprises a histidine kinase acceptor domain, a histidine kinase-like ATPase domain, a receiver domain and six HAMP domains. It lacks a transmembrane segment and is presumably localized in the cytoplasm. We designated this protein Two-component system protein C (TcsC) following the nomenclature of the previously studied *Aspergillus* TCS sensor kinases TcsA (Fos-1; AFU6G10240) and TcsB (AFU2G00660) [7,8,13].

Generation and Characterization of a ΔtcsC Mutant

To analyze the function of TcsC, we deleted the gene and complemented the mutant by ectopic insertion of the tcsC gene under control of its native promoter. The complementation procedure and the analysis of the genotype of the resulting strain are shown in Figure S1. On AMM, YG or Sabouraud medium the mutant grew well, but the colonies had a distinct appearance characterized by a broader white rim and fewer extending hyhae at the periphery (Figure 1A to D), whereas the complemented strain was indistinguishable from the wild type (data not shown). At 48°C growth of the mutant was comparable to the controls (Figure 1E) demonstrating that it is not particularly sensitive to temperature stress. Radial growth of the ΔtcsC mutant was slightly slower on AMM supplemented with ammonium tartrate (Figure 1E), whereas a remarkable reduction in growth was found on AMM plates supplemented with $NaNO_3$ instead of ammonium tartrate. This defect was not observed for the complemented strain indicating that TcsC is required for normal growth with nitrate as sole nitrogen source (Figure 1F).

In *A. nidulans* deletion of the homologous nikA gene had severe consequences for the production and viability of asexual spores [14,15,16]. We therefore compared sporulation and conidial viability of the ΔtcsC mutant and its parental strain. After four days at 37°C both strains produced a confluent and sporulating mycelial layer. No obvious difference in sporulation was apparent and this was confirmed by determining the conidial yield per cm^2 (mutant: $9.7\pm0.8x10^7$, parental strain: $9.3\pm0.8x10^7$). Conidia of the ΔnikA gene lose their viability within a few days when stored in water at 4°C. In contrast, conidia of the ΔtcsC mutant remained fully viable after storage for one month (mutant: 93.7%±3.0%, parental strain: 95.5%±3.0%). Thus, deletion of the Group III HHK gene in *A. fumigatus* does not affect sporulation or conidial viability, thus disclosing a remarkable difference between the two homologous sensor kinases in *A. fumigatus* and *A. nidulans*.

Conidial viability in *A. nidulans* was recently shown to depend on the presence phosphorylated SakA in resting conidia [17]. Several Group III HHK have been linked to the HOG pathway and shown to influence the phosphorylation state of HOG proteins, like *Aspergillus* SakA. In immunoblot experiments we detected only a slight decrease in the level of SakA phosphorylation in resting conidia of the ΔtcsC mutant when compared to its parental strain (Figure 2A), demonstrating that TcsC is not essentially required for SakA phosporylation in resting conidia.

Group III HHK have been shown to be required for resistance to osmotic stress in several fungi, but not in *A. nidulans*. Our data revealed a strong growth inhibition of the ΔtcsC mutant under hyperosmotic stress, e.g. on plates containing 1.2 M sorbitol (Figure 3B), 1 M KCl (Figure 3C) and 1 M NaCl (data not shown). This demonstrates that TcsC is clearly important for adaptation to high osmolarity. Immunoblot analysis revealed that SakA phosphorylation is much weaker in germlings than in resting conidia (Figure 2A and B). However, both 1.2 M sorbitol and the antifungal agent fludioxonil induced SakA hyper-phosphorylation in a TcsC-dependent manner (Figure 2B). Thus, TcsC is required for activation of the HOG pathway by hyperosmotic stress and the phenylpyrrole antifungal agent fludioxonil.

We found no evidence for an enhanced sensitivity of the ΔtcsC mutant to calcofluor white, several clinically relevant antifungals (amphotericin B, posaconazol and caspofungin), pH (pH 5-9), temperature (20°C–48°C) or oxidative stress (H_2O_2 and t-BOOH) (data not shown). In fact, the mutant turned out to be slightly more resistant to the cell wall stressor congo red and UV light (Figure 3D and data not shown). Thus, TcsC activity is required for adaptation to hyper-osmotic stress, but is not essential for the general stress response.

TcsC is Essential for the Fungicidal Acitivity of Fludioxonil and Related Compounds

An interesting feature of Group III HHK mutants is their resistance to fludioxonil and related fungicides. Accordingly, the ΔtcsC mutant grew normally in liquid medium containing 10 μg/ml fludioxonil, whereas growth of the wild type was completely abrogated at 1 μg/ml fludioxonil (data not shown). This phenotype was also evident in drop dilution assays on plates supplemented with fludioxonil (1 μg/ml; Figure 3E) or the functionally related fungicides quintozene (25 μg/ml) and iprodione (25 μg/ml) (Figure S2 B and C, respectively).

To obtain more information on the impact of fludioxonil at the level of individual cells, germlings were incubated in the presence of 1 μg/ml fludioxonil. No obvious morphological changes were apparent after 2 h (Figure 4A and B), but 4 h and 6 h after addition of fludioxonil growth of the wild type (Figure 4D and F) and the complemented mutant (data not shown) stopped and the cells began to swell, whereas the growth and morphology of the ΔtcsC mutant remained normal (Figure 4C and E). Similar results were obtained with 25 μg/ml iprodione (data not shown). DAPI staining of germlings treated with fludioxonil for 6 h revealed a normal distribution of nuclei in hyphae of the mutant.

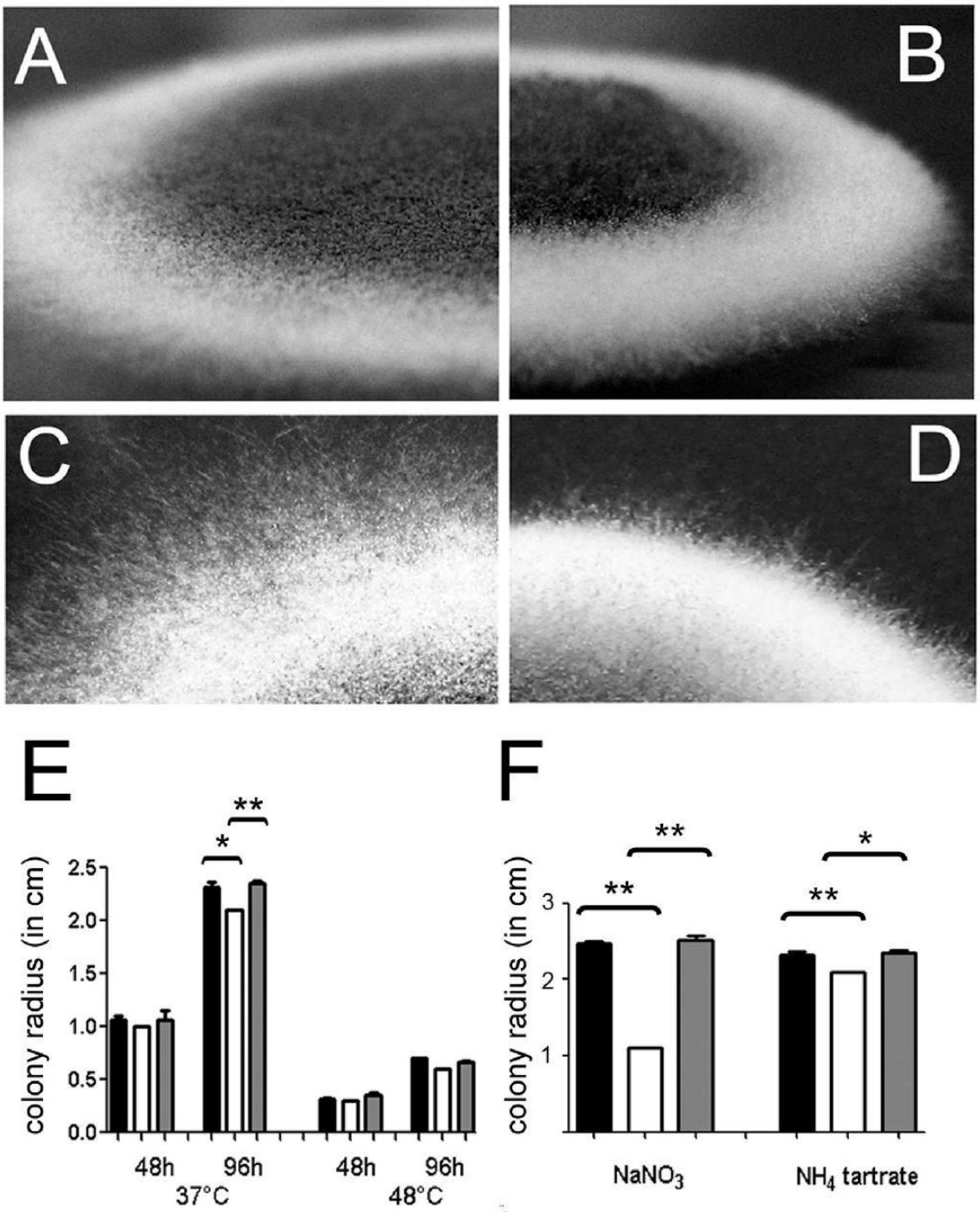

Figure 1. Growth of the Δ*tcsC* mutant. Colonies of the AfS35 wild type and the ΔtcsC mutant grown for 72 h on AMM plates are shown in panels A/C and B/D, respectively. Magnifications of the edge of the colonies are depicted in panels C and D. Note the reduced number of extending hyphae in the mutant. Panel E: Quantification of the radial growth of AfS35 (black), ΔtcsC mutant (white) and complemented mutant colonies (gray) on AMM plates after 48 h and 96 h at 37˚C or 48˚C. Panel F: Quantification of the radial growth after 96 h of AfS35 (black), ΔtcsC mutant (white) and complemented mutant colonies (gray) on AMM plates supplemented with 1.4 M $NaNO_3$ or 0.2 M ammonium tartrate at 37˚C. The experiments shown in panels E and F were done in triplicate. Standard deviations are indicated. Student's t-test: *p,0.005; **p,0.001. doi:10.1371/journal.pone.0038262.g001

(Figure 4G), but an unusually high number of nuclei in the swollen cells of the wild type (Figure 4H) and the complemented mutant (data not shown).

We also analyzed the impact of fludioxonil on the germination of resting conidia. Spores were incubated in medium supplemented with 1 μg/ml fludioxonil. After 28 h, the wild type produced only small germlings (Figure S3 A and B), while abundant hyphae were found in the fludioxonil-treated ΔtcsC mutant and an untreated wild type control (data not shown). Thus, germination of wild type spores was impaired, but not completely abolished by fludioxonil. An additional 18 h incubation in fludioxonil yielded cells whose growth was arrested and these exhibited irregular, swollen morphologies (Figure S3 C-F).

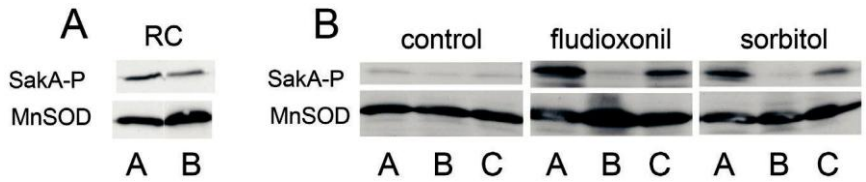

Figure 2. The role of TcsC in the phosphorylation of SakA. Protein extracts of resting conidia (RC)(panel A) and germlings (panel B) were analyzed by immunoblot using specific antibodies to phosphorylated SakA and as a loading control mitochondrial MnSOD. Extracts were prepared from germlings treated with 10 µg/ml fludioxonil and 1.2 M sorbitol for 2 and 20 min, respectively. A: parental strain AfS35, B: ΔtcsC mutant, C: complemented mutant.
doi:10.1371/journal.pone.0038262.g002

As observed for germlings, fludioxonil treatment during germination resulted in unusually high numbers of nuclei that were often clustered in the cytoplasm (Figure S3 A, C and E). Only few fludioxonil-treated cells showed signs of leakage after 46 h (data not shown). We therefore replaced the medium and incubated the cells for another 15 h without fludioxonil to analyze their ability to recover. Although fludioxonil had induced severe morphological changes the cells were able to restore growth and the resulting hyphae had a normal appearance and a normal distribution and number of nuclei (Figure S3 G and H).

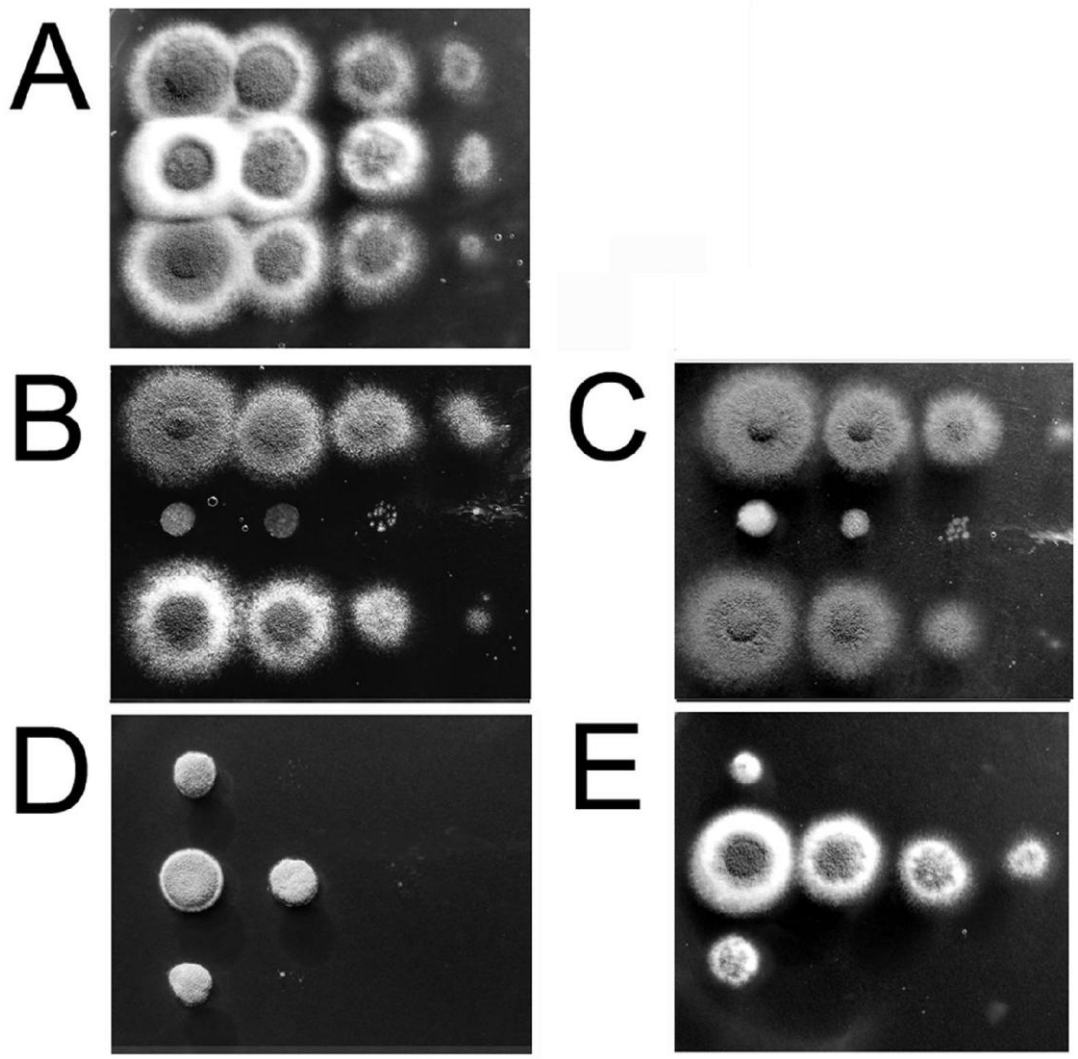

Figure 3. The *ΔtcsC* mutant is sensitive to hyperosmotic stress and resistant to fludioxonil. Drop dilution assays were performed on AMM plates (supplemented with ammonium). Panel A: control; B: 1.2 M sorbitol; C: 1 M KCl; D: 100 µg/ml congo red; E: 1 µg/ml fludioxonil. The depicted colonies were obtained after 48 h at 37°C. Top: AfS35; middle: ΔtcsC; bottom: complemented strain.
doi:10.1371/journal.pone.0038262.g003

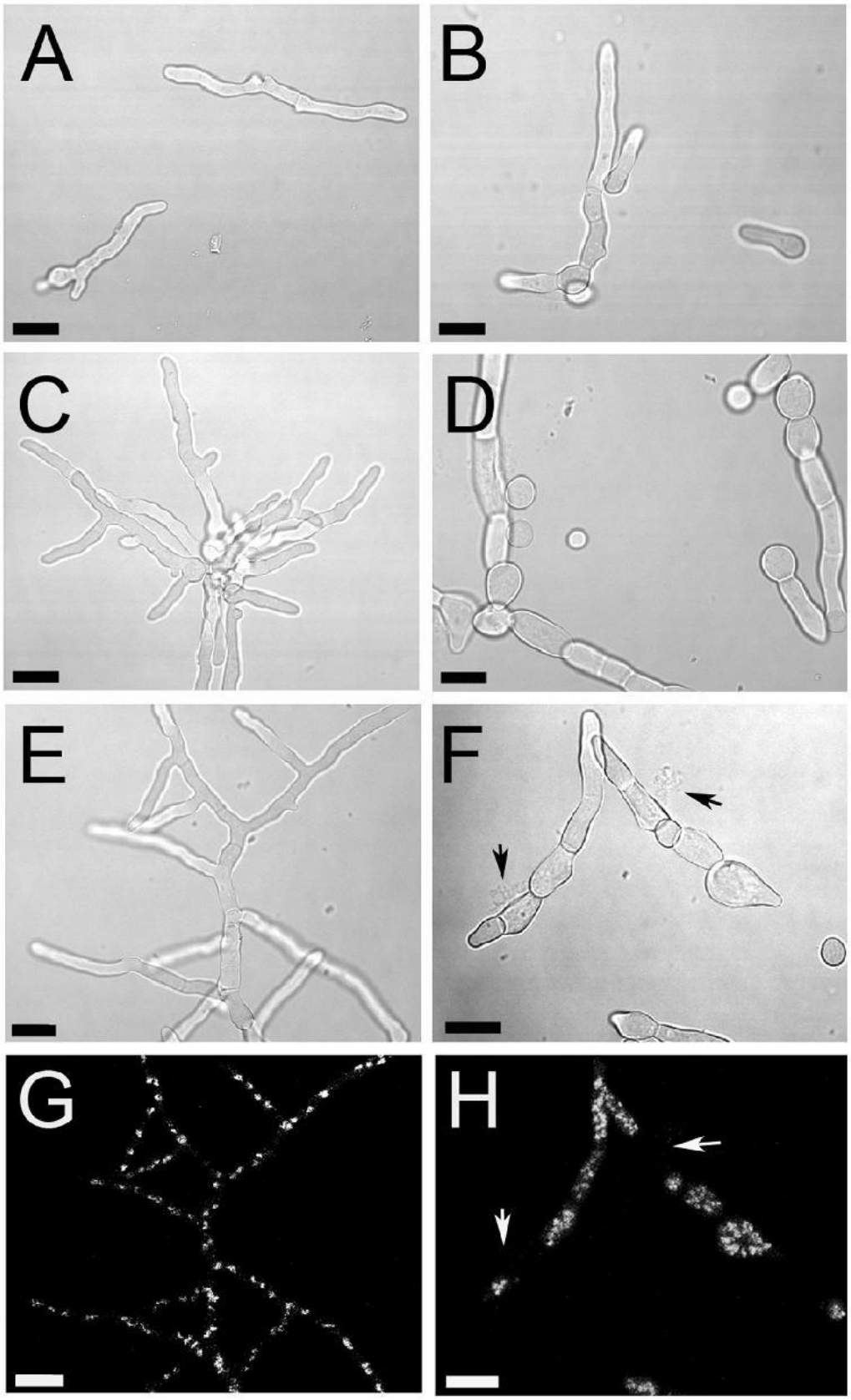

Figure 4. Impact of fludioxonil on *A. fumigatus* germ tubes. Conidia of the ΔtcsC mutant (panels A, C, E, G) and its parental strain AfS35 (panels B, D, F, H) were seeded on glass cover slips and incubated overnight in AMM at 30°C. The resulting germ tubes were treated with 1 µg/ml fludioxonil for 2 h (A, B), 4 h (C, D) and 6 h (E–H) at 37˚C. A DAPI staining is shown in panels G and H. Arrows indicate lysed cells that lack intracellular nuclei and are associated with amorphous extracellular material. All bars represent 10 µm.
doi:10.1371/journal.pone.0038262.g004

The Role of TcsC in the fluffy Growth Phenotype in *A. fumigatus*

Tco1, the Group III HHK of *Cryptococcus neoformans*, is required for growth under hypoxic conditions [18]. Oxygen limitation is also encountered by *A. fumigatus* during infection and it was recently shown that its ability to grow under hypoxic conditions is a prerequisite for virulence [19]. Adaptation of *A. fumigatus* to 1% oxygen results in colonies that are characterized by a massive production of aerial hyphae, resulting in a dome-shaped morphology, and a complete lack of sporulation (Figure 5A). At 1% oxygen the ΔtcsC mutant was indistinguishable from the control strains with respect to growth and colony morphology. At 2% oxygen flat and sporulating colonies were found for the control strains, whereas the mutant colonies remained white and dome-shaped (Fig. 5B). Similar *A. nidulans* colonies, also characterized by the formation of abundant aerial hyphae and the lack of sporulation, were described previously as having a 'fluffy' developmental phenotype [20]. Thus, oxygen limitation seems to activate a specific morphogenetic program and the threshold level of hypoxic stress required to trigger this developmental process is clearly lower in the ΔtcsC mutant.

A fluffy phenotype is also apparent in the presence of 2 mM of the acyclic sesquiterpene alcohol farnesol (Figure 5C and E; [21]). Titration of farnesol revealed that at lower concentrations the fluffy growth was restricted to the ΔtcsC mutant (Figure 5D). Thus, the absence of TcsC renders *A. fumigatus* more sensitive to oxygen limitation and farnesol. Further experiments revealed a third trigger for fluffy growth in *A. fumigatus*. White, dome-shaped colonies of the mutant, but not of the control strains were obtained on plates containing 100 mM $CaCl_2$ and 100 mM $MgCl_2$ (Figure 5G and data not shown). This phenotypic switch was also induced by 100 mM $MgSO_4$ (Figure 5F and H), but not by 200 mM NaCl (data not shown), indicating that divalent cationic ions, but not the slight increase in osmolarity or elevated chloride concentration induced the fluffy growth. The phenotypic differentiation was already obvious with 50 mM $CaCl_2$ (Figure 5I), and could be enforced by addition of 20 μM farnesol, which *per se* had no impact on the colony morphology (data not shown), suggesting a synergistic mode of action for these stimuli. A further increase of the calcium concentration to 500 mM induced the fluffy growth phenotype in the control strains, but concomitantly abrogated growth of the mutant (Figure 5J). Thus, oxygen limitation, farnesol and divalent cations activate the fluffy developmental program and the lack of tcsC renders cells more susceptible to this developmental reprogramming.

The fluffy growth phenotype in *A. nidulans* is regulated by a heterotrimeric G protein that has been functionally linked to the cAMP-dependent protein kinase pathway [22,23]. For *A. fumigatus*, addition of 5 mM cAMP partially rescued the sporulation defect caused by farnesol (Figure 6A), but not that triggered by 100 mM $CaCl_2$ or hypoxia (1% oxygen) (data not shown). We also tested the influence of light that stimulates sporulation in many fungi. Exposure of colonies to white light rescued the sporulation defect induced by 1% oxygen in the parental and the complemented strain, but not in the ΔtcsC mutant. Moreover, light also reduced the formation of aerial hyphae and resulted in colonies with a normal appearance (Figure 6B). 100 mM $CaCl_2$ or 2% oxygen are weaker activators of the fluffy program. They only influence the growth of the ΔtcsC mutant and this effect can also be prevented by light (Figure 6B and data not shown). The impact of light on the farnesol-induced sporulation defect could not be analyzed due to the known sensitivity of this agent to light. Thus, light and cAMP can antagonize the development towards a fluffy growth phenotype. In doing so cAMP was only able to neutralize the effect of farnesol, whereas light seems to have a broader impact.

Analysis of the Virulence of the DtcsC Mutant

The ability to respond to certain kinds of stress is clearly impaired in the ΔtcsC mutant. In order to investigate whether this negatively affects its virulence potential, cortisone-acetate treated mice were infected via the intra-nasal route. Survival of mice infected with the ΔtcsC mutant was comparable to those infected with the control strains (Figure 7) and the histological analysis of samples from the lungs of mice that succumbed to infection also revealed no apparent differences (data not shown). A normal virulence was furthermore observed in a alternative infection model using embryonated eggs [24](data not shown).

Discussion

In an often hostile environment pathogenic microorganisms rely on the ability to sense and respond to environmental changes. Two-component signaling (TCS) systems are sensing entities that are abundant in bacteria, but also found in fungi and plants. Because they are absent in mammals, TCS systems and their hybrid histidine kinases (HHK) are potential targets for novel anti-microbial strategies. Group III HHK are predicted to localize in the cytoplasm, but are nevertheless supposed to sense changes in the environment. The resulting signals are then transferred via a phospho-relay system to two response regulators that directly or indirectly trigger an appropriate transcriptional response [4]. In this study we have analyzed TcsC, the only Group III HHK of the pathogenic mold *A. fumigatus*. Deletion of the homologous nikA gene in *A. nidulans* has been reported to cause a significantly reduced growth on solid medium [14,15], whereas the ΔtcsC mutant grows normally on complex media and on minimal medium (AMM) supplemented with ammonium. Growth was however impaired on AMM supplemented with nitrate, suggesting that TcsC is required for efficient nitrogen assimilation. In this context it is noteworthy that the growth defect of the ΔnikA mutant was observed using minimal medium with nitrate as the sole nitrogen source [15] and it would be interesting to test the growth of this mutant on a medium containing ammonium.

Although Group III HHK are often linked to the high osmolarity glycerol (HOG) pathway, their relevance for adaptation to hyperosmotic stress seems to vary in different fungi. While the ΔnikA mutant showed a normal ability to adapt to hyperosmotic stress [15], the ΔtcsC mutant turned out to be highly sensitive. Another striking difference between both mutants exists with respect to their conidial viability. Conidia of the ΔnikA mutant showed a dramatic loss of viability when stored in water for several days [14,15], whereas conidia of the ΔtcsC mutant remained fully viable upon storage for several weeks. Thus, TcsC and NikA although closely related, appear to differ in their biological activities.

A characteristic feature of mutants lacking Group III HHK is their resistance to fungicides, like fludioxonil. These compounds are currently used in agriculture, but are also of potential interest for the development of novel therapeutic anti-fungals. Their mode

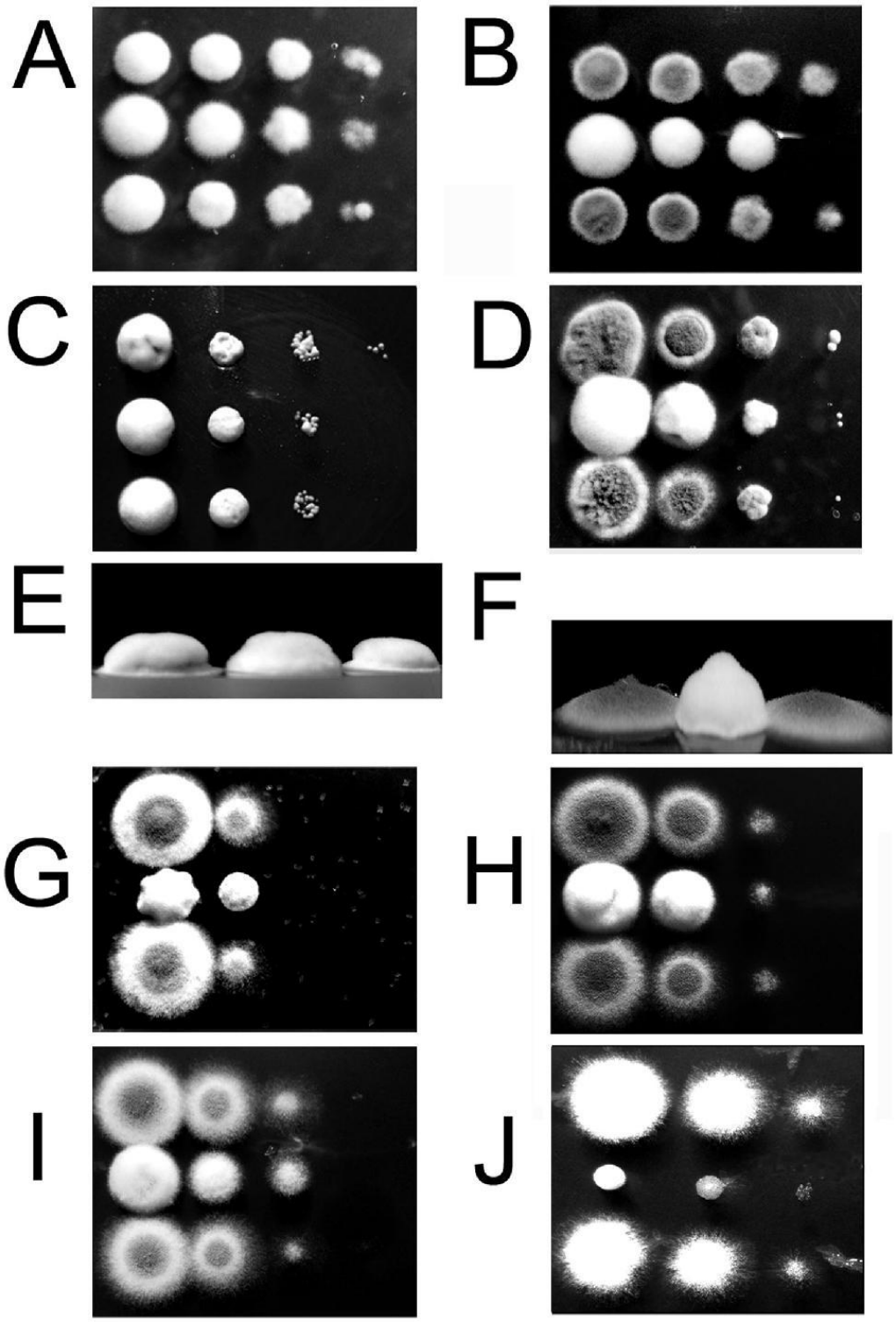

Figure 5. The role of TcsC in the stress-induced developmental program leading to a fluffy growth phenotype. Drop dilution assays were performed on AMM plates (supplemented with ammonium). Panel A: 1% oxygen; B: 2% oxygen; C: 2 mM farnesol; D: 200 µM farnesol; E: 2 mM farnesol; F: 100 mM $MgSO_4$; G: 100 mM $CaCl_2$; H: 100 mM $MgSO_4$; I: 50 mM $CaCl_2$; J: 500 mM $CaCl_2$. Side views of colonies from C and D are shown in panels E and F. The depicted colonies were photographed after 48 h at 37°C. AfS35 (top/left); ΔtcsC (middle); complemented strain (bottom/right). doi:10.1371/journal.pone.0038262.g005

of action is unique in that they activate a fungal signaling process, the HOG pathway. The hallmark of this activation is the phosphorylation and subsequent translocation of SakA/Hog1 to the nucleus [10]. In *A. fumigatus* fludioxonil induces a rapid, transient phosphorylation and translocation of the MAP kinase SakA that leads to a tremendous cellular swelling. Fludioxonil

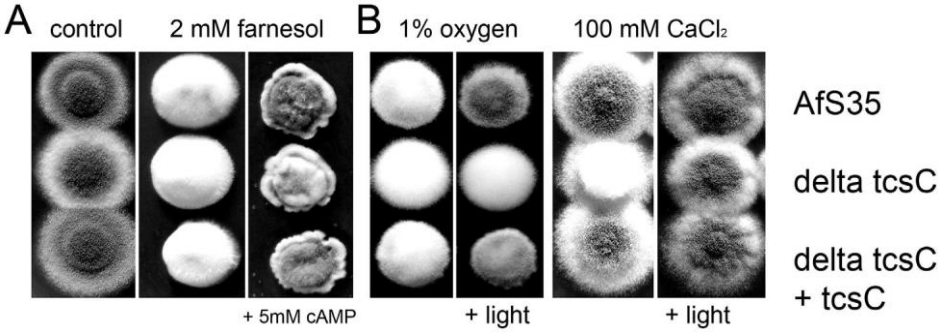

Figure 6. The impact of cAMP and light on the fluffy growth phenotype. Drop dilution assays were performed on AMM plates (supplemented with ammonium). The plates were supplemented or treated as indicated and incubated in incubator. When indicated plates were incubated under white light produced by an LED light source. Pictures were taken after 48 h at 37°C.
doi:10.1371/journal.pone.0038262.g006

blocks growth of germ tubes and hyphae, but it is unable to completely prevent germination of resting conidia. Prolonged incubation in the presence of fludioxonil results in rather odd cellular morphologies. These phenotypic changes are stable as long as the agent is present, but normal growth can be restored after removal of the agent. Apart from their swelling, fludioxonil-treated *A. fumigatus* cells are remarkable because of their large number of nuclei. A block in nuclear division, as recently suggested for fludioxonil-treated *A. nidulans* [17], was not detectable; instead cytokinesis and mitosis seem to be transiently uncoupled, resulting in the accumulation of many more nuclei per cell than normal. These fludioxonil-induced phenotypic changes are dependent on TcsC, since they do not occur in the ΔtcsC mutant.

The complete resistance of the ΔtcsC mutant to fludioxonil and related fungicides correlates with its high sensitivity to hyperosmotic stress. It has been shown for several plant-pathogenic fungi that fludioxonil mediates its anti-fungal effect by activating the HOG pathway via a Group III HHK [9]. It is therefore conceivable that the characteristic swelling of fludioxonil-treated *A. fumigatus* cells results from a hyperactivation of SakA. This is already detectable after 2 minutes and seems to trigger an uncontrolled increase in the intracellular osmotic pressure. In *A. fumigatus*, TscC is clearly required for the activation of the HOG pathway by both, fludioxonil and hyperosmotic stress. Thus, the inability of the ΔtcsC mutant to adapt to hyperosmotic stress and its resistance to fludioxonil both reflect the important role of the TcsC-SakA signaling axis in the control of the internal osmotic pressure of *A. fumigatus*.

The life cycle of *A. fumigatus* is tightly controlled by environmental cues. In contact with air hyphae initiate the formation of conidiophores and the production of conidia. The 'fluffy' developmental program impedes sporulation and leads to the massive formation of aerial hyphae and the appearance of white, dome-shaped colonies. Fluffy *A. nidulans* colonies were initially described after treatment with 5-azacytidine [20]. The phenotypical stability of these mutants indicates that a developmental program is permanently activated in these cells. We have recently identified the sesquiterpene alcohol farnesol as a trigger for transient fluffy growth in *A. fumigatus* [21]. In the current study, we observed similar phenotypic switches in response to hypoxia and elevated concentrations of certain divalent cations. The fluffy growth type likely provides an advantage enabling the fungus to survive under certain kinds of stress. The ΔtcsC mutant shifts earlier towards this phenotype than the wild type. White, dome-shaped colonies appeared at lower concentrations of farnesol and divalent cations and at less pronounced hypoxia. The earlier adaptation of the mutant does not result in a higher robustness,

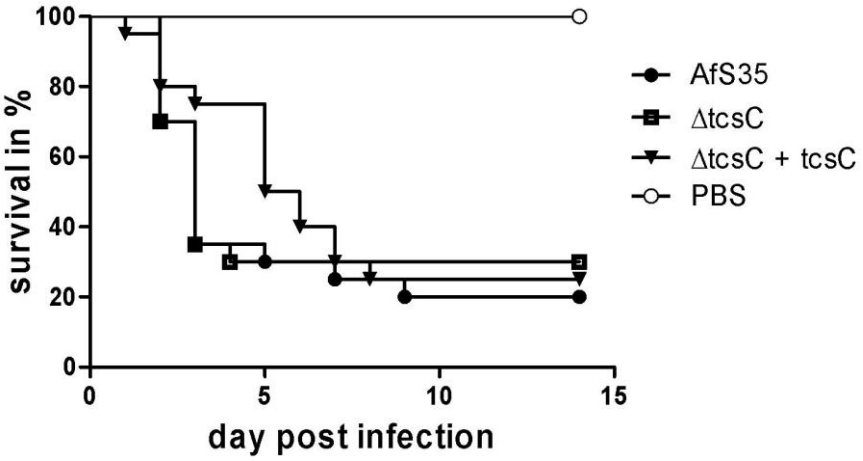

Figure 7. Infection of immuno-compromized mice. Intranasal infection of cortisone-acetate treated mice infected with $1x10^6$ conidia of the ΔtcsC mutant (n = 20), the parental strain AfS35 (n = 20) and the complemented strain (n = 20). Controls received PBS only. Survival of mice is shown over time.
doi:10.1371/journal.pone.0038262.g007

but seems to be the consequence of a reduced stress resistance. The limited compensatory potential of the fluffy growth was in particular evident at elevated calcium concentrations. The mutant shifts already at 50 mM calcium chloride, but its growth is abolished at 500 mM calcium chloride, when the wild type is still growing well.

So far, little is known about the mechanisms that underlie the fluffy growth phenotype and cause its peculiar morphological changes. In *A. nidulans* the fluffy growth seems to be controlled by a heterotrimeric G protein that is linked to the cAMP-dependent protein kinase pathway [22,23]. This and the recent finding that farnesol blocks adenylyl cyclase activity in *Candida albicans* [25] prompted us to study the relevance of the intracellular cAMP level. Addition of cAMP abrogated the farnesol-induced block in sporulation in the wild type, but cAMP was unable to rescue the sporulation defect caused by hypoxia or elevated calcium concentrations. Light is an environmental signal that stimulates sporulation in many fungi. Exposure to light restored normal growth and sporulation under hypoxic conditions and in the presence of elevated concentrations of divalent cations. Thus, light, cAMP and the TcsC protein are factors that impede an activation of the fluffy growth program caused by hypoxia, farnesol or divalent cations (Figure 8).

A stable fluffy *A. fumigatus* mutant secretes more proteases and has an increased angioinvasive growth capacity [26]. This suggests that fluffy hyphae may be well adapted to the specific requirements during infection. In line with this hypothesis, we identified oxygen limitation as another trigger for a fluffy growth. It will be interesting to analyze to what extent the fluffy growth program observed in vitro resembles the morphogenetic program that is active during infection.

The ΔtcsC mutant shows a normal sensitivity to oxidative, temperature and pH stress as well as clinically relevant anti-fungal agents. On the other hand, TcsC activity is important for the response to a limited array of stress signals including hypoxia (Figure 8). The ability to adapt to oxygen limitation is an essential characteristic of many pathogenic microorganisms. Tco1, the homologous group III HHK in *Cryptococcus neoformans* regulates growth under hypoxic conditions and is also required for virulence [27]. In *A. fumigatus* the situation seems to be different, since we observed no significant attenuation in virulence for the ΔtcsC mutant. However, TcsC is required for the anti-fungal activity of fludioxonil and related compounds and may therefore be an attractive target for new therapeutic anti-fungals. Further studies are underway to define the precise mode of action of the TcsC stress sensing pathway and the impact of fludioxonil on growth and survival of *A. fumigatus*.

Materials and Methods

Strains Media and Growth Conditions

The *A. fumigatus* strain AfS35, a derivative of strain D141, has been described in [28]. AMM and YG medium were prepared as described [29]. AMM was either supplemented with 1.4 M $NaNO_3$ [30] or 0.2 M ammonium tartrate. For hypoxic growth plates were incubated at 37˚C in a HERAcell 150i incubator (Thermo Fisher Scientific) adjusted to 5% CO_2 and the desired oxygen concentration.

Sequence Analysis and Data Base Searches

Domains were predicted using SMART (http://smart.embl-heidelberg.de/) and alignments were performed using CLUSTAL (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Construction of the ΔtcsC Mutant Strain

All oligonucleotides used in this study are listed in Table S1. To construct a suitable replacement cassette a 3.5 kb hygromycin resistance cassette was excised from pSK346 using the SfiI-restriction enzyme. The flanking regions of the tcsC gene (approx. 900 bp each) were amplified by PCR from chromosomal DNA using the oligonucleotide pairs tcsC-upstream and tcsC-down-stream. These oligonucleotides harbor ClaI and Sfi sites. After

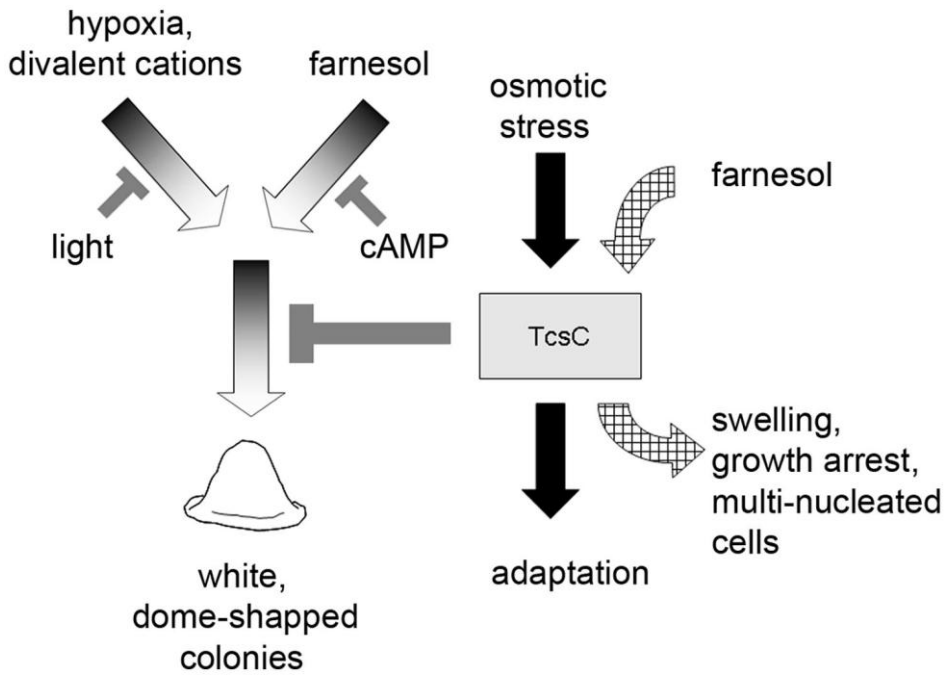

Figure 8. Schematic model of the biological activities of *A. fumigatus* TcsC.
doi:10.1371/journal.pone.0038262.g008

digestion with ClaI and SfiI, ligation of the three fragments (resistance cassette and flanking regions) yielded a 5.3 kb deletion cassette that was purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The fragment was cloned into the pCR2.1 vector (Invitrogen) using oligonucleotide-derived ClaI sites. A 9.2 kb fragment from the resulting plasmid was linearized with SpeI and used for transformation of *Aspergillus*. The construct used for complementation of ΔtcsC was generated by amplifying tcsC and its native promoter (1.5 kb region upstream of the gene) from chromosomal DNA using the oligonucleotides tcsC + native promoter-forward and tcsC-reverse. The gpdA promoter was excised from the pSK379 vector using EcoRV and NsiI; the latter enzyme generates sticky ends compatible with those generated by PstI. The amplified tcsC + native promoter fragment was cloned into this modified version of pSK379 using oligonucleotide-derived NsiI sites. The resulting plasmid was purified as above and used for transformation of the ΔtcsC mutant.

A. fumigatus protoplasts were generated and fungal transformation was performed essentially as described previously [29]. The resulting protoplasts were transferred to AMM plates containing 1.2 M sorbitol and either 200 μg/ml hygromycin (Roche, Applied Science) or 0.1 μg/ml pyrithiamine (Sigma-Aldrich).

Genomic DNA Analysis

A. fumigatus clones which showed the expected resistance on selective plates were further analyzed by PCR. The correct integration of the deletion cassette was analyzed at the 5' end using oligonucleotides tcsC-upstream-forward and hph-3-SmaI (PCR1) and at the 3' end using oligonucleotides trpCt-forward and tcsC-downstream-reverse (PCR2) (Figure S2). To detect the presence of tcsC in the complementation mutant, the entire tcsC gene was amplified using primers at the 5' and 3' ends of the gene (tcsC-forward and tcsC-reverse, PCR3). Primer sequences are listed in Table S1.

Quantification of Sporulation Efficiency

For each strain tested, three small tissue culture flasks (25 cm^2; Sarstedt, Nürnbrecht, Germany) with YG agar were inoculated with $4x10^6$ conidia per flask. After incubation for 4 days at 37°C conidia were harvested and counted using a Neubauer chamber.

Spore Viability Assay

To determine their viability, $2x10^4$ resting conidia were transferred to 1 ml YG medium in a 24 well plate. After overnight incubation at 37°C samples were fixed by addition of 100 μl 37% formaldehyde. The percentage of germinated cells was determined microscopically. These experiments were done in triplicate.

Protein Extraction and Western Blot

For protein extractions from resting conidia, 75 cm^2 flasks containing YG agar were inoculated with AfS35 or ΔtcsC conidia (in triplicate) and grown at 37°C for 3 days. Conidia were harvested in sterile water and the pellet frozen overnight at –20°C. Frozen conidia pellet was lyophilized overnight at 6°C. The dry pellet was ground with a mortar and pestle in liquid nitrogen. The ground conidia powder was added to 300 μl Laemmlie buffer (2% [w/v] SDS, 5% [v/v] mercaptoethanol, 60 mM Tris/Cl pH 6.8, 10% [v/v] glycerol, 0.02 [w/v] bromophenol blue), heated at 95°C and immediately extracted twice using a Fast Prep 24 (M.P. Biomedical, Irvine, CA) with a speed of 5.5 m/s for 20 s, followed by a final heat denaturation at 95 °C for five minutes. 20 μl protein extract was used for SDS-PAGE on 12% SDS gel. Proteins were blotted onto 0.45 μm nitrocellulose membranes and labeled with an α-phospho-p38 MAP kinase antibody (Cell Signaling Tech-nology [#9211], MA, USA). A monoclonal antibody directed against mitochondrial MnSOD (P118-H3) kindly provide by Bettina Bauer was used as a loading control. For protein extractions from germ tubes, $4x10^7$ resting conidia were in-oculated in 10 ml AMM and incubated 9 h at 37°C. The germ tubes were treated with 10 μg/ml fludioxonil or 1.2 M sorbitol for 2 min or 20 min, respectively, at 37°C. Protein was then extracted from the cell pellet as above and used for SDS-PAGE and immunoblot in the same manner.

Phenotypic Plate Assays

Isolated conidia were counted using a Neubauer chamber. For drop dilution assays, a series of tenfold dilutions derived from a starting solution of $1x10^8$ conidia per ml were spotted in aliquots of 1 μl onto plates. These plates were supplemented with the indicated agents and incubated at the indicated temperatures. For quantification of the radial growth, 3 μl containing $3x10^4$ conidia were spotted in the centre of a 9 cm Petri dish. The radius of the colonies was determined over time.

E-test strips of voriconazole, amphotericin B and caspofungin were obtained from Inverness Medical (Cologne, Germany). Each E-test strip was placed onto an AMM agar plate spread with $8x10^5$ conidia. Plates were incubated 36–48 h at 37°C.

Paper disk assays were performed by spreading $8x10^5$ conidia on AMM, Sabouraud, or YG agar plates and placing a sterile paper disk containing fludioxonil, iprodione, or quintozene (Sigma-Aldrich; 46102, 36132 and P8556, respectively), or H_2O_2 or tert-butyl hydroperoxide (t-BOOH; Sigma-Aldrich). Plates were incubated 36–48 h at 37°C. Fludioxonil and iprodione were dissolved at 100 mg/ml stock concentrations in DMSO and quintozene was dissolved at 10 mg/ml stock concentration in chloroform. The influence of light was analyzed using an LED light (Osram DOT-it, Osram, Munich, Germany) that was affixed 15 cm above the Petri dish.

Microscopic Analysis

To visualize the effects of fludioxonil on germ tubes and resting conidia, AfS35 or ΔtcsC resting conidia were inoculated in 24-well plates containing 1 ml AMM and glass cover slips. Germ tubes were generated by incubating at 30°C overnight before adding 1 μg/ml fludioxonil. After incubation at 37°C for the indicated times, cells were fixed in 3.7% formaldehyde for five minutes at room temperature. Cover slips were mounted to glass slides in Vecta Shield containing DAPI (Vector Laboratories, Burlingame, California, USA). Cells were then visualized using a Leica SP-5 microscope (Leica Microsystems).

Infection Experiments

To analyze the impact of TcsC we used an intranasal infection model using immunocompromized female outbred CD-1 mice. Mice were immunosuppressed by intraperitoneal injection of cortisone acetate (25 mg/mouse, Sigma-Aldrich) on days –3 and 0. On day 0 the mice were anesthetized with fentanyl (0.06 mg/kg, Janssen-Cilag, Germany), midazolam (1.2 mg/kg, Roche, Germany) and medetomidin (0.5 mg/kg, Pfizer, Germany) and infected intranasally with $1x10^6$ conidia in 20 μl PBS. Controls received PBS only. Survival was monitored for 14 days. During this period, mice were examined clinically at least twice daily and weighed individually every day. Kaplan-Meier survival curves were compared using the log rank test (SPSS 15.0 software). Mice were cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European

Treaty Series, no. 123; http://conventions.coe.int/Treaty/en/Treaties/Html/123). All animal experiments were in compliance with the German animal protection law and were approved (permit no. 03-001/08) by the responsible Federal State authority and ethics committee.

Supporting Information

Figure S1 (A) Schematic drawing of the genomic tcsC gene and the deleted tcsC::hph/tk locus. Approximately 1 kb of the 5' and 3' regions of tcsC gene were used for construction of the deletion cassette. The positions of the primers employed for the PCR amplifications and the resulting PCR products (PCR 1-3) are indicated. (B) Equal amounts of genomic DNA of AfS35, ΔtcsC and ΔtcsC+tcsC were used as template for PCR amplification of the regions indicated in panel A (PCR 1-3). (TIF)

Figure S2 Resistance of the ΔtcsC mutant to iprodione and quintozene. The sensitivity to iprodione and quintozene was analyzed in drop dilution assays. AfS35 (top) and its ΔtcsC mutant (bottom) were spotted on plates without fungicides (panel A) or plates containing either 25 μg/ml quintozene (panel B) or 25 μg/ml iprodione (panel C). Pictures were taken after 48 h at 37˚C. (TIF)

Figure S3 Impact of fludioxonil during germination of *A. fumigatus* conidia. Conidia of *A. fumigatus* strain AfS35 were seeded on glass cover slips and incubated at 37˚C in the presence of 1 μg/ml fludioxonil for 28 h (A, B) and 46 h (C to F). After 46 h the medium was replaced by fresh medium. Fungal cells fixed after another 15 h in the absence of fludioxonil are shown in G and H. DAPI stainings are shown in panels A, C, E and G. All bars represent 10 μm. (TIF)

Table S1 Oligonucleotides used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: FE AM. Performed the experiments: AM IDJ MB JB. Analyzed the data: FE AM JH. Wrote the paper: FE AM.

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7. Discussion

The incidence of aspergillosis has continued to rise over the past few decades, and additional diagnosis and treatment options are continuously sought. While many of these have proven beneficial, scientists, medical doctors, and patients still require ulterior solutions to combat this deadly disease. *Aspergillus fumigatus*, as a pathogen, remains an enigma. The sheer ubiquity of the fungus and the body's ability to avoid infections on a daily basis makes the relatively rare case all the more baffling. We have attempted to unravel part of the mystery of *A. fumigatus* pathogenicity by first exploring defense mechanisms of the innate immune system, by expounding upon the role of NETs and IFN-γ from the host, and then by clarifying the role of a conserved fungal stress response pathway, not only in adaptation and growth, but in virulence as well.

7.1 NETs are formed in response to A. fumigatus

The innate immune system is the host's first line of defense against inhaled conidia, and when functioning properly, together these cells easily rid the body of *A. fumigatus*. Leading the initial attack, and thought to be most important in fending off *A. fumigatus* infection, are the polymorphonuclear neutrophils (PMN) (Mircescu *et al.*, 2009). Conidia that manage to escape resident alveolar macrophages must resist attacking PMNs, which like alveolar macrophages, can phagocytose resting and swollen conidia and even small germlings. Hyphae, however, present a much larger problem. The hyphae not only break off and disseminate through the blood stream, but can penetrate tissues, which becomes especially dangerous in the brain (McCormick *et al.*, 2010b). Long ago it was shown that PMNs align themselves along the surface of a growing hypha (Diamond *et al.*, 1978) so that degranulation and the oxidative burst can occur in close proximity to the hyphal surface, supposedly damaging the fungal cell with toxic granule contents and reactive oxygen species (ROS) (Hasenberg *et al.*, 2011).

A third mode of neutrophil attack has recently been discovered, called neutrophil extracellular traps (NETs) (Brinkmann *et al.*, 2004). During NETosis, the chromosomal DNA decondenses and the neutrophil bursts, releasing a sticky mass of DNA decorated with antimicrobial peptides and certain cellular proteins, e.g., elastase. NETs have been found to degrade virulence factors and kill bacteria, via the attached antimicrobial peptides, while the sticky chromatin prevents microbes from spreading (Brinkmann and

Zychlinsky, 2007; Papayannopoulos and Zychlinsky, 2009). It has been shown by our group (unpublished data) and others (Urban *et al.*, 2009b; Urban *et al.*, 2006) that NETs are formed in response to *Candida albicans* infection. However, while Urban *et al.* showed that NETs *per se* can kill *C. albicans*, we have found no evidence of such (unpublished data). In the current study we found that NETs are also released in response to *A. fumigatus* conidia and hyphae. It was suggested in a parallel study that more NETs are released in response to hyphae than conidia (Bruns *et al.*, 2010); we found the same and provide qualitative evidence in our manuscript. Additionally, we report this process of NETosis as a form of cell death altogether different than apoptosis or necrosis. However, in contrast to the function of NETs during bacterial and even *Candida* infection (at least as reported by others), we found no evidence that NETs alone can kill *A. fumigatus*, neither conidia nor hyphae (McCormick *et al.*, 2010a).

7.1.1 NETs do not inhibit conidial germination but do inhibit hyphal elongation

We showed that PMNs can inhibit germination of resting conidia by phagocytosing the spores. Ingested conidia are then degraded within the phagosome (Hasenberg *et al.*, 2011; Nauseef, 2007) or intracellular germination is at least delayed. In the presence of NETs alone (PMNs induced with phorbol myristate acetate [PMA]), resting conidia swell and germinate as normal. We confirmed these findings by inhibiting phagocytosis with cytochalasin D, a substance that blocks polymerization of the actin cytoskeleton. Also under these conditions, resting conidia germinated as normal.

While we found that NETs, namely the associated calprotectin (and perhaps other NETs-associated proteins not yet analyzed), can inhibit germ tube elongation by depleting the surrounding media of essential zinc, we found no evidence that NETs alone can effectively kill *A. fumigatus* hyphae. We determined that calprotectin, and not lactoferrin, is responsible for inhibiting germ tube elongation. Calprotectin is a chelator of zinc and manganese; lactoferrin is an iron chelator known to inhibit the growth of *A. fumigatus* conidia (Zarember *et al.*, 2007; Savchenko *et al.*, 2011). Both metal chelators are associated with NETs (Urban *et al.*, 2009b). Supplementing the media with Zn^{2+}, but not iron or Mn^{2+} rescued the growth inhibition in the presence of NETs. Interestingly, we found that NETs inhibit growth of *C. albicans* hyphae in a similar way, but we could only partially rescue this growth inhibition by adding zinc to the media (unpublished data).

For both *A. fumigatus* and *C. albicans* growth inhibition assays we measured germ tube elongation at the cellular level using a method adapted from Wozniok *et al*., rather than the more common plating assay (Wozniok *et al.*, 2008). Plating assays, though often used to determine bacterial killing, are not as reliable a tool to detect fungal killing. Conidia and hyphae (and yeast) are very sticky and often grow as large conglomerates. So that when plated on agar a single colony may not necessarily represent a single fungal cell. Use of the plating assay could be the reason Urban *et al*. found NETs capable of killing *C. albicans,* while we did not find such evidence in our microscopic growth assay.

To confirm that NETs could not kill *A. fumigatus*, we used a strain in which the mitochondria were labelled with GFP. Fungal mitochondria are arranged in dynamic tubular networks that move throughout the cell, and the constant reorganization reflects cellular vitality (Okamoto and Shaw, 2005). We used live-cell microscopy to visualize the GFP-tagged mitochondrial dynamics in *A. fumigatus* hyphae in the presence of NETs (PMNs pre-treated with PMA). Under these conditions, no change in mitochondrial movement or morphology was detectable, indicating that the hyphae are certainly still alive (*Supplemental Figure 1*, McCormick *et al.*, 2010a).

7.1.2 Proposed purpose and mechanism of NETosis during *A. fumigatus* infection

There must be further factors that, *in vivo*, can manage infection. The neutrophils themselves may be activated by soluble factors released from other leukocytes, or the PMNs may have help from contributory recruited immune cells. Neutrophils are short-lived cells and it has long been believed that cytokine production is therefore minimal. However, it is now becoming clear that not only is their role as effector expanding—to include the production of NETs—but neutrophils may also be key recruiters (Ellis and Beaman, 2004). *In vivo*, a whole armament of leukocytes is available to a healthy host, and while NETs alone cannot kill the fungus, they serve an important purpose nonetheless. They slow and contain fungal growth, perhaps even opsonizing the ensnared fungus with pentraxin 3 (PTX3), a well documented opsonin and NETs-associated protein (Bottazzi *et al.*, 2009; Jaillon *et al.*, 2007; Moalli *et al.*, 2010). PTX3 is thought to bind galactomannan in the *Aspergillus* cell wall, though this was shown experimentally using resting conidia, where galactomannan is masked by hydrophobic rodlet proteins (Heesemann *et al.*, 2011). Regardless of the binding partner, PTX3 does

indeed bind *A. fumigatus* resting conidia (Moalli *et al.*, 2010), thereby enhancing phagocytosis by incoming PMNs, as well as dendritic cells and macrophages (Bottazzi *et al.*, 2006). We have seen that PTX3 also binds the hyphal surface (unpublished data). Additionally, high blood levels of PTX3 correlate with neutrophil recruitment to sites of inflammation (Deban *et al.*, 2010). The acronym—NETs—is no accident. Like a true 'net,' neutrophil extracellular traps contain microbes while allowing the movement and accessibility of other molecules, namely antimicrobial peptides and osponins.

The mechanism of NETosis was originally thought to be dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the production of ROS (Brinkmann and Zychlinsky, 2007). However, we have found that neutrophils collected from patients who have chronic granulomatous disease (CGD) are still capable of NETs production in response to *A. fumigatus* (unpublished data), suggesting the presence of an alternative pathway. CGD is caused by mutations in genes encoding subunits of NADPH oxidase, rendering these cells incapable of ROS production. CGD patients are therefore less able to kill invading microbes and so suffer from recurring life-threatening infections (Seger, 2008). NADPH oxidase-dependent NETosis signalling is transduced via Syk (spleen tyrosine kinase) through PKC, MEK, and ERK (protein kinase C, mitogen-activated kinase kinase, extracellular signal-regulated kinase, respectively) to the transmembrane NADPH oxidase (see **Figure 5**). As a result, ROS accumulate and NETs are released. Myeloperoxidase, an enzyme that catalyzes the production of hypochlorous acid from hydrogen peroxide is required for NETosis in this setting (Metzler *et al.*, 2011). Activation of MEK was found in turn to activate Mcl-1, an apoptosis inhibitor (Hakkim *et al.*, 2011), confirming our finding that regulated cell death by NETosis is clearly different than apoptosis. B-integrins, and to a lesser extent dectin-1, were found to activate NADPH oxidase production of ROS via Syk (Boyle *et al.*, 2011). Dectin-1 is a receptor on the surface of many immune cells that recognizes β-glucan present in the *Aspergillus* cell wall (Dennehy and Brown, 2007). The involvement of dectin-1 could explain an NADPH oxidase-dependent mode of NETosis in response to *A. fumigatus*. Additional receptors involved in *Aspergillus* recognition, TLR2 or 4 for example, may also be involved in NETosis, but to our knowledge this has not yet been explored. In fact, whether NETosis requires specific recognition of a microbe or is a general infection phenomenon has not yet been decided. We and others have evidence for an alternative, NADPH oxidase-independent mechanism of NETosis. Marcos *et al*. showed that CXCR2 binding on the neutrophil surface activates Src kinases and NETs are produced even in the presence of NADPH oxidase inhibitors (Marcos *et al.*, 2010). CXCR2 was previously found to be critical

to PMN recruitment during *Aspergillus* response (Phadke and Mehrad, 2005), and CXCR2-mediated NETosis could be the means by which neutrophils from CGD patients produce NETs in response to *A. fumigatus*. Non-CGD neutrophils may also utilize this pathway. During infection, oxygen concentrations in inflamed tissues rapidly decrease from healthy levels (0.5 – 11%) to hypoxic levels (0.5 – 3%) (Dietz *et al.*, 2012). NADPH oxidase requires oxygen to produce ROS. We have evidence that neutrophils can also undergo NETosis during *Aspergillus* infection *in vitro* in hypoxia, and in fact produce even more NETs in these conditions (unpublished data), again suggesting an NADPH oxidase-independent mechanism. The transcription factor HIF-1α is activated in hypoxia but also during infection and regulates a wide array of immune response genes, including NF-κB and Toll-like receptors (Rius *et al.*, 2008; Werth *et al.*, 2010; Kuhlicke *et al.*, 2007). We have evidence that HIF-1α is activated in PMNs both in hypoxia and during *A. fumigatus*

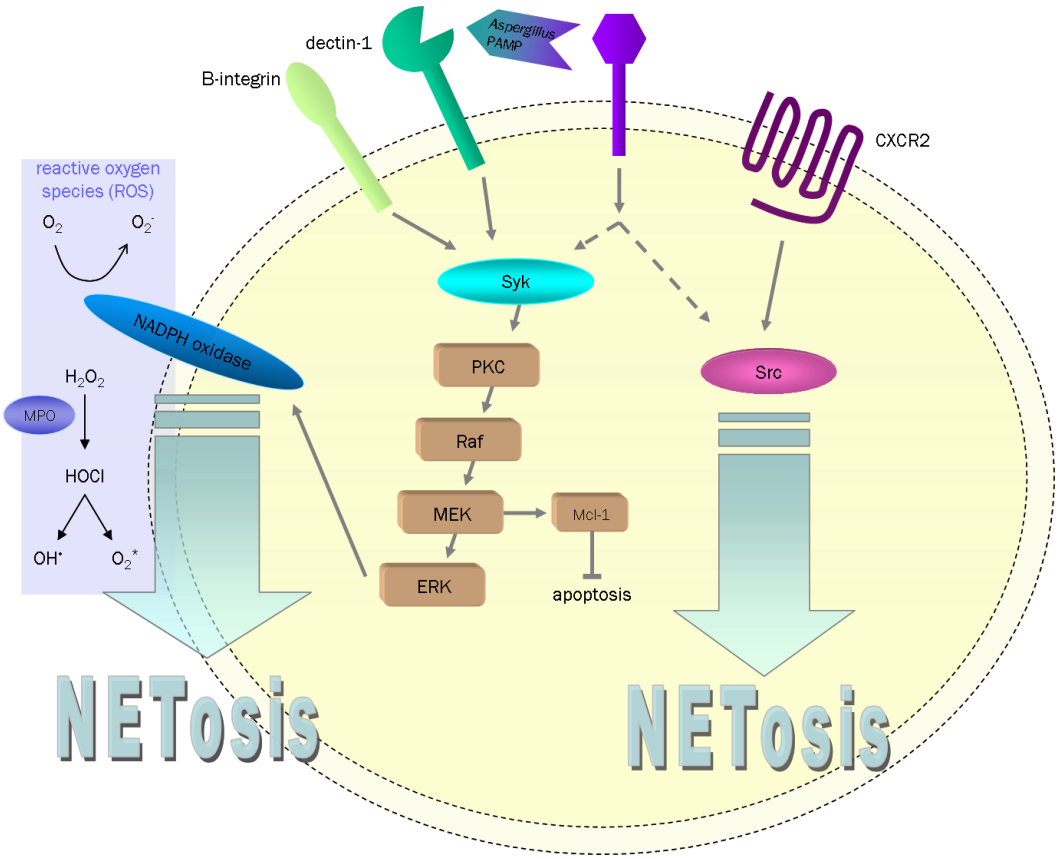

Figure 5. NADPH oxidase-dependent and -independent mechanisms of NETosis

NETosis can occur via NADPH oxidase by binding β-integrins and to a lesser extent dectin-1 on the neutrophil surface and transducing the signal through Syk. Reactive oxygen species (ROS) are produced and myeloperoxidase (MPO) converts H_2O_2 to highly reactive antimicrobial molecules. Activation of MEK also activates Mcl-1, an apoptosis inhibitor. NADPH oxidase, ROS, and MPO are all required components of this pathway. In CGD patients, NETosis occurs independent of NADPH oxidase, by binding of the CXCR2 receptor on the neutrophil surface. The signal is transudced via Src family kinases. This could also be a mechanism of NETosis in hypoxic environments, for example in inflamed tissue during infection. The involvement of additional receptors also involved in *Aspergillus* recognition is still unknown.

infection (unpublished data). Whether HIF-1α also plays a part in NETosis has not yet been explored, but presents an additional intriguing alternative to the classical NADPH oxidase-dependent pathway.

7.2 IFN-γ from NK cells acts directly against A. fumigatus *hyphae*

The activity of neutrophils, including NETosis, though unable to alone kill *A. fumigatus* hyphae, is important in abating fungal growth while the remaining innate immune arsenal arrives. Belonging to this arsenal are the natural killer (NK) cells. The role of NK cells during infection is not as well understood as their role against tumor cells, but it is becoming increasingly evident that NK cells also play a crucial role in protecting the host from a variety of pathogens (Newman and Riley, 2007). Stimulated NK cells are known to release high levels of interferon gamma (IFN-γ), a potent cytokine with a multitude of immunoregulatory functions (Boehm *et al.*, 1997; Ellis and Beaman, 2004). Our collaborators from the Universitätsklinikum in Würzburg found that IFN-γ is released by NK cells in contact with *A. fumigatus* hyphae in a time- and morphotype-dependent manner (Bouzani *et al.*, 2011). IFN-γ released by NK cells, but also in recombinant form, can directly damage *A. fumigatus* hyphae but not conidia, i.e., metabolically active fungal cells. Hyphae incubated with NK cell supernatant (the supernatant collected after NK cells were infected with *A. fumigatus* hyphae) or recombinant IFN-γ, showed decreased metabolic activity using an XTT assay and decreased polar growth using an assay to measure the elongation of individual hypha microscopically (Bouzani *et al.*, 2011). In the presence of IFN-γ, the hyphae grew with a strange morphology: long, thin, and often even in spiral-shaped curls. Using the *A. fumigatus* strain with GFP-tagged mitochondria in live-cell microscopy, we noticed that though the mitochondria remained active, they had lost their usual tubular morphology and appeared rather short and round (unpublished data). The effect was reduced if an anti-IFN-γ antibody was used to deplete the NK cell supernatant. This suggests not only a previously unknown means of attack for NK cells, but also a previously unknown method of harming hyphae and a surprising new role for IFN-γ as more than just an immunoregulatory molecule.

The mechanism of IFN-γ activity against *A. fumigatus* hyphae is still unclear. The finding that recombinant IFN-γ alone damages the fungus is quite astounding, suggesting the ability of this cytokine to directly target *A. fumigatus* hyphae. If another factor is involved,

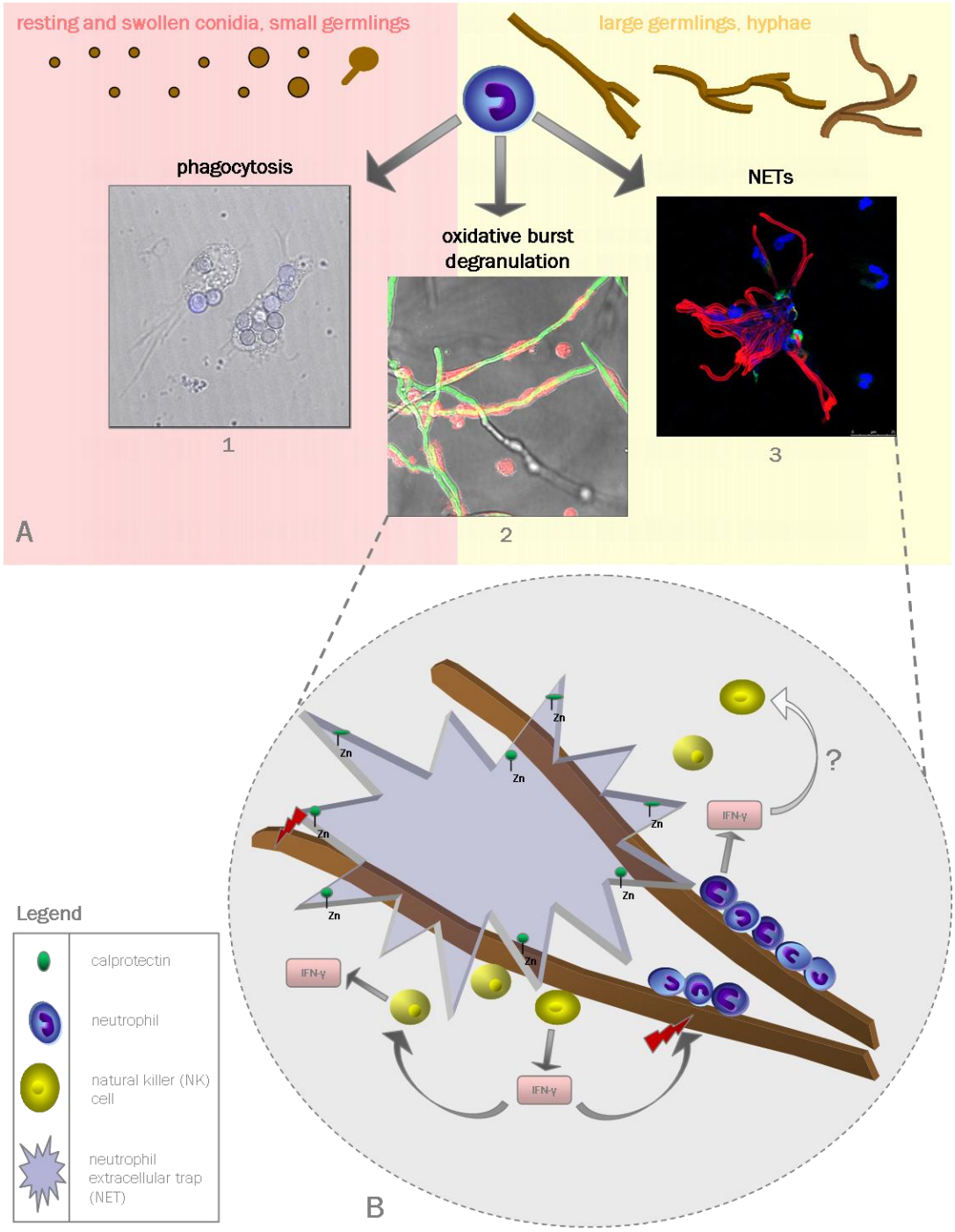

Figure 6. The neutrophil and natural killer cell responses to *Aspergillus fumigatus* infection: formation of NETs and release of IFN-γ

Neutrophils act in three ways to defend the body from *Aspergillus* infection: by phagocytosing resting and swollen conidia and even small germlings (**A1**); by degranulation and oxidative burst (**A2**), both of which release antimicrobial molecules in close proximity to the growing hyphae; or by NETosis (**A3**). Neutrophils and natural killer (NK) cells both release IFN-γ upon stimulation, which may function by directly damaging the hyphae and also by activating incoming NK cells (**B**). NETs are believed to physically contain microbes, preventing dissemination while bringing the microbe in close or direct contact with the antimicrobial NETs-associated proteins. One such protein is calprotectin, which chelates proximal zinc, starving the fungus of this nutrient and thus inhibiting polar growth. **A1**: The bright field image shows two neutrophils that have ingested resting conidia (strain ATCC46645). The conidia are dyed blue with Blankophor, a chitin-binding dye. **A2**: *A. fumigatus* strain AfS35 expressing GFP in the cytoplasm is in green. Neutrophils are dyed red with tetramethylrhodamine and are aligned along the hyphal surface, presumably prior to degranulation, oxidative burst, or even NETosis. Photo taken together with Julia Beck. **A3**: *A. fumigatus* strain ATCC46645 shown in red, using α-galactomannan antibody; NETs DNA stained in blue with DAPI; neutrophil elastase shown in green.

(Hu *et al.*, 2001). Our group published that *A. fumigatus* does in fact secrete significant amounts of ribotoxin, namely mitogillin (Schwienbacher *et al.*, 2005), which could theoretically act in cooperation with IFN-γ, but whether this hypothesis is correct remains to be tested.

7.3 Cooperation between innate immune cells is necessary for A. fumigatus protection

We have not yet found a leukocyte capable of single-handedly killing *A. fumigatus* hyphae. The innate immune system's initial methods of attack, namely phagocytosis, can rid the host of conidia, but extending hyphae present a unique dilemma to responding immune cells. A hypha is too large to be engulfed, is protected by a tough cell wall, and has evolved mechanisms of isolating damaged portions to protect the remaining undamaged organism (Latge, 2007). **Figure 6** summarizes the work presented in this study—the proposed interactions between neutrophils and NK cells in managing *A. fumigatus* hyphae. When phagocytosis is no longer possible, PMNs align themselves along the length of a hypha. From this advantageous position, they can undergo oxidative burst, degranulate, or produce NETs. Bruns *et al*., suggested that only a subpopulation of neutrophils can produce NETs (Bruns *et al.*, 2010), a finding we agree with based on our own unpublished data. Distinguishing and characterizing this subpopulation has not yet been done. But one could speculate that while a percentage of PMNs undergo NETosis, others remain intact, perhaps as recruiters or as phagocytes of slow-germinating conidia. We have even seen, using live-cell microscopy, that a subpopulation of neutrophils responds to both conidia and hyphae. If incubated with resting conidia, for example, some neutrophils actively migrate towards the fungus, engulfing numerous conidia while others remain in place, seemingly unaffected by the presence of the fungus. Similarly, if co-incubated with hyphae, some neutrophils actively migrate towards individual hypha, even wrapping the cell membrane around the girth of the hypha, while others don't show any motion (though alive) or move in a seemingly undirected manner (unpublished data). IFN-γ is both produced by and bound by PMNs. In fact, PMN responses may change over time with the changing cytokine environment, therefore not only enticing other immune cells but also acting on themselves (Ellis and Beaman, 2004). Non-autologous responders to IFN-γ could of course be NK cells. Whether NK cells actively migrate towards PMNs that are actively responding to *A. fumigatus* infection has not yet been

tested, but it is known that early recruitment of NK cells to the lung is critical in the host response to asperillosis (Morrison *et al.*, 2003). We hypothesize here that neutrophils do more than release NETs and ROS to fight *A. fumigatus* hyphae, they must also recruit help from additional leukocytes, and activate these responders. Responding NK cells, in conjunction with activated PMNs, could together attack growing hyphae.

The immune response to *A. fumigatus* is a complex network, involving many subsets of immune cells, each of which carries out a very specific set of functions. Though studying the interaction of each subset with the fungus has been helpful in the past, a more complete picture of the body's clearance mechanisms, especially with regards to this rather complex opportunistic pathogen, is necessary for future advancements, not only in diagnosis and treatment, but for the general understanding of the scientific community. *Aspergillus fumigatus* then serves as a tool from which we can learn the complex workings of the human innate immune response.

7.4 The A. fumigatus *response: signalling via the hybrid histidine kinase TcsC*

During infection, the host immune system must manage severe stress caused by invading *Aspergillus* conidia and hyphae, but the fungus also must cope with a hostile tissue environment. Several signalling cascades have been identified in fungal stress response, including the cyclic AMP (cAMP) signalling pathway, Ca^{2+}/calcineurin signalling pathway, protein kinase C/mitogen-activated protein kinase pathway, and the Hog1 MAPK pathway (Bahn, 2008). In the present study, we investigated the role of the sensor histidine kinase of the HOG signalling pathway in *Aspergillus fumigatus*, TcsC, in stress response and virulence. We generated a *tcsC* knockout mutant after identifying the putative Group III hybrid histidine kinase (AFU2G03560). The predicted structure (using SMART online software) contains six HAMP domains arranged as a poly-HAMP chain (see **Figure 7**), along which the stress signal is presumably transduced as consecutive HAMP domains alternate between two stable conformations (Airola *et al.*, 2010; Dunin-Horkawicz and Lupas, 2010). TcsC lacks a transmembrane domain, but it is becoming increasingly clear that cytosolic sensor kinases are more important in regulating responses in filamentous fungi than transmembrane histidine kinases (Bahn, 2008).

7.4.1 *A. fumigatus* TcsC is part of the HOG pathway

We showed that TcsC is involved in the osmotic stress response, as has been shown for other yeast and fungal mutants of the HOG pathway (Kruppa and Calderone, 2006), the exception is the homologous *A. nidulans* NikA mutant, which is not sensitive to osmotic stress (Vargas-Perez *et al.*, 2007). The ΔtcsC mutant was also resistant to fludioxonil, a phenolpyrrole fungicide thought to activate the HOG pathway by acting against hybrid histidine sensor kinases (Bahn *et al.*, 2007). The addition of either 1.2M sorbitol (causing osmotic stress) or fludioxonil caused a transient translocation of SakA (the *A. fumigatus* Hog1 homolog) to the nucleus in wild type *A. fumigatus* hyphae (unpublished data). Western blot analysis proved that SakA is phosphorylated in its active form in the wild type but not in the mutant and that phosphorylation takes place after treatment with fludioxonil or osmotic stressors. Phosphorylation and presumably translocation of SakA, therefore, requires TcsC signalling, implicating this histidine kinase as the upstream sensor in the HOG pathway of *A. fumigatus*.

We and others are now finding evidence that the arrangement of poly-HAMP domains in hybrid sensor kinases could determine the functionality of these proteins. Meena *et al*. systematically deleted HAMP domains from the *Deboryomyces hansenii* Group III sensor kinase DhNIK1 and found that alternative interaction among consecutive domains regulates activity, like an “on-off switch” (Meena *et al.*, 2010). We found that if this poly-HAMP chain is interrupted, as is the case with the putative TcsC homolog in *A. terreus* (see **Figure 7**), the sensitivity to fludioxonil is abolished, although the species can still adapt to osmotic stress (unpublished data). Whether the missing HAMP domain or the missing REC (receiver) domain in the TcsC homolog in *A. terreus* is responsible for the aberrant fludioxonil response is not yet clear. However, these findings provide additional evidence that fludioxonil acts on TcsC homologs in the HOG pathway. TcsC homologs without the ability to properly sense this fungicide (via a continuous poly-HAMP chain) or pass the signal to a response regulator (from the phosphorylated REC domain) bypass the induced toxicity. Interestingly, *A. fumigatus* TcsC seems functionally more similar to other fungal homologs than to the homologs within the genus, namely in *A. nidulans* or *A. terreus*. In *A. nidulans*, it was shown that the downstream response regulator SrrA can mediate fludioxonil response independently of the HOG pathway (via the second response regulator downstream of TcsC, SskA), i.e., independently of the osmotic stress response (Vargas-Perez *et al.*, 2007). It is reasonable to hypothesize that *A. terreus* uses

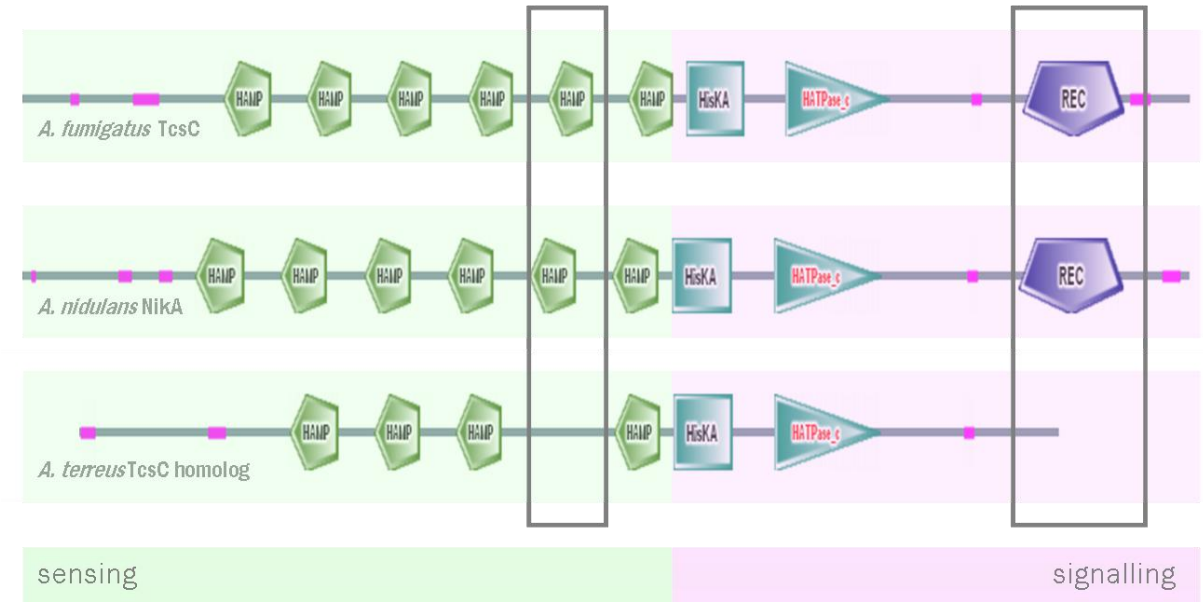

Figure 7. TcsC of *A. fumigatus*, *A. nidulans*, and *A. terreus*: secondary structures as predicted by SMART

A. fumigatus TcsC and *A. nidulans* NikA contain six consecutive HAMP (histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins, phosphatases) domains, a HisKA (histidine kinase) domain, an HATPase_c domain, and a receiver domain (REC). The *A. terreus* putative homolog contains only four HAMP domains and is missing a REC domain. A stress signal is sensed at the N-terminus and passed to the C-terminus via phosphorylation and then through the HOG MAPK pathway. Either of the two missing domains in the *A. terreus* TcsC homolog could be responsible for the species' inherent resistance to fludioxonil.

a completely different mechanism to adapt to hyperosmotic stress, though this has not yet been shown experimentally.

In the presence of fludioxonil (and also the functionally related fungicides iprodione and quitozene), wild type *A. fumigatus* cannot grow on agar plates, even at very low concentrations, while the mutant is completely resistant. When viewed microscopically in liquid media plus fludioxonil (or iprodione, data not shown), wild type conidia and germlings swell significantly, often to the point of bursting. These compounds are thought to act at TcsC homologs, stimulating glycerol synthesis in wild type strains (Ochiai *et al.*, 2002; Zhang *et al.*, 2002). As glycerol accumulates, turgor increases and the cells swell as a consequence (Lew, 2010). In addition, we found that these swollen cells contain an increased number of nuclei compared to untreated cells. Not only are fludioxonil-treated cells suffering from increased turgor, but also a seemingly unchecked cell cycle. Perhaps the response regulators downstream of TcsC, SrrA and SskA, mediate different toxic responses to fludioxonil. We attempted to explore this idea further by creating SrrA and SskA knockout mutants in *A. fumigatus*. However, transformation of *Aspergillus* is performed by stabilizing protoplasts on 1.2M sorbitol, an osmotic condition we found too stressful to HOG pathway mutants. In fact, we found that others had similar troubles

creating a ΔtcsC mutant (personal communication with Nir Osherov at the University of Tel Aviv). Though we clearly showed that TcsC is upstream of SakA in the HOG pathway, knocking out the downstream response regulator genes (*srrA* and *sskA*) will be an important step in truly identifying the role of TcsC, not only in the HOG pathway, but in other cellular response systems as well.

Fludioxonil is currently used to control pathogenic fungi in agriculture, and has been shown to activate the Hog1 homolog in the plant pathogen *Colletotrichum lagenarium* (Kojima *et al.*, 2004). We have shown that fludioxonil induces SakA phosphorylation and translocation to the nucleus in *A. fumigatus* wild type; it therefore seems plausible that fludioxonil or related agents could also be used to treat fungal pathogens of human hosts. Initial experiments in an embryonic egg model proved that fludioxonil-treated eggs were significantly more likely to survive *A. fumigatus* infection than controls, but only with repetitive treatment (unpublished data). This correlates with our finding that upon removal of fludioxonil-containing media, surviving conidia and germlings can resume normal growth. Clearly, more research must be done to establish the use of fludioxonil or related agents in the clinic, but preliminary results are quite promising and provide the framework from which to continue.

7.4.2 Linking TcsC and the HOG pathway to other regulatory modules in *Aspergillus*

The ΔtcsC mutant also exhibited a strong "fluffy" phenotype under hypoxic stress, in the presence of high concentrations of Ca^{2+}, and on farnesol. The mutant and the wild type strains showed the same phenotype to all three stressors, namely a tremendous production of white aerial hyphae and lack of differentiation, i.e., conidiation, but this occurred in the mutant under less stress than the wild type. In *A. nidulans*, such fluffy growth is under the control of a heterotrimeric G protein linked to the cAMP-dependent protein kinase pathway (Shimizu and Keller, 2001; Yu *et al.*, 1996). With this in mind, as well as the finding that farnesol blocks adenylyl cyclase activity in *Candida albicans* (Hall *et al.*, 2011), we attempted to rescue the fluffy phenotype in both *A. fumigatus* wild type and the ΔtcsC mutant. The addition of exogenous cAMP rescued the differentiation defect in the wild type entirely on 2mM farnesol. However, such rescue was not possible in high Ca^{2+} concentrations or hypoxia. A quarter century ago, Tamame *et al*. found a compound, 5-azacytidine, that induced a mutation resulting in a remarkably similar fluffy

phenotype in *A. nidulans* and *A. niger*. They were able to restore differentiation by simply exposing the mutant to light (Tamame *et al.*, 1988; Tamame *et al.*, 1983). Ben-Ami *et al*. have since tried the same with *A. fumigatus* and subsequently suggested the existence of a light-sensing pathway involving G-protein signalling (Ben-Ami *et al.*, 2010). It is known that light is necessary for normal *Aspergillus* development and metabolism (Bahn *et al.*, 2007; Ruger-Herreros *et al.*, 2011). Accordingly, we found that by incubating the ΔtcsC mutant in white light, we could restore conidiation in hypoxia and on 100mM $CaCl_2$. Evidence for cross-talk between the HOG pathway and other signalling pathways is not new (Bahn, 2008). Here we have provided additional evidence that the HOG pathway responds to the well documented signals—osmotic and fludioxonil stress—but also plays a role in regulating certain G-protein signalling pathways (McCormick, *et al*., 2012).

The fluffy phenotype of the ΔtcsC mutant on farnesol perhaps links HOG signalling to yet another *A. fumigatus* signalling cascade. Farnesol is a quorum-sensing molecule known to inhibit cell wall integrity signalling in *A. fumigatus* (Dichtl *et al.*, 2010). Its aggrandized effect on the ΔtcsC mutant suggests that TcsC inhibits the farnesol effector, thus implying cross talk between the HOG and cell wall integrity (CWI) pathways. Our unpublished finding that the CWI mutant, ΔmpkA, shows increased sensitivity to fludioxonil suggests a link between the two signalling pathways. Additionally, we found that the ΔmpkA mutant, when grown on fludioxonil, is far less likely to produce spontaneously fludioxonil-resistant mutants than the wild-type (unpublished data). Other groups have also found evidence of cross-talk between the HOG and CWI pathways (Arana *et al.*, 2005; Hahn and Thiele, 2002), and such a proposition certainly seems plausible. The first line of fungal defense is of course the cell wall, which is also the point of contact between the fungus and exogenous stressors.

HOG two-component signalling in *Cryptococcus neoformans* regulates not only stress response and drug sensitivity, but also sexual development (Bahn *et al.*, 2006). Our unpublished data suggest a similar role for the HOG pathway in *A. fumigatus*. Preliminary crossing experiments give evidence that ΔtcsC and wild type crosses produce fewer cleistothecia than wild type control crosses, and the cleistothecia appear much larger, are more loosely woven, and are always buried under abnormally large piles of hyphae. These giant cleistothecia contain nearly ten times more ascospores than wild type. Both ΔtcsC strains crossed with the wild type produce these large cleistothecia, though their development is delayed, but when the two ΔtcsC strains are crossed, no cleistothecia are visible at all, but enormous amounts of aerial hyphae are present (unpublished data in

collaboration with Edita Szewczyk and Jorge Amich at the Research Center for Infectious Diseases, Julius-Maximilians-University, Würzburg). Our unpublished findings are in agreement with data that *C. neoformans hog1* mutations influence mating. The same group also found that enhanced mating occurs by an increase in the production of mating pheromones, which is repressed under control of the HOG pathway in nonmating conditions (Bahn *et al.*, 2006; Bahn *et al.*, 2005). In *A. nidulans*, SakA activity has been linked to the transcription factor, SteA, which regulates pheromone production (Dyer and O'Gorman, 2012; Kawasaki *et al.*, 2002). Whether the ascospores from the ΔtcsC

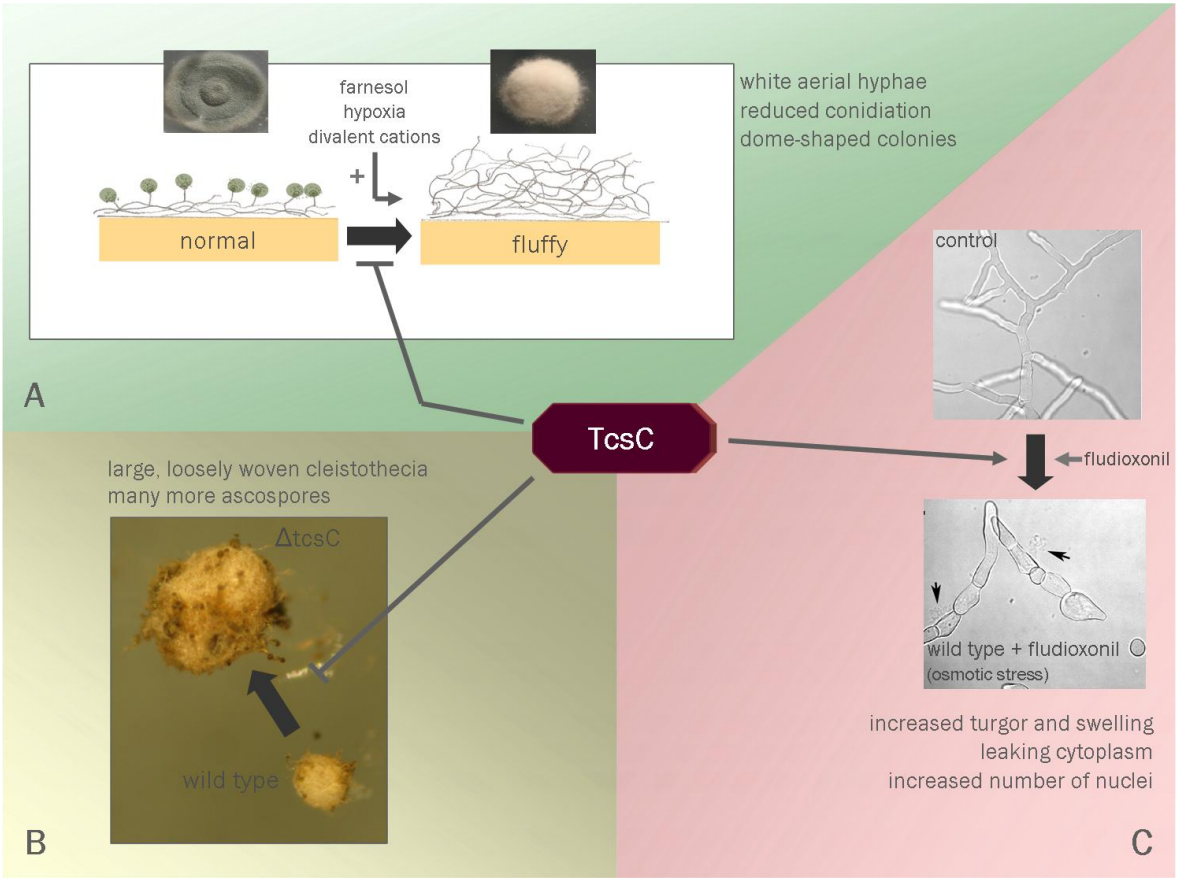

Figure 8. The various roles of TcsC.

Lack of TcsC results in a switch to the "fluffy" developmental program under less stress than wild type (**A**). Hypoxia, farnesol, and high concentrations of divalent cations induce this fluffy phenotype. Fludioxonil activates the HOG pathway via TcsC and causes tremendous swelling, cytoplasmic leakage, and an increased number of nuclei (**C**). Lack of TcsC also influences sexual reproduction in *A. fumigatus*. Heterokaryons produce large, loosely woven cleistothecia that contain ten times more ascospores than wild type crosses (**B**), but ΔtcsC crosses produce no cleistothecia at all.

crosses are viable remains to be tested, as does the possibility of crossing two *A. fumigatus* HOG mutants. Though the precise role of TcsC in *A. fumigatus* sexual development is not yet elucidated, the link between fungal sexual development and the two-component HOG pathway is clearly suggested by our work and that done with other filamentous fungi. The roles of TcsC reported in this study are summarized in **Figure 8**.

7.4.3 TcsC is not required for *A. fumigatus* virulence

Though the HOG pathway has been implicated in virulence in several fungal pathogens (Viaud *et al.*, 2006; Chun *et al.*, 2007; Bahn *et al.*, 2006; Jiang *et al.*, 2011), we found no difference in virulence between the ΔtcsC mutant and the wild type, in both a mouse model and embryonic egg model of infection (data of the latter unpublished). However, this should not exclude TcsC or other components of the HOG pathway as drug targets. The ΔtcsC mutant was indeed robust, showing no sensitivity to oxidative, temperature, or pH stress or to clinically relevant anti-fungal agents. However, the mutant did show, not only involvement in a variety of different signalling cascades, but sensitivity to a set of stress factors relevant in an infection environment, especially hypoxia. As mentioned above, hypoxia in the host is not only a result of inflamed (e.g., infected) tissue, but often a necessary trigger, activating a HIF-1-regulated immune response. *Aspergillus* must be able to maintain growth within this environment to be successful as a pathogen. We found the ΔtcsC mutant to have increased sensitivity to hypoxic stress, suggesting a point of weakness that, perhaps in combination with appropriate drug therapy could open up a new therapeutic option.

7.5 Aims for the future

The treatment of aspergillosis requires a delicate balance between maintaining immune integrity and destroying the invading pathogen. The challenge is that the former relies on the latter. Effective treatment might involve both immune and antifungal therapy. However, such an approach requires a more in-depth understanding of the interaction between the innate immune system in the host and stress-response networks in this opportunistic pathogen. We have attempted such a two-pronged exploration in the current study. The work presented here represents merely a starting point, and further experiments are needed to really unravel the mystery of *Aspergillus* infection. Expanding *in vitro* experiments to include additional innate immune cells, such as macrophages and dendritic cells, and different cytokine milieus may explain the system's mechanism of killing the fungus. Determining the role of response regulators downstream of TcsC in the HOG pathway and indeed the role of TcsC itself in additional signalling cascades may elucidate the means by which the fungus evades immune detection or attack. We have begun by reporting the function of NETs on *A. fumigatus*, the effect of IFN-γ released by NK cells on hyphae, and the role of TcsC in virulence and adaptation to stress.

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9. Abbreviations

CGD	chronic granulomatous disease
CWI	cell wall integrity
ERK	extracellular signal-regulated kinase
HAMP	histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins, phosphatases
HK	hybrid sensor kinase
HOG	high osmolarity glycerol
HPt	histidine-containing phosphotransfer protein
IFN-γ	interferon gamma
IRAK	IL-1 receptor-associated kinase
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated kinase kinase (MAPKK)
MyD88	myeloid differentiation primary response gene (88)
NADPH	nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NK	natural killer
PAMP	pathogen associated molecular pattern
PKC	protein kinase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear neutrophil
PRR	pattern recognition receptor
PTX3	pentraxin 3
REC	response regulator receiver
ROS	reactive oxygen species
RR	response regulator
Syk	spleen tyrosine kinase
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
TRAF6	TNF receptor associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand

10. Permission to use published figures

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I give permission to Allison McCormick to use an adapted portion of Figure 1, published in the manuscript referenced below, in her PhD thesis. The figure has been adapted and will be used as detailed below.

Original publication:

Dyer,P.S., O'Gorman,C.M., 2012. Sexual development and cryptic sexuality in fungi: insights from Aspergillus species. FEMS Microbiol Rev. 36, 165-192.

Adapted version:

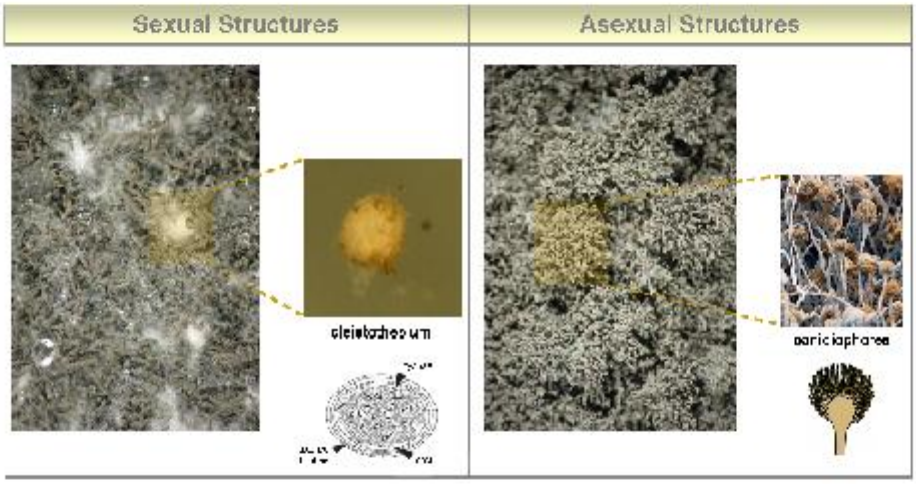

Figure 1. Morphology of the *Aspergilli*: sexual and asexual reproductive structures.

The name *Aspergillus* comes from the Latin *aspergillum*, a mop for distributing holy water, obviously in reference to the appearance of the conidiophore. The conidiophores produce and release large numbers of asexual spores called conidia, which are constantly dispersed into the air. It was very recently discovered that *Aspergillus fumigatus* can also reproduce sexually. Meiosis yields haploid asci from the diploid ascus. The asci develop within a closed structure called the cleistothecium. The lower magnification photos are of *A. fumigatus* strains AfS35 crossed with D141 on oatmeal agar after 4 months growth in the dark at 30°C. The cleistothecium was taken from the plate as indicated and photographed under higher magnification. Aforementioned photos taken in collaboration with Edita Szewczyk at the Research Center for Infectious Diseases, Julius-Maximilians-University Würzburg. Cartoon cleistothecium adapted from (Dyer and O'Gorman, 2012).

Paul Dyer

PAUL S. DYER 10/5/12

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Original publication:

Dennehy,K.M., Brown,G.D., 2007. The role of the beta-glucan receptor Dectin-1 in control of fungal infection. J Leukoc. Biol. 82, 253-258.

Adapted version:

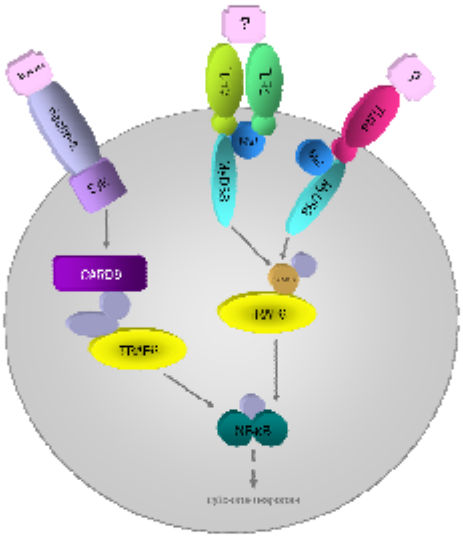

Figure 3. Dectin-1 and TLR signalling pathways.

Both dectin-1 and TLR signalling lead to NF-κB activation and cytokine production. However, dectin-1 uses the Syk kinase and CARD9 adaptor protein to couple to the TRAF6 complex and activate NF- κB, while TLRs use the MyD88 adaptor protein and IRAK (IL-1 receptor-associated kinase). Dectin-1 binds β-glucan, a component of the *Aspergillus* cell wall. The binding partners for the TLR receptors involved in *Aspergillus* response are currently not known [adapted from (Dennehy and Brown, 2007;Netea *et al.*, 2008)].

10 May 2012

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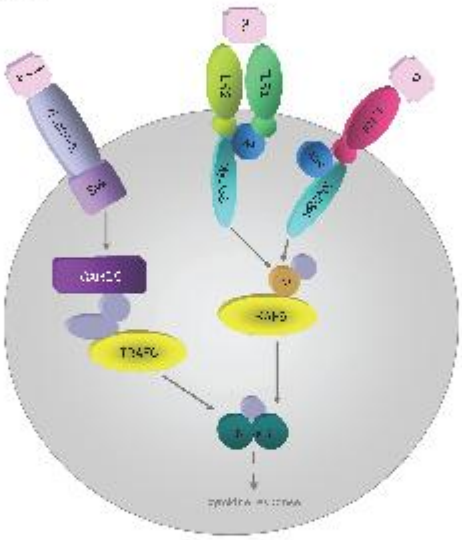
Netea,M.G., van der Meer,J.W., Kullberg,B.J., 2006. Role of the dual interaction of fungal pathogens with pattern recognition receptors in the activation and modulation of host defence. Clin. Microbiol Infect. 12, 404-409.

Adapted version:



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Original publication:

Bahn,Y.S., 2008. Master and commander in fungal pathogens: the two-component system and the HOG signaling pathway. Eukaryot. Cell 7, 2017-2036.

Adapted version:

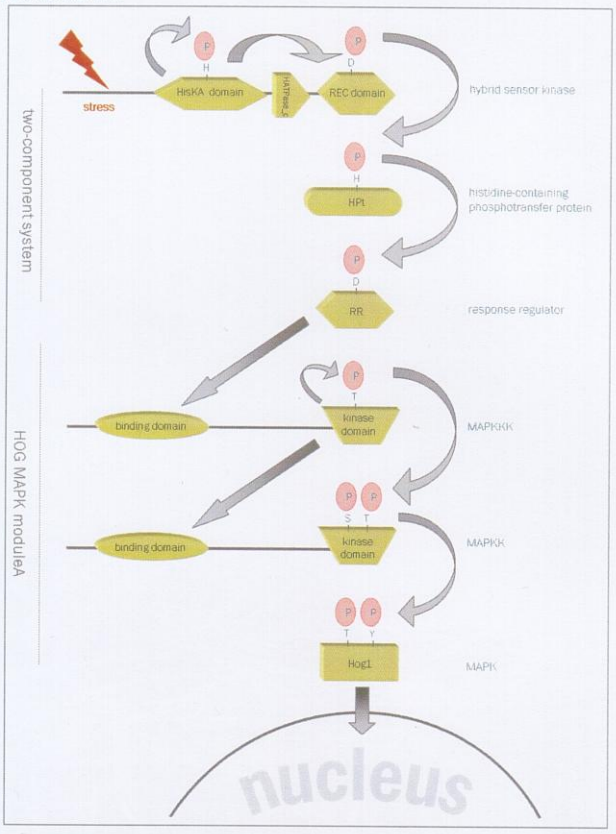

Figure 4. The two-component HOG pathway.

In bacteria and fungi a stress signal is sensed by an HK, which autophosphorylates and then transfers a phosphate through the two-component and HOG MAPK modules to the Hog1 MAPK. Phosphorylated Hog1 translocates to the nucleus where it activates the expression of stress-defensive genes [adapted from (Bahn, 2008)].

(Signature and date)

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12. Curriculum Vitae

12.1 Education

Ph.D. in Medical Microbiology, Ludwig-Maximilians-Universität Munich, 2012
B.S. in Cellular/Molecular Biology, University of San Francisco, 2007
B.A. in Applied Mathematics, University of San Francisco, 2007
High School Diploma – Valedictorian; Douglas High School, Nevada, USA, 2000
Carson Valley Middle School secondary education; Nevada, USA
Jacks Valley Elementary School primary education; Nevada, USA

12.2 Publications

- McCormick, A., Jacobsen, I., Broniszewska, M., Beck, J., Heesemann, J., Ebel, F. The role of the *Aserpgillus fumigatus* two-component sensor kinase TcsC in stress resistance and virulence. *PLoS ONE*. 2012; 7(6).
- Bouzani, M., Ok, M., McCormick, A., Ebel, F. Human Natural Killer cells display important antifungal activity against *Aspergillus fumigatus*, which is directly mediated by IFN-γ release. *J Immunol*. 2011; 3:1369-76.
- McCormick, A., Heesemann L., Wagener, J., Marcos, V., Hartl, D., Loeffler J., *et al*. NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbe Infect*. 2010; 12:928-36.
- McCormick, A., Loeffler J., Frank E. *Aspergillus fumigatus*: Contours of an Opportunistic Human Pathogen. *Cell Microbiol*. 2010; 11:1535-43.

12.3 Presentations

- Kongress für Infektionskrankheiten und Tropenmedizin (KIT)
  Poster presented by Maria Bouzani: Interferon-γ released by human natural killer cells demonstrates direct fungicidal properties against *Aspergillus fumigatus*.
  April 25-28, 2012: Köln, Germany
- Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM) – Eukaryotic Pathogen Division
  Talk given: Characterization of the *Aspergillus fumigatus* two-component sensor kinase TcsC and elucidation of its role in the HOG pathway.
  February 17-18, 2012. Berlin, Germany
- Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM)
  Poster Presented: Characterization of the two-component histidine kinase TcsC of the pathogenic mold *Aspergillus fumigatus*.
  September 25-28, 2011. Essen, Germany
- Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM) – Eukaryotic Pathogen Division
  Talk: The role of neutrophil extracellular traps (NETs) in antifungal immunity
  March 25-26, 2011; Düsseldorf, Germany
- Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM)
  Poster presented: Studies of the *Aspergillus fumigatus* ribotoxin mitogillin produced in *Pichia pastoris*
  September 20-23, 2009; Göttingen, Germany

12.4 Experience

Max-von-Pettenkofer Institute, Ph.D. candidate	since 3/2009
California Pacific Medical Center, Clinical Research Coordinator (Neurology)	4/2008 – 9/2008
California Pacific Medical Center, Research Associate	8/2007 – 4/2008
Genentech, Laboratory Technician (Research and Development)	2/2006 – 5/2007
Bayer, Media Preparation Operator	9/2002 – 8/2005
Humboldt State University, Laboratory Assistant	8/2001 – 5/2002

12.4.1 Professional Summary

- Over ten years laboratory experience in the clinic, in industry, and in academia
- Superior interpersonal and communication skills – ability to effectively communicate, written and orally, with a socially, economically, and culturally diverse group of people under pressure
- Published in peer-reviewed journals and presented to international audiences
- Highly self-motivated, creative problem solver, strong work ethic

12.4.2 Qualifications

In the Clinic

- Acted as liaison between drug company and doctor in clinical trials
- Familiar with FDA regulations and capable of maintaining compliant laboratory practices and clinical records
- Established strong working relationships with doctors, nurses, researches, regulatory agencies, drug companies, and patients

In Industry

- Familiar with biotech production and research laboratory practices, protocols, and regulations
- Written and edited numerous SOPs and BPRs used in production facilities
- Acted as trainer for incoming employees and substitute shift manager (biotech production)
- Lead several laboratory reorganization projects

In Academia

- Studied PRRs on human macrophages and PAMPs on *A. fumigatus* to determine the participation of TLR2, TLR4, and dectin-1 in fungal recognition and response
- Created *A. fumigatus* knockout mutants to explore the proteins involved in fungal stress response, virulence, and cell wall integrity
- Characterized the release of NETs by primary human and murine neutrophils with *A. fumigatus* in response to *A. fumigatus* and *Candida albicans*
- Studied HIF-1α activation in cells of the innate immune system
- Used a panel of genetic markers to screen patient tissue samples for early markers of colon cancer and other diseases of the digestive tract