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The infectious propagules of *Aspergillus fumigatus* are coated with antimicrobial peptides

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Abstract

Fungal spores are unique cells that mediate dispersal and survival in the environment. For pathogenic fungi encountering a susceptible host, these specialised structures may serve as infectious particles. The main causative agent of the opportunistic disease aspergillosis, *Aspergillus fumigatus*, produces asexual spores, the conidia, that become dissipated by air flows or water currents but also serve as propagules to infect a susceptible host. We demonstrate that the *defX* gene of this mould encodes putative antimicrobial peptides resembling cysteine-stabilised (CS) $\alpha\beta$ defensins that are expressed in a specific spatial and temporal manner in the course of asexual spore formation. Localisation studies on strains expressing a fluorescent proxy or tagged *defX* alleles expose that these antimicrobial peptides are secreted to coat the conidial surface. Deletion mutants reveal that the spore-associated *defX* gene products delay the growth of Gram-positive *Staphylococcus aureus* and demonstrate that the *defX* gene and presumably its encoded spore-associated defensins confer a growth advantage to the fungal opponent over bacterial competitors. These findings have implications with respect to the ecological niche of *A. fumigatus* that serves as a ‘virulence school’ for this human pathogenic mould; further relevance is given for the infectious process resulting in aspergillosis, considering competition with the host microbiome or co-infecting microorganisms to break colonisation resistance at host surfaces.

KEYWORDS

aspergillosis, defensin-like peptide, fungal virulence

1 | INTRODUCTION

Fungal virulence is a complex and multi-factorial trait. While most bacterial pathogens express distinct, host-damaging virulence factors, this attribute is less defined for fungal pathogens. Given that most infections of fungal origin are opportunistic in nature (Brown

et al., 2012), it is now commonly agreed on that, to some extent, fungal virulence results from the combination of various determinants that act on growth and invasion and that have evolved at the primary ecological niche to serve as dual-use factors, supporting ready-made pathogenicity (Casadevall, Steenbergen, & Nosanchuk, 2003; Tekaia & Latgé, 2005). Moreover, toxic compounds and antimicrobial activities

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may be produced by a fungus in the wild to gain advantage over competitors, which may also be of relevance in the context of infection. For the opportunistic human pathogenic mould *Aspergillus fumigatus*, a variety of cellular characteristics have been identified to determine its virulence potential (Krappmann, 2016; Latgé & Chamilos, 2019), among them metabolic versatility, stress resistance, or the production of gliotoxin, a mycotoxin resulting from its secondary metabolism with immunomodulatory but also amoebicidal properties (Scharf, Brakhage, & Mukherjee, 2016). The infectious disease caused by *A. fumigatus*, aspergillosis (Segal, 2009) is commonly initiated by the inhalation of its omnipresent asexual spores. In the absence of an appropriate immune response, these infectious propagules will germinate to invade the surrounding tissue at the site of infection; from there, haematogenous dissemination may occur to eventually result in systemic disease. Besides the aforementioned invasive form of aspergillosis, several others exist that may result in chronic or hyper-inflammatory lung diseases (Kosmidis & Denning, 2015) or even cutaneous affection (Darr-Foit, Schliemann, Scholl, Hipler, & Elsner, 2017), which makes this fungal infection remarkable in terms of progression. Considering the existence of a pulmonary microbiome or the scenario of co-infection during aspergillosis, the capability of *A. fumigatus* to produce antimicrobial agents needs to be taken into account when defining its human virulome (Casadevall, 2006).

In search for such activities other than mycotoxins that are produced by *A. fumigatus* and that may act as virulence determinants in competition with the host microbiota, we focused on a recently identified class of fungal effector molecules: defensin-like peptides (DLPs). These short and generally amphiphilic peptides resemble host antimicrobial peptides (AMP) of animal and plant origin and belong to the family of defensins. They are characterized by at least three conserved intramolecular disulfide bonds and further structural elements. The superfamily of defensins that were originally identified in invertebrates and plants contain a cysteine-stabilised alpha beta (CS $\alpha\beta$) motif, where an alpha-helix is followed by two antiparallel beta sheets, connected by loop regions, and stabilised by at least three disulfide bonds. These CS $\alpha\beta$ defensins can further be distinguished into three major types (Wu, Gao, & Zhu, 2014). For the fungal kingdom, the existence of CS $\alpha\beta$ DLPs was largely unknown until plectasin of the saprophyte *Pseudoplectania nigrella* became the first identified fungal peptide to structurally resemble these microbicidal agents (Mygind et al., 2005; Schneider et al., 2010). By subsequent sophisticated alignment analyses, a variety of DLPs encoded in the genome of fungal hosts were identified in silico (Wu et al., 2014; Wu, Gao, & Zhu, 2017; Zhu, 2008), most of them awaiting functional characterisation. Besides plectasin and the *Eurotium amstelodami*-derived eurocin (Oemig et al., 2012), the CS $\alpha\beta$ DLPs, micasin (Zhu, Gao, Harvey, & Craik, 2012) and copsisin (Essig et al., 2014) from a dermatophyte, *Microsporum canis*, and the basidiomycete *Coprinopsis cinerea*, respectively, have been characterized with respect to their mode of bactericidal action to reveal that these structurally conserved molecules bind to the lipid II intermediate of bacterial peptidoglycan synthesis, thereby interfering with cell wall formation. Moreover recently, the induced expression

of the CS $\alpha\beta$ DLPs from *C. cinerea* in the presence of bacteria was demonstrated to illustrate an intimate fungal–bacterial interplay (Kombrink et al., 2018). For the *Aspergillus* genus, preliminary data had been collected for the DLP of *A. nidulans*, anisin1, that imply a functional role in fitness of this mould (Eigentler, Pocsí, & Marx, 2012).

To scrutinise the function of DLPs expressed by the opportunistic fungal pathogen *A. fumigatus* and to further address the lack of knowledge about any distinct spatio-temporal expression pattern executed for fungal DLPs, we took efforts to generate mutant strains deleted for the encoding gene combined with detailed expression analyses. Our finding that CS $\alpha\beta$ defensins coat the asexual spores of *A. fumigatus* adds a novel and unrecognised component to the infectious particle of aspergillosis, where it has the capacity to support virulence by breaking colony resistance of the host microbiome or modulating the immune response.

2 | RESULTS

2.1 | The *defX* gene of *A. fumigatus* has the capacity to encode two CS $\alpha\beta$ -DLPs

With the aim to complement existing gaps in the preliminary knowledge about fungal DLPs, we took efforts to investigate the two putative DLPs of the opportunistic pathogen *A. fumigatus* as they had been annotated in the seminal study by Zhu (2008). For one of these, an antimicrobial activity had recently been demonstrated testing the recombinantly expressed peptide (Contreras, Braun, Schäfer, & Wink, 2018). In the light of this finding, we became interested to characterize the encoding gene and to assess any biological function of its products in the relevant context of microbial interaction and competition.

The two putative DLPs of *A. fumigatus* are encoded by the annotated gene locus AfuA_7G05180 that we named *defX*. The *defX* transcript comprises 888 nucleotides (nt) with – as identified by 5'RACE – an untranslated 5' region (5'UTR) of 319 nt, followed by a coding sequence of 441 nt that results in a deduced gene product of 146 amino acids (aa), and a 3'UTR of 128 nt (Figure 1a). Comparison of cDNA-derived sequences with the genomic locus revealed the presence of an intron of 57 nt that precedes the open reading frame 8 nt upstream of the translational start codon. From the deduced protein sequence, a signal peptide of 19 aa for secretory processing can be identified by sequence similarity to established consensus sequences (Gierasch, 1989), as well as a 40 aa pro-peptide that separates two DLPs, AfusinN (49 aa) and AfusinC (38 aa), with 27% identity. Upon secretion and post-translational processing, the N-terminal signal peptide is cleaved from the precursor peptide and the separating pro-peptide fragment is removed to release the two mature defensins. Distinct motifs can be deduced from the primary sequence of these to predict structurally conserved elements (Figure 1b), such as the consensus cysteine framework as well as α -helical and β -sheet elements connected by loop regions, therefore classifying the *defX*

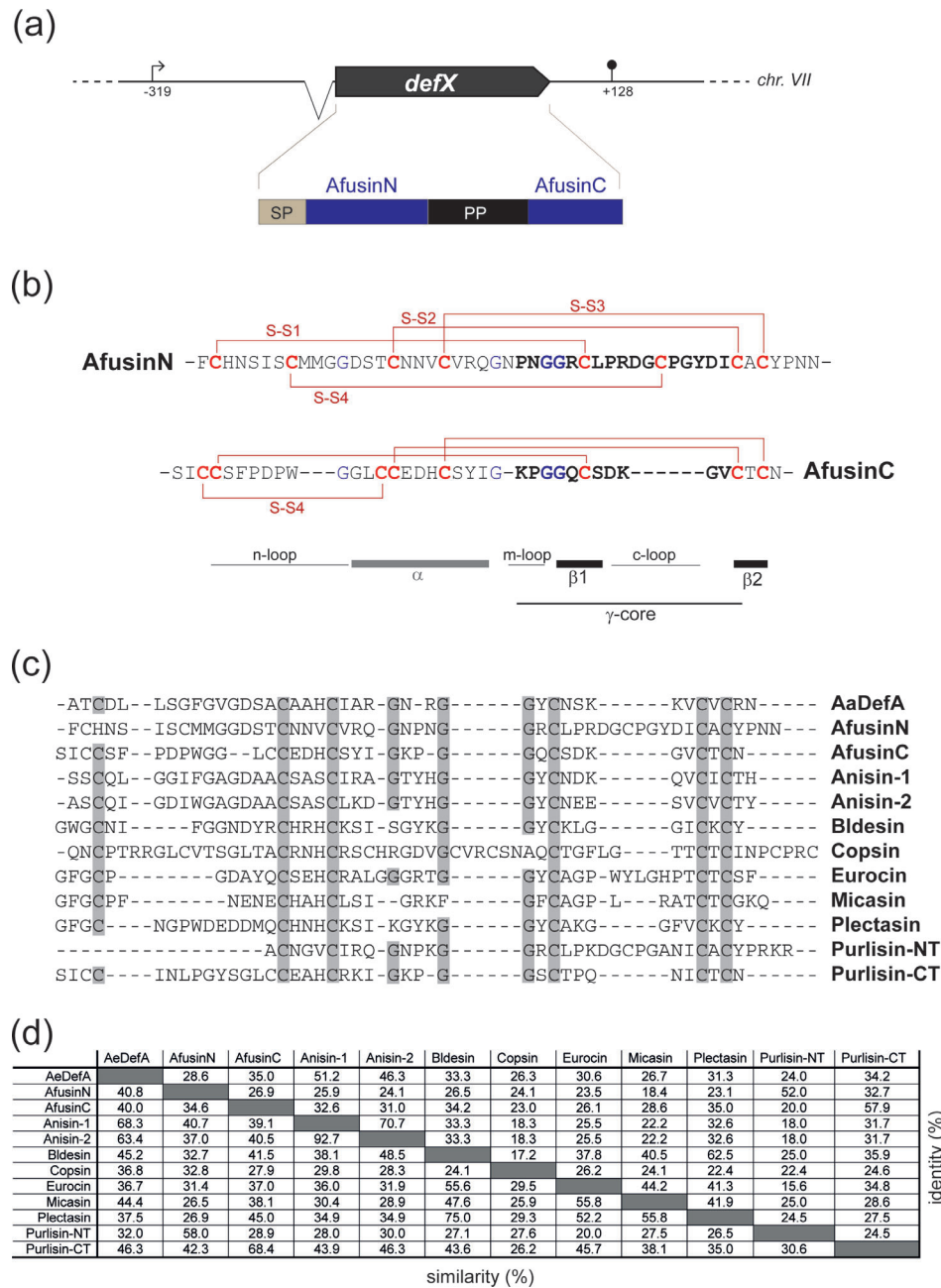


FIGURE 1 The *defX* gene of *A. fumigatus* has the capacity to encode two CS α β defensin-like peptides. (a) Schematic overview on the annotated *defX* gene locus on chromosome VII of the *A. fumigatus* genome (AfuA_7G05180), with positions of transcriptional initiation and termination indicated relative to the start and stop codon, respectively, of the coding sequence. The deduced gene product comprises a signal peptide (SP) and two defensin-like peptides, AfusinN and AfusinC that are processed from the primary translation product after removal of a pro-peptide (PP) spacer region. (b) Sequence and connectivity between conserved cysteines of the deduced CS α β -defensin-like peptides. Cysteine residues forming disulfide bridges are highlighted in red, conserved glycine residues in blue, and structural elements are illustrated below with corresponding residues of the γ -core segment in bold. (c) Global alignment of selected fungal defensin-like peptides and the originating insect defensin AaDefA of the mosquito *Aedes aegypti*, with conserved (>50%) residues – the disulfide bridging cysteines and two glycine residues – highlighted in grey. Aligned peptides with sequence identifiers are AaDefA of *A. aegypti* (ASY93145), AfusinN and AfusinC of *A. fumigatus* (XP_748991), Anisin-1 and Anisin-2 of *A. nidulans* (XP_662650 and CBF69423), Bldesin of *Blastomyces dermatitidis* (EQL35841), Copsin of *Coprinopsis cinerea* (AIU55999), Eurocin of *Eurotium amstelodami* (K7N5L0), Micasin of *Microsporium canis* (AEM44801), Plectasin of *Pseudoplectanania nigrella* (Q53106), and Purlisin-NT and Purlisin-CT of *Purpureocillium lilacinum* (PWI75532). (d) Tabular overview on similarities and identities between above selected fungal DLPs as deduced from pairwise global alignments (Needleman-Wunsch algorithm)

gene products AfusinN and AfusinC as fungal DLPs belonging to family IV and III (Zhu, 2008), respectively.

Aligning the amino acid sequences of AfusinN and AfusinC with selected, characterized fungal DLPs and the originating insect defensin from *Aedes aegypti* reveals, with the exception of disulfide-bridging cysteines, the absence of conservation on the level of primary structure while underscoring structural similarities to some degree (Figure 1c). Direct comparison of the DLPs, however, illustrates distinct similarities emerging from evolutionary relationships (Figure 1d).

Yet to determine functional properties related to the DLPs in a genuine context, a mutant *A. fumigatus* strain deleted for the entire *defX* gene was generated by gene replacement and subsequent marker excision, followed by reconstitution of the *defXΔ* genetic lesion for control purposes (Figure S1a). Genotypes of the resulting set of strains with respect to the *defX* locus were confirmed by diagnostic PCRs (not shown) and Southern blot hybridizations (Figure S2). Extensive analyses regarding growth or stress resistance did not reveal any prominent phenotypes that would result from the loss of the *defX* gene products (Figure S3).

2.2 | Expression of the *defX* gene products follows a distinct spatio-temporal pattern

We further investigated expression of the *A. fumigatus* DLPs by monitoring *defX* transcript steady-state levels under defined culturing conditions (Figure 2a). While almost undetectable transcript levels were produced during vegetative hyphal growth, an increase in *defX* transcription became evident in the presence of specific stressors, such as oxidative stress imposed by hydrogenperoxide or hyperosmolarity by high salt concentrations; also, slightly elevated transcript levels were detected upon starvation for sources of carbon (not shown). We

furthermore followed *defX* transcription in the course of conidiation as it is triggered by a transfer of vegetative mycelia to a solid culture surface (Adams, Wieser, & Yu, 1998). Significantly elevated levels of *defX* transcripts were detected as soon as 12 hr after the induction of conidiophore formation and became even more abundant during prolonged asexual spore formation. Increased *defX* transcript levels under conditions of osmotic and oxidative stress and in the course of asexual sporulation were confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) (Figure 2b). Given the specific temporal expression pattern under the latter experimental condition, we also became interested in spatial distribution of the *defX* gene products. The coding sequence of an adapted green fluorescent protein (GFP) (Fernandez-Abalos, Fox, Pitt, Wells, & Doonan, 1998) was fused to the *defX* promoter region directly or in frame with the signal peptide-encoding sequence and according gene alleles were used to replace the genuine DLP-encoding sequence in the *A. fumigatus* genome. Inspection of the resulting reporter strains revealed a distinct temporal and spatial expression pattern of the *PdefX*-driven GFP (Figure 3a). While fluorescence was virtually absent in germlings and in vegetative hypha grown in submerged culture, a clear signal could be detected in reproductive structures that strongly correlated with the stage of conidiosporogenesis. In the absence of a secretory signal peptide, *PdefX*-driven GFP expression was initiated in the conidiophore vesicles and became prominent inside the phialides budding from them, while conidia were apparently not associated with the fluorescent reporter protein. When the signal peptide was present in the reporter construct, a strict correlation of GFP expression with distinct stages of conidiophore development and eventual spore formation became evident. Fluorescence could be detected early in hyphal compartments that became exposed to an aerial interface, which serves as environmental trigger for the formation of conidiophores (Law & Timberlake, 1980). In the course of conidiation, GFP expression became prominent in stalks and vesicles but then shifted to the tier of

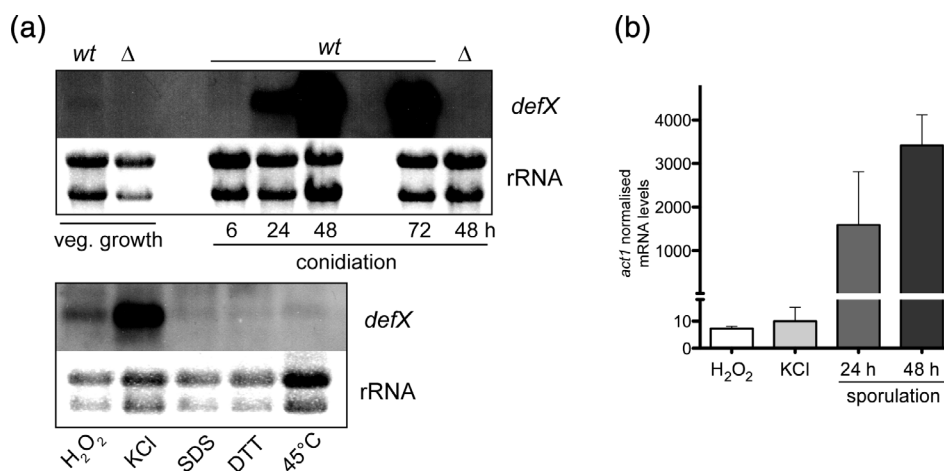


FIGURE 2 Transcription of *defX* coincides with asexual spore formation of *A. fumigatus* and is increased under stress conditions. (a) Northern blot hybridizations with a *defX*-specific probe on RNA samples derived from varying culture conditions reveal significantly increased steady-state transcript levels during asexual spore formation and under oxidative or osmotic stress. (b) Quantitative real-time PCR analyses confirm the significant upregulation of *defX* transcription in the course of conidiation and a subtle increase under conditions of osmotic or oxidative stress

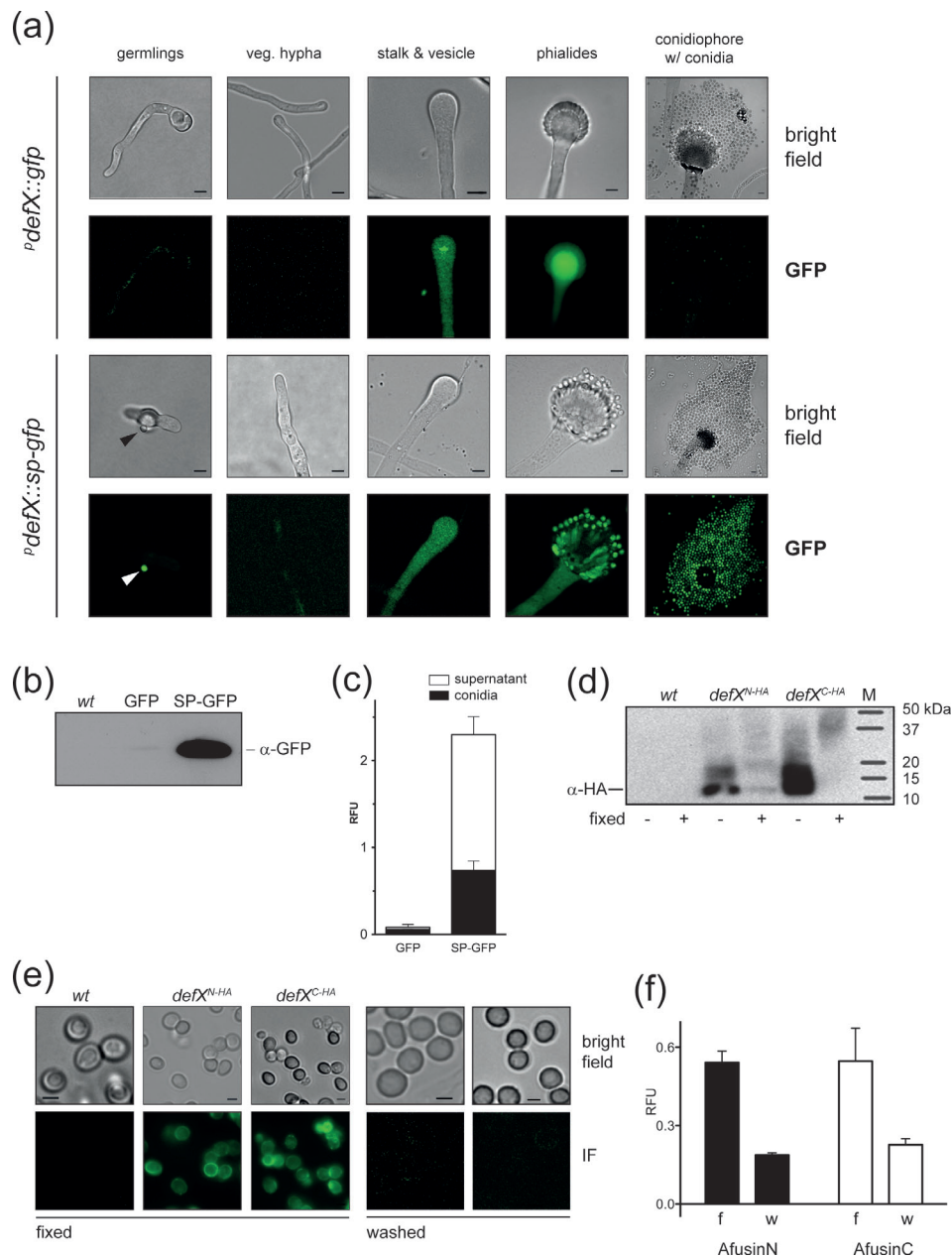


FIGURE 3 The *defX* gene products are expressed in a specific temporal and spatial fashion to coat the asexual spores of *A. fumigatus*. (a) Fluorescence emitted from GFP reporter strains in which the *defX*-coding sequence had been replaced completely or only for the Afusin-encoding part by the codon-optimised *gfp2-5* allele. Expression of the GFP proxy coincides with asexual sporulation and is restricted to the reproductive structures, with apparent translation of the GFP to mature conidia only in when preceded by the *defX*-derived signal peptide sequence. Arrowheads indicate a fluorescent conidium besides a germinated spore, scale bars equal 5 μ m. Release of the P_{defX} -driven SP-GFP proxy from spores as demonstrated by Western blot analysis of conidial suspensions prepared from 10^6 conidia of the GFP reporter strains (b) and fluorescence levels emitted from cleared supernatants and residual conidia derived from $1 \cdot 10^7$ spores (c). (d) Analysis of strains expressing HA-tagged Afusin alleles by probing conidial supernatants with a monoclonal antibody against the HA epitope demonstrates that the HA-fused Afusins can be washed off the spores to a significant extent that is reduced when fixing the conidial proteins prior to harvesting. Immunofluorescence of spores derived from strains expressing the HA-tagged Afusins supports their localisation on the conidial surface. Spores were probed with an HA-cross-reacting antibody to reveal significantly diminished signal intensities after washing as evident upon microscopic inspection (e, scale bars equals 2 μ m), or quantification from washed (w) and fixed (f) conidia harvested from both strains (f)

reproductive phialide cells. As soon as the first conidia budded from the sterigmata, there was significant labelling of the asexual spores by the fluorescent proxy while conidiophore compartments appeared less marked (Figure 3a).

To monitor whether this terminal localisation pattern was consistent with extracellular secretion of the *defX* gene products onto the conidial surface, spores were washed directly from the mycelia of the GFP reporter strains and the cleared supernatants were probed for

their GFP content by Western blot analysis (Figure 3b). While GFP expressed from the $P_{defX}::gfp$ strain was not detected in the supernatants, a clear cross-reaction of the GFP-directed antibody could be verified for the supernatant generated from conidia-forming mycelia of the $P_{defX}::sp-gfp$ reporter strain. Quantification of the fluorescence emitted from the harvested conidia and their supernatants revealed that about one third of the *defX* promoter-driven GFP expression is retained in the conidia (Figure 3c). In the absence of a signal sequence for secretion, significantly less fluorescence becomes associated with the fungal spores and negligible amounts are released in suspension.

In order to rule out artefacts generated by the use of GFP as proxy, recombinant strains in which each DLP had been fused to a human influenza haemagglutinin (HA) tag sequence were generated, replacing the original *defX* coding region by these alleles after transformation of the respective deletion strain. Western immunoblots on adjusted supernatants of spores harvested from the respective set of strains clearly support the idea of *defX* gene product secretion onto the conidial surface, based on successful detection of either Afusin-HA peptide (Figure 3d). Fixing the conidial proteins by formaldehyde treatment before harvesting and washing resulted in reduced amounts of detectable AfusinN-HA or AfusinC-HA. To further investigate the localisation of the *defX*-derived peptides, detection of their HA-tagged fusions on formaldehyde-fixed or washed conidia was carried out by immunofluorescence (Figure 3e). A uniform binding of the HA-cross-reacting antibody to the surface of conidia harvested from the Afusin-HA-tagged strains was evident after fixation but virtually absent when spores had been washed. Quantification of immunofluorescence emitted from spores of the HA-tagged strains further support the idea of DLPs' release from the conidial surface, with lower levels detected after washing the spores prior to the labelling protocol (Figure 3f). All these studies imply a distinct release of the *defX*-encoded peptides, being secreted onto the conidial surface from where they are being easily washed off.

2.3 | The *defX* gene products provide the conidia of *A. fumigatus* with antimicrobial activity to confer a competitive advantage

Aiming to validate a biological function of the spore-associated *defX* gene products, we determined any antimicrobial activity linked to spores of *A. fumigatus* by direct confrontation of bacterial cell suspensions with supernatants that had been generated from conidia harvested from the *defXΔ* deletion strain, its wild-type progenitor, or a reconstituted isolate (Figure 4). A significant delay in growth became evident for Gram-positive *Staphylococcus aureus*, but not Gram-negative *Escherichia coli*, when solutions rinsed off from a defined number of wild-type spores were added to bacterial cultures, and this antibacterial effect was not seen for *defXΔ*-derived extracts (Figure 4a,b). The reconstituted strain had regained the capacity to delay proliferation of the tested *S. aureus* isolate, thereby confirming the *defX*-linked phenotype of bacterial growth inhibition by *A. fumigatus* conidia. To quantify the *defX*-dependent antimicrobial

activity that is linked to *A. fumigatus* conidia, differences between areas under the growth curves from independent culturing experiments were calculated (Figure 4c). With respect to *S. aureus* proliferation, supernatants of conidial suspensions from the deletion strain had a substantially less inhibitory effect in comparison to wild-type-derived ones or ones generated from the reconstituted isolate, while this effect could not be monitored to that extent for cultures of an *E. coli* isolate.

To evaluate the spore-associated antimicrobial activity conferred by the Afusins in a competitive setting, conidia of *A. fumigatus* wild type, the *defXΔ* deletion mutant, and its reconstituted isolate were placed on solid culture medium together with cells of *S. aureus*. Spore germination and hyphal extension was followed and quantified by time lapse microscopy to reveal a significant reduction in fungal growth when the bacterial competitor was present (Figure 4d). This reduction in hyphal elongation, however, was more pronounced when the conidia lacked the *defX*-encoded AMP, indicating that the Afusins confer an advantage to *A. fumigatus* when challenged with a bacterial competitor.

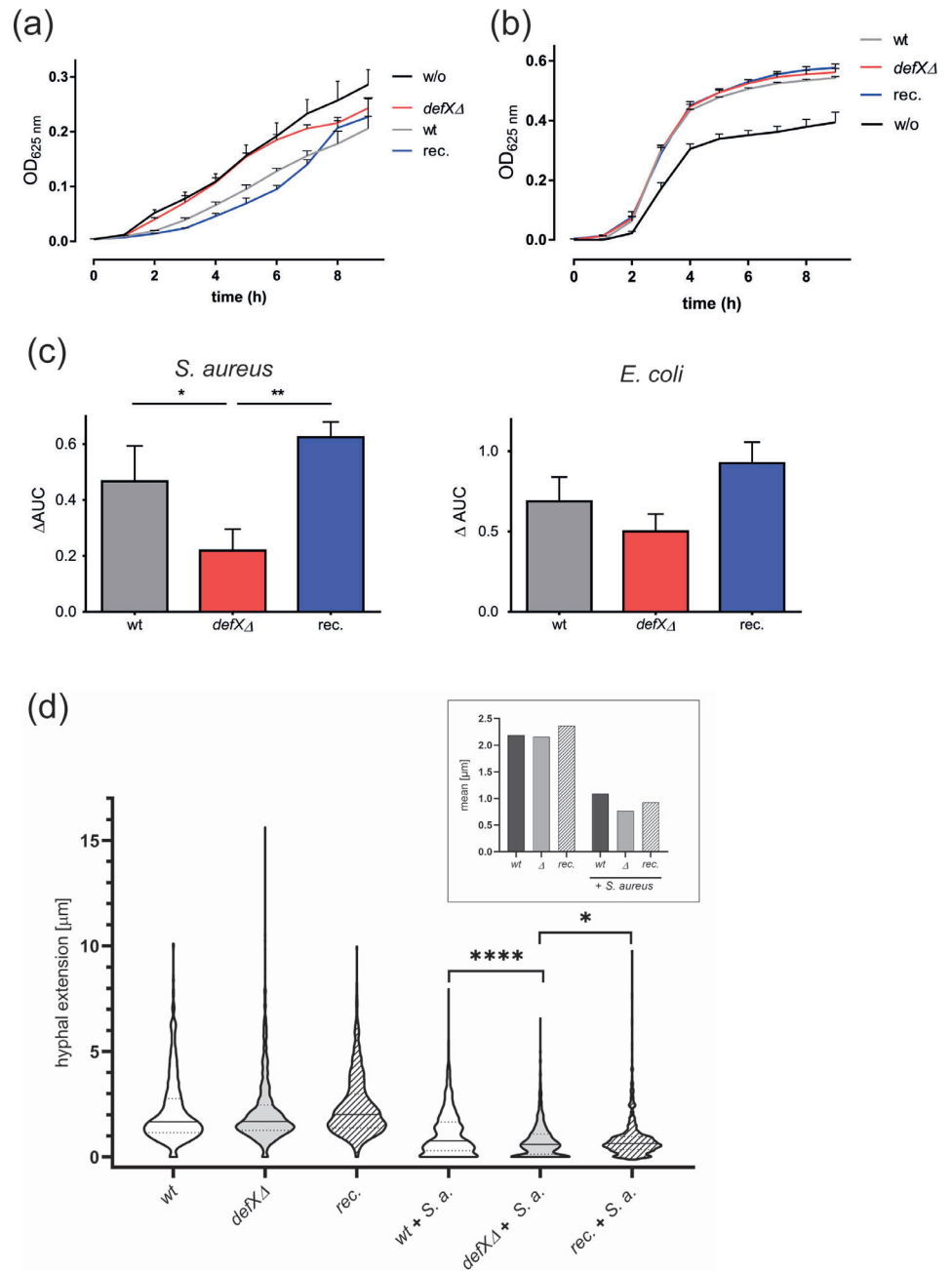
In conclusion, the asexual spores of *A. fumigatus* appear to be coated by AMPs encoded by the *defX* gene that become secreted onto the conidial surface in the course of sporulation to confer a growth advantage in a competitive setting.

3 | DISCUSSION

The fungal spore is a unique cellular entity that enables these sessile microorganisms to efficiently disperse to distant territories and to colonise these in the quest for nutrients. Besides this ecological facet, spores of fungal pathogens may serve as infectious propagules to encounter the host environment in the first instance. In the context of aspergillosis, the conidia of the major causative agent *A. fumigatus* have been extensively studied for specific components that may interfere with host-driven means of defence, such as the protective dihydroxynaphthalene-melanin layer (Langfelder, Streibel, Jahn, Haase, & Brakhage, 2003; Thywissen et al., 2011) or coating hydrophobins that serve as immunological stealth proteins (Aimanianda et al., 2009; Aimanianda & Latgé, 2010; Valsecchi et al., 2017). Recently, the amphiphilic conidial cell wall protein A (CcpA) could be identified and characterized as further structural component that shields the spores of *A. fumigatus* from host recognition and eradication by impacting their immunogenicity (Volders et al., 2018). Our findings add a novel component to the infectious particles: DLPs conferring antimicrobial activity that are secreted in a precise temporal and spatial manner to eventually cover the conidial surface. The observation that the corresponding deletion strain has no prominent phenotype, which is in contrast to its *A. nidulans* counterpart (Eigentler et al., 2012), indicates that the *defX* gene products do not have a structural function in spore formation or resistance of *A. fumigatus*. Together with low-weight antimicrobial compounds that commonly emerge from fungal secondary metabolism (Bignell, Cairns, Throckmorton, Nierman, & Keller, 2016), the DLPs of *A. fumigatus*

FIGURE 4 Afusins interfere with bacterial growth and confer a fungal advantage under competitive conditions. Growth curves from representative cultures of *Staphylococcus aureus* (a) and *Escherichia coli* (b) spiked with adjusted aliquots of cleared conidial suspensions from an *A. fumigatus* wild-type (wt) strain, its *defX* Δ deletion derivative, and the reconstituted (rec.) isolate. Growth was monitored over a time frame of 9 hr to reveal a reproducible, *defX*-dependent interference with growth of the Gram-positive bacterium *S. aureus* but not the Gram-negative one *E. coli*. (c) Absolute differences between areas under the growth curves (Δ AUC) from independent cultures ($n = 3$) and using data resulting from unspiked suspensions as reference substantiate the antimicrobial effect by the *defX* gene products against *S. aureus*.

(d) Distribution of hyphal extension measured within a 80 min time frame for *A. fumigatus* germlings grown on solid culture medium in the absence or presence of *S. aureus* cells, with mean values presented in the inset



form a highly efficient chemical armoury to fight off environmental competitors at the primary ecological niche.

Defensins of fungal origin were first isolated from saprophytic as well as dermatophytic isolates (Mygind et al., 2005; Zhu et al., 2012), which led to the identification of putative others in several species based on structural conservation (Wu et al., 2014; Zhu, 2008) to be followed by functional characterisation of only a few (Contreras et al., 2018; Eigentler et al., 2012; Essig et al., 2014). The postulated structure and connectivity of cysteine bridges of the deduced AfusinN and AfusinC peptide sequences fits to the CS α β fold that is common in fungal DLPs and places them in family 4 and 3, respectively, according to a recently proposed classification scheme (Tarr, 2016). The Afusins' expression pattern and their highly coordinated secretion

during asexual sporulation onto the conidia of *A. fumigatus* appears to be an unprecedented observation, as for no other defensin of fungal origin such as specific localisation has been monitored. Given that DLP expression is triggered by environmental clues correlating to the onset of conidiophore formation and sporulation, a link of *defX* regulation to the *bristle-abacus-wet* genetic cascade that hardwires the conidiation pathway in *Aspergillus* (Timberlake, 1990) seems reasonable and awaits further investigation. The observed changes in *defX* gene product localisation in the course of spore formation appear to be a coordinated cellular process, shifting from intracellular expression in the stalk and vesicle to extracellular secretion that emerges from phialide cells from which coated conidia are budding; its underlying mechanisms of translocation and processing, however, remain elusive

and may serve as a promising area of research to elucidate cell-specific secretion in a multicellular microorganism.

Once coating the fungal spore, DLPs are to be released to the nearby vicinity, as monitored for the GFP proxy expressed from the *defX* promoter and the HA-tagged versions of Afusin N and AfusinC, where they expose their antimicrobial capacities to interfere with the environmental microbiota. The assumption that the *defX*-encoded peptides indeed bear antimicrobial activity is supported by a recent *in vitro* study with recombinant AfusinC (Contreras et al., 2018) that is in accordance with our observation when adding conidial extracts to bacterial cultures. As demonstrated for such peptides, interference with cell wall biosynthesis of bacterial competitors might be the predominant mode of bactericidal action against the Gram-positive bacteria. A clear advantage might emerge from equipping reproductive and disseminating cells with toxic compounds such as AMPs, and given the primary ecological niche of saprobic fungi like *Aspergillus*, this highly competitive environment might have driven the evolution of such an aggressive conidial coating to fight off bacterial rivals that might hamper the germination process. This point is supported by our data resulting from confrontation of *A. fumigatus* spores with *S. aureus* cells serving as direct competitor. The recent discovery of an antifungal peptide that is cell wall-associated with conidia of the entomopathogenic fungus *Beauveria bassiana* (Tong et al., 2020) underscores the relevance of fungal spores as antimicrobial entities further. Here, this aspect translates into the context of fungal virulence and pathogenesis of aspergillosis when considering the human pulmonary microbiome as competing entity in the course of host colonisation that precedes invasion (Kolwijck & van de Veerdonk, 2014; Yatera, Noguchi, & Mukae, 2018). Especially the pulmonary microbiota might restrict access to colonisation sites or nutritional supply before conidial germination can be executed, and eliminating bacterial competitors in the infection site vicinity might support fungal outgrowth and therefore virulence. This scenario is of special relevance in the context of allergic bronchopulmonary aspergillosis (ABPA), a T_H2 -driven hypersensitivity response to *A. fumigatus* that affects patients suffering from asthma or cystic fibrosis (Tracy, Okorie, Foley, & Moss, 2016). Especially the latter condition is often associated with airway colonisation by various bacteria (Lipuma, 2010), such as *Pseudomonas aeruginosa* or *S. aureus*. Given the significant activity of the *A. fumigatus* DLP(s) against the latter, their role in the pathogenesis of ABPA, but also other forms of aspergillosis, might be a significant one that has not been recognised to date.

A further relevant aspect emerges from the recently discovered activity of a homologous fungal from a clinical isolate of the pathogenic fungus *Purpureocillium lilacinum* (Shen et al., 2020): the purlisin precursor is split into two peptides that are highly similar to the deduced Afusins of *A. fumigatus* (Figure 1c). Remarkably, a potassium channel inhibitory activity could be revealed for the N-terminal one, Purlisin-NT. Fungal defensins targeting voltage gated Kv1.3 channels have been described before (Luo et al., 2019; Xiang et al., 2015), and considering the high degree of conservation between Purlisin-NT and AfusinN, the latter might be one of those as well. Such channels are expressed in numerous human cells, among them macrophages and T

lymphocytes in which they are involved in cell proliferation and activation as well as cytokine production (reviewed by Feske, Wulff, & Skolnik, 2015). To what extent the conidia-associated *defX* gene product AfusinN might interfere with such host cell activities remains to be demonstrated.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains, media and culture conditions

Escherichia coli strain DH5 α (Woodcock et al., 1989) was used for plasmid DNA preparation and propagated in LB medium (1% peptone, 0.5% yeast extract, and 1% NaCl) under selective condition (100 μ g/ml ampicillin); for growth on solid culture medium, 1.5% agar was added. *E. coli* cells were grown at 37°C with shaking at 200 rpm when propagated in liquid culture, and bacterial transformations were performed using calcium/manganese-treated cells according to the protocol of Hanahan, Jessee, and Bloom (1991). Bacterial cultures of *S. aureus* strain ATCC 25923 and the *E. coli* isolate ATCC 25922 were propagated in LB. The *defX*-dependent effect of conidial extracts on these bacteria was monitored by adding $2 \cdot 10^5$ cells in 10 μ l LB to 200 μ l of cleared and filtered LB extracts from $1 \cdot 10^8$ fungal spores and following growth at 37°C using the optical density at 625 nm as readout as determined in a SpectraMax 340PC microplate reader (Molecular Devices). *A. fumigatus* strains were cultured at 37°C on Sabouraud agar or in/on nitrate-based minimal medium (Scott & Käfer, 1982) with shaking of liquid cultures at 150 rpm. Selection for the presence of the hygromycin B resistance marker was carried out by adding 200 μ g/ml of this compound. Phenotypes of the *defX* deletion strain, its wild-type progenitor and the reconstituted descendant were monitored and scored on solid culture plates 72 hr post inoculation. Vegetative fungal mycelia serving as source for nucleic acid extractions to monitor transcript levels were harvested from liquid shaken cultures 8 hr after shifting them to defined experimental conditions, unless otherwise stated. *A. fumigatus* hyphal extension after spore germination in the presence or absence of *S. aureus* cells was qualified by time lapse microscopy after inoculating blocks of solidified Terrific Soy Broth culture medium with diluted bacterial cell suspensions and dryly transferred fungal conidia. Images were taken every 20 min and hyphal extension of at least 100 fungal germlings in close vicinity to bacterial cells was measured, analysing between six and eight experimental replicates.

4.2 | Generation of recombinant strains and cloning procedures

Strains generated in this study (Table 1) derived from isolate Afs77, a non-homologous recombination-deficient derivative of the *A. fumigatus* clinical isolate ATCC 46645 (Hearn & Mackenzie, 1980; Krappmann et al., 2006) that served as wild-type

TABLE 1 Fungal strains used in this study

Name	Description	Relevant genotype	References
Afs77	wild-type reference strain, ATCC 46645 derivative	<i>akuA::loxP</i>	Hearn and Mackenzie (1980), Krappmann, Sasse, and Braus (2006)
Afs172	<i>defX</i> deletion strain	<i>defX::six^{-P}xylP::β-rec::trpC^t, hygro^R-six</i>	This study
Afs173	Marker-less <i>defX</i> deletant	<i>defX::six</i>	This study
Afs174	Reconstituted <i>defX</i> deletant	<i>defX<six^{-P}xylP::β-rec::trpC^t, hygro^R-six></i>	This study
Afs175	Marker-less reconstituted strain	<i>defX<six></i>	This study
Afs192	Reporter strain with <i>defX</i> coding sequence replaced by <i>gfp2-5</i> allele	<i>^PdefX::gfp2-5<six^{-P}xylP::β-rec::trpC^t, hygro^R-six></i>	This study
Afs193	Reporter strain with <i>defX</i> coding sequence, except for secretion signal, replaced by <i>gfp2-5</i> allele	<i>^PdefX::sp-gfp2-5<six^{-P}xylP::β-rec::trpC^t, hygro^R-six></i>	This study
Afs239	Strain expressing HA-tagged AfusinN	<i>^PdefX::sp-afusinN^{HA3}-pp-afusinC<six^{-P}xylP::β-rec::trpC^t, hygro^R-six></i>	This study
Afs240	Strain expressing HA-tagged AfusinC	<i>^PdefX::sp-afusinN-pp-afusinC^{HA3}<six^{-P}xylP::β-rec::trpC^t, hygro^R-six></i>	This study

recipient for gene targeting constructs (Figure S1). Cloning of gene replacement cassettes was essentially carried out as described following standard protocols of recombinant DNA technology by fusing homologous arms to a recyclable selection marker module (Hartmann et al., 2010; Szweczyk et al., 2006). For gene targeting, reconstitution, or promoter replacement, recipient strains were transformed by induced protoplast fusion (Dümig & Krappmann, 2015) and selection of primary transformants was carried out in the presence of respective antifungal substances. After colony purification, recombined isolates were screened by diagnostic PCR to become eventually validated by Southern analyses (Southern, 2006) using a 5'-specific probe amplified with oligonucleotides Sv1211 and Sv1212 (Table S1). A detailed description of the used cloning strategies and construction of plasmids is provided as Supporting Information.

4.3 | Isolation of nucleic acids

Plasmid DNA isolated from *E. coli* for diagnostic PCR and sequencing was prepared by applying the Machery-Nagel NucleoSpin plasmid DNA purification kit or for fungal transformation using the NucleoBond Xtra Midi plasmid DNA purification kit. *A. fumigatus* genomic DNA was prepared from ground mycelia as described earlier (Wu et al., 1998). Small amounts of total RNA for qRT-PCR were extracted from ground mycelia by applying the innuPREP Plant RNA kit (Analytik Jena). Larger amounts of total RNA for Northern hybridization and RNA-sequencing were extracted from ground mycelia with the TRI reagent (Sigma) and cleaned with the peqGOLD Phase Trap Eppendorf tube (peqlab). DNase I (Thermo Scientific) digestion was then carried out according to the manufacturer's instructions, followed by purification of RNA employing the Qiagen RNeasy mini kit.

4.4 | Monitoring of transcript levels

For *defX* transcript steady-state level quantification by Northern blot hybridization, 10 µg of total RNA samples were separated in formaldehyde-containing agarose gels by electrophoresis, blotted onto Amersham Hybond-N nylon membranes (GE Healthcare), and hybridized with a digoxigenin (DIG)-labelled probe that was amplified from ATCC 46645 genomic DNA with the primer pair Sv861 (5'-ATG CGT TTC ACC ACC ATC GTC ACT CC-3') and Sv862 (5'-CTA GTT GCA GGT GCA AAC ACC CTT G-3') covering the entire *defX* coding region and labelled during amplification by applying the PCR DIG labelling mix (Roche). For quantitative real-time PCR (qRT-PCR), DNase I-digested and purified RNA was reverse transcribed into cDNA using the SuperScript III first-strand synthesis SuperMix for qRT-PCR (Invitrogen) according to manufacturer's protocol, using 500 ng RNA template by default for each reaction in a total volume of 20 µl. Reactions for quantification of transcript levels were run in the 7900HT fast real-time PCR system with a 384-well block module (Applied Biosystems) according to the manufacturer's instructions. All reactions were performed in MicroAmp optical 384-well reaction plates (Applied Biosystems) with qPCR adhesive seal sheets (4titude). The standard volume of each individual reaction was 10 µl containing 10 ng cDNA as a template, two primers at a concentration of 250 nM each, and 2 µl of the 5x EvaGreen qPCR mix (Rox, Bio & Sell). The primer pair used for qRT-PCR in this study was MD014 (5'-CTG CTA CCC GAA CAA CAA GC-3') and MD015 (5'-AGC TTC CAA CTT CTC CAC GG-3'). All reactions were performed in technical triplicates from at least three independent biological samples, and dissociation curves were plotted to determine the specificity of PCR runs. For analyses of the qRT-PCR results, the SDS software (version 2.4, Applied Biosystems) was used. Quantitative analyses were performed according to the comparative threshold cycle method ($2^{-\Delta\Delta C_T}$ method, (Livak & Schmittgen, 2001), using

transcript levels of the actin-encoding gene (*act1*, AFUA_6G04740) as internal, constitutive reference.

4.5 | Immunoblot analysis and immunofluorescence microscopy

Detection of GFP in supernatants of washed conidia derived from the reporter strains Afs192 and Afs193 was carried out by Western blot following a standard experimental procedure (Guo, Huang, Cha, & Liu, 2010) and using a polyclonal rabbit anti-GFP IgG antibody (A-6455 from Thermo Fisher Scientific) together with an HRP-conjugated goat anti-rabbit IgG (H + L) secondary antibody (Invitrogen, A-16096). Afusins fused to the HA tag were detected in supernatants using the rat monoclonal anti-HA high affinity antibody (clone 3F10, Roche). For direct immune-fluorescence detection, the monoclonal anti-HA high affinity antibody (clone 3F10, Roche) was used on fixed conidia harvested from formaldehyde-purged mycelia or on conidia suspended directly in Tween 80-supplemented saline to be followed by a standard protocol. An Axio Observer7 fluorescence microscope (Zeiss) was used for imaging purposes together with the ZEN 2.3 software suite.

4.6 | Quantification of fluorescence emitted from supernatants or spores

Conidia of the GFP reporter strains Afs192 and Afs193 were washed away from sporulating mycelia with Tween 80-supplemented (0.02%) saline and suspensions were adjusted to $1 \cdot 10^8$ conidia/ml. Fluorescence emitted from cleared supernatants and resuspended spores of 100 μ l aliquots was directly quantified in a Fluoroskan Ascent FL fluorometer (Labsystems). Conidia from strains Afs239 and Afs240 expressing the HA-fused Afusin alleles were fixed for 24 hr by formaldehyde purging before suspending them in 4% paraformaldehyde (PFA) or washed three times with saline containing 0.02% Tween 80 before fixation in 4% PFA. After standard washing and blocking steps, $1.5 \cdot 10^8$ conidia were mixed with the fluorescein-conjugated monoclonal anti-HA high affinity antibody (clone 3F10, Roche), washed after incubation overnight, and their fluorescence determined in a Fluoroskan Ascent FL fluorometer (Labsystems).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Michaela Dümig designed and conducted experiments and analysed data; Jasmin Binder conducted experiments; Anastasia Gaculenko conducted experiments; Franziska Daul conducted experiments; Lex Winandy conducted experiments; Mike Hasenberg conducted experiments and analysed data; Matthias Gunzer analysed data; Reinhard Fischer analysed data; Markus Künzler co-wrote the manuscript; Sven Krappmann designed the study, analysed data, and co-wrote the manuscript.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

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