ADVANCES IN ASPERGILLUS FUMIGATUS PATHOBIOLOGY

EDITED BY: Frederic Lamoth, Praveen R. Juvvadi and William J. Steinbach

PUBLISHED IN: Frontiers in Microbiology

frontiers Research Topics

Frontiers Copyright Statement

© Copyright 2007-2016 Frontiers Media SA. All rights reserved.

All content included on this site, such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of or is licensed to Frontiers Media SA ("Frontiers") or its licensees and/or subcontractors. The copyright in the text of individual articles is the property of their respective authors, subject to a license granted to Frontiers.

The compilation of articles constituting this e-book, wherever published, as well as the compilation of all other content on this site, is the exclusive property of Frontiers. For the conditions for downloading and copying of e-books from Frontiers' website, please see the Terms for Website Use. If purchasing Frontiers e-books from other websites or sources, the conditions of the website concerned apply.

Images and graphics not forming part of user-contributed materials may not be downloaded or copied without permission.

Individual articles may be downloaded and reproduced in accordance with the principles of the CC-BY licence subject to any copyright or other notices. They may not be re-sold as an e-book.

As author or other contributor you grant a CC-BY licence to others to reproduce your articles, including any graphics and third-party materials supplied by you, in accordance with the Conditions for Website Use and subject to any copyright notices which you include in connection with your articles and materials.

All copyright, and all rights therein, are protected by national and international copyright laws.

The above represents a summary only. For the full conditions see the Conditions for Authors and the Conditions for Website Use.

ISSN 1664-8714
ISBN 978-2-88919-789-7
DOI 10.3389/978-2-88919-789-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

ADVANCES IN ASPERGILLUS FUMIGATUS PATHOBIOLOGY

Topic Editors:
Frederic Lamoth, Lausanne University Hospital, Switzerland
Praveen R. Juvvadi, Duke University Medical Center, USA
William J. Steinbach, Duke University Medical Center, USA

Aspergillus fumigatus is a human fungal pathogen that causes invasive aspergillosis (IA), a major infectious cause of death in the expanding population of immunocompromised individuals such as cancer patients and transplant recipients. The mortality of IA remains high (30-70%) and emerging resistance to triazoles, the first-line antifungal drug class, is of particular concern. Second-line therapies for IA are limited by their toxicity (polyenes) or their lack of fungicidal activity (echinocandins). Identification of novel antifungal targets is an urgent need for improving the outcome of IA.

A. fumigatus is a filamentous fungus exhibiting a complex developmental cycle and elaborated mechanisms of adaptation to allow the initiation and progression of infection in the human host. The fungal cell wall, with its unique and dynamic structure, is crucial for protecting cell integrity and evading the host immune system, also contributing to biofilm formation and virulence, and thus representing an ideal antifungal target. The emergence of azole resistance implies various and complex mechanisms that need to be further elucidated. Other important processes, such as biosynthetic pathways and toxin/metabolite production are important for fungal survival and propagation in the host environment, ultimately leading to disease. Moreover, the host immune response is a determinant factor in influencing the course of infection.

The objective of this topic issue is to provide an overview of the recent advances in our understanding of *A. fumigatus* pathobiology and of IA pathogenesis to outline future research.

Citation: Lamoth, F., Juvvadi, P. R., Steinbach, W. J., eds. (2016). Advances in *Aspergillus fumigatus* Pathobiology. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-789-7

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 03 February 2016
doi: 10.3389/fmicb.2016.00043

Editorial: Advances in *Aspergillus fumigatus* Pathobiology

Frédéric Lamoth[1,2,3]*, *Praveen R. Juvvadi*[1] *and William J. Steinbach*[1,4]

[1] Division of Pediatric Infectious Diseases, Department of Pediatrics, Duke University Medical Center, Durham, NC, USA, [2] Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Lausanne, Switzerland, [3] Institute of Microbiology, Lausanne University Hospital, Lausanne, Switzerland, [4] Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA

Keywords: invasive aspergillosis, antifungal resistance, *Aspergillus fumigatus*, fungal virulence, fungal proteins

The Editorial on the Research Topic

Advances in Aspergillus fumigatus Pathobiology

Aspergillus fumigatus and other *Aspergillus* spp. are ubiquitous in our environment. However, their potential to cause severe disease in humans was ignored for many centuries. The first reported case of presumed human aspergillosis is from 1791 during the French revolution. A 22-year old soldier sought medical attention for painful cheek swelling because of a fungus ball of the maxillary sinus invading the mouth and orbit (Plaignaud, 1791). The patient ultimately recovered after surgery. A few decades earlier, in 1729, the Italian priest and botanist Pier Antonio Micheli provided the first description of the fungal genus that he named "*Aspergillus*" because of the similarity of the conidial head and spores to the aspergillum, the liturgical implement used to sprinkle holy water in the Catholic church (Micheli, 1729). During the first half of the twentieth century, *Aspergillus* spp. were considered common laboratory contaminants and only an occasional cause of human diseases with some case reports of chronic bronchopulmonary aspergillosis among farmers, cerebral abscesses, meningitis, and bone infections (Cawley, 1947). The introduction of steroid therapy in the 1950s, the later development of anti-neoplastic chemotherapy, and the first hematopoietic stem cell transplantations during the following decades revealed the devastating potential of these fungi in patients with severely depressed immune systems.

Invasive aspergillosis (IA) has now emerged as a major infectious threat and the prevalence and spectrum of the disease has progressed in parallel with advances in medicine and the advent of new therapies with potent immunosuppressive effects. The cumulative 12-month incidence of IA is estimated at 1.6% in hematopoietic stem cell transplant recipients and 0.7% in solid organ transplant recipients, with an overall 1-year mortality of 40–75% (Kontoyiannis et al., 2010; Pappas et al., 2010). Moreover, IA is increasingly reported in populations with other underlying conditions, such as intensive-care unit patients, or patients with autoimmune or chronic bronchopulmonary diseases (Meersseman et al., 2004; Garbino et al., 2011).

Despite a slight improvement in survival rates (Steinbach et al., 2012), the mortality of IA remains high, and little significant progress has been made in the management of the disease over the last several decades. Amphotericin B was historically the pillar of antifungal therapy, but included an unacceptably high rate of failure due to toxicity. At the beginning of this century, voriconazole demonstrated a better efficacy and safety profile and became the preferred first-line therapy of IA (Herbrecht et al., 2002). However, emergence of resistance to triazoles as a probable consequence of the widespread use of fungicides in the agriculture and industry (Vermeulen et al., 2013) has led to the need for second-line antifungal agents. Echinocandins, such as caspofungin or micafungin, are now considered as salvage therapy of IA (Maertens et al., 2004), but their lack of fungicidal activity limits their efficacy. Posaconazole is active against most *Aspergillus* spp. However,

OPEN ACCESS

Edited and reviewed by:
Maurizio Del Poeta, Stony Brook University, USA

***Correspondence:**
Frédéric Lamoth
frederic.lamoth@chuv.ch

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *16 December 2015*
Accepted: *11 January 2016*
Published: *03 February 2016*

Citation:
Lamoth F, Juvvadi PR and Steinbach WJ (2016) Editorial: Advances in Aspergillus fumigatus Pathobiology. Front. Microbiol. 7:43. doi: 10.3389/fmicb.2016.00043

the most frequent mechanism of voriconazole resistance (i.e., mutations of the *Cyp51A* gene) often confers pan-azole resistance and the increasing use of posaconazole prophylaxis in patients with hematologic malignancies raises concern about breakthrough infections due to resistant *Aspergillus* spp. or other fungi (Auberger et al., 2012). Combination antifungal therapy, such as the association of triazoles and echinocandins, has led to inconclusive results (Marr et al., 2015).

Considerable effort from the research community is dedicated to the discovery of new antifungal targets. An effective antifungal agent must be fungal-specific to avoid unacceptable human toxicity, but this is difficult to achieve as both fungi and humans are eukaryotes. Indeed, all currently approved antifungal drugs target the specific components of the fungal cell membrane (ergosterol) or cell wall (β-1,3-D-glucan). A better understanding of the molecular pathways involved in fungal metabolism, virulence, stress response, and resistance are therefore important steps toward the discovery of novel therapeutic approaches.

The purpose of this research topic is to provide an overview on the current state of research and to strengthen the links within the *Aspergillus* community. Scientists purposefully chosen from various countries and continents have contributed to this special issue dealing with all the medical aspects of IA, including taxonomy, genetics, epidemiology, pathogenesis, antifungal resistance, and novel therapeutic perspectives. Multiple species have now been identified within the *Aspergillus* section *Fumigati* and the possible relationship between their metabolite profiles and pathogenicity are discussed (Frisvad and Larsen). The worldwide problem of emerging azole resistance among *Aspergillus* spp. is illustrated by an interesting epidemiological study and update of the situation in Asia (Chowdhary et al.). In addition, the existence of mechanisms of azole resistance other than mutations of the *Cyp51A* gene, such as increased expression of ATP-binding cassette (ABC) transporters, is highlighted (Moye-Rowley). To counteract emerging resistance, novel potential antifungal targets are being investigated and several research groups present their latest updates on the cell wall integrity signaling pathway (Valiante et al.), the Ras pathway (Al Abdallah and Fortwendel), the Hsp90-calcineurin network (Juvvadi et al.; Lamoth et al.), and the regulation of zinc and iron homeostasis (Schafferer et al.; Vicentefranqueira et al.). Finally, because innate and adaptive immunity are key determinants in the development of IA, the analysis of host-pathogen interactions represents a promising research area. The current knowledge about the immune responses mediated via T-helper cells is presented in two reviews (Amarsaikhan and Templeton; Thakur et al.). The recent identification of host genetic determinants of IA, such as TLRs polymorphisms, also opens perspectives for preventive strategies (Oliveira-Coelho et al.).

This overview on the recent advances in *A. fumigatus* pathobiology suggests that we are entering a new era in the approach and management of IA. The epidemiology and pathophysiology of the disease has become more complex, with emerging resistance to triazoles and the increased diversity of immunosuppression types and host susceptibilities. Combination therapies of existing compounds or novel molecules that may enhance their activity or modulate the pattern of host immune recognition, as well as personalized diagnostic and therapeutic strategies based on individual susceptibility profiles of high risk patients, may change our conventional approach of IA and hopefully result in better outcomes.

AUTHOR CONTRIBUTIONS

FL: Design and writing; PJ: Design and writing; WS: Design and writing.

REFERENCES

Auberger, J., Lass-Flörl, C., Aigner, M., Clausen, J., Gastl, G., and Nachbaur, D. (2012). Invasive fungal breakthrough infections, fungal colonization and emergence of resistant strains in high-risk patients receiving antifungal prophylaxis with posaconazole: real-life data from a single-centre institutional retrospective observational study. *J. Antimicrob. Chemother.* 67, 2268–2273. doi: 10.1093/jac/dks189

Cawley, E. P. (1947). Aspergillosis and the Aspergilli: report of a unique case of the disease. *Arch. Intern. Med.* 80, 423–434. doi: 10.1001/archinte.1947.00220160002001

Garbino, J., Fluckiger, U., Elzi, L., Imhof, A., Bille, J., and Zimmerli, S. (2011). Survey of aspergillosis in non-neutropenic patients in Swiss teaching hospitals. *Clin. Microbiol. Infect.* 17, 1366–1371. doi: 10.1111/j.1469-0691.2010.03402.x

Herbrecht, R., Denning, D. W., Patterson, T. F., Bennett, J. E., Greene, R. E., Oestmann, J. W., et al. (2002). Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N. Engl. J. Med.* 347, 408–415. doi: 10.1056/NEJMoa020191

Kontoyiannis, D. P., Marr, K. A., Park, B. J., Alexander, B. D., Anaissie, E. J., Walsh, T. J., et al. (2010). Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin. Infect. Dis.* 50, 1091–1100. doi: 10.1086/651263

Maertens, J., Raad, I., Petrikkos, G., Boogaerts, M., Selleslag, D., Petersen, F. B., et al. (2004). Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clin. Infect. Dis.* 39, 1563–1571. doi: 10.1086/423381

Marr, K. A., Schlamm, H. T., Herbrecht, R., Rottinghaus, S. T., Bow, E. J., Cornely, O. A., et al. (2015). Combination antifungal therapy for invasive aspergillosis: a randomized trial. *Ann. Intern. Med.* 162, 81–89. doi: 10.7326/M13-2508

Meersseman, W., Vandecasteele, S. J., Wilmer, A., Verbeken, E., Peetermans, W. E., and Van Wijngaerden, E. (2004). Invasive aspergillosis in critically ill patients without malignancy. *Am. J. Respir. Crit. Care Med.* 170, 621–625. doi: 10.1164/rccm.200401-093OC

Micheli, P. (1729). *Nova Plantarum Genera Juxta Tournefortii Methodum Disposita*. Florence.

Pappas, P. G., Alexander, B. D., Andes, D. R., Hadley, S., Kauffman, C. A., Freifeld, A., et al. (2010). Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clin. Infect. Dis.* 50, 1101–1111. doi: 10.1086/651262

Plaignaud, M. (1791). Observation concerning a fungus in the maxillary sinus. *J. Chir*. 87, 244–251.

Steinbach, W. J., Marr, K. A., Anaissie, E. J., Azie, N., Quan, S. P., Meier-Kriesche, H. U., et al. (2012). Clinical epidemiology of 960 patients with invasive aspergillosis from the PATH Alliance registry. *J. Infect.* 65, 453–464. doi: 10.1016/j.jinf.2012.08.003

Vermeulen, E., Lagrou, K., and Verweij, P. E. (2013). Azole resistance in *Aspergillus fumigatus*: a growing public health concern. *Curr. Opin. Infect. Dis.* 26, 493–500. doi: 10.1097/QCO.0000000000000005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Lamoth, Juvvadi and Steinbach. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

MINI REVIEW
published: 07 January 2016
doi: 10.3389/fmicb.2015.01485

Extrolites of *Aspergillus fumigatus* and Other Pathogenic Species in *Aspergillus* Section *Fumigati*

Jens C. Frisvad and Thomas O. Larsen*

Section of Eukaryotic Biotechnology, Department of Systems Biology, Technical University of Denmark, Kongens Lyngby, Denmark

Aspergillus fumigatus is an important opportunistic human pathogen known for its production of a large array of extrolites. Up to 63 species have been described in *Aspergillus* section *Fumigati*, some of which have also been reliably reported to be pathogenic, including *A. felis*, *A. fischeri*, *A. fumigatiaffinis*, *A. fumisynnematus*, *A. hiratsukae*, *A. laciniosus*, *A. lentulus*, *A. novofumigatus*, *A. parafelis*, *A. pseudofelis*, *A. pseudoviridinutans*, *A. spinosus*, *A. thermomutatus*, and *A. udagawae*. These species share the production of hydrophobins, melanins, and siderophores and ability to grow well at 37°C, but they only share some small molecule extrolites, that could be important factors in pathogenicity. According to the literature gliotoxin and other exometabolites can be contributing factors to pathogenicity, but these exometabolites are apparently not produced by all pathogenic species. It is our hypothesis that species unable to produce some of these metabolites can produce proxy-exometabolites that may serve the same function. We tabulate all exometabolites reported from species in *Aspergillus* section *Fumigati* and by comparing the profile of those extrolites, suggest that those producing many different kinds of exometabolites are potential opportunistic pathogens. The exometabolite data also suggest that the profile of exometabolites are highly specific and can be used for identification of these closely related species.

Keywords: *Aspergillus*, gliotoxin, fumagillin, extrolites, proxy-exometabolites

OPEN ACCESS

Edited by:
Frederic Lamoth, Lausanne University Hospital, Switzerland

Reviewed by:
Vito Valiante, Leibniz-Institute for Natural Product Research and Infection Biology - Hans Knöll Institute, Germany
William Charles Nierman, J. Craig Venter Institute, USA
Miroslav Kolarik Kolarik, Institute of Microbiology, Czech Republic

****Correspondence:***
Jens C. Frisvad
jcf@bio.dtu.dk

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *05 August 2015*
Accepted: *09 December 2015*
Published: *07 January 2016*

Citation:
Frisvad JC and Larsen TO (2016) Extrolites of Aspergillus fumigatus and Other Pathogenic Species in Aspergillus Section Fumigati. Front. Microbiol. 6:1485. doi: 10.3389/fmicb.2015.01485

INTRODUCTION

The genus *Aspergillus* comprises 344 species (Samson et al., 2014), and some of these can cause human diseases. *A. fumigatus* is the most important species (Latgé, 1999), but several other species in *Aspergillus* section *Fumigati* have been shown to be pathogenic in humans and animals with an inefficient immune system, including *A. lentulus* (Balajee et al., 2005a; Alhambra et al., 2008; Alcazar-Fuoli et al., 2014; Howard, 2014), *A. fumisynnematus* (Alcazar-Fuoli et al., 2014), *A. fumigatiaffinis* (Alcazar-Fuoli et al., 2014), *A. novofumigatus* (Peláez et al., 2013), *A. felis* (Barrs et al., 2013), *A. fischeri* (Kano et al., 2015), *A. viridinutans* (Vinh et al., 2009a; Coelho et al., 2011; Alcazar-Fuoli et al., 2014), *A. pseudofelis, A. pseudoviridinutans,* and *A. parafelis* (Sugui et al., 2014), *A. thermomutatus* (Toskova et al., 2013; Alcazar-Fuoli et al., 2014; Howard, 2014; Khare et al., 2014), *A. laciniosus* (Malejczyk et al., 2013), *A. hiratzukae* (Guarro et al., 2002; Alcazar-Fuoli et al., 2014), *A. spinosus* (Sutton et al., 2002); and *A. udagawae* (Kano et al., 2008; Vinh et al., 2009b; Sugui et al., 2010; Posteraro et al., 2011; Gyotoku et al., 2012; Kano et al., 2013). The taxonomy and identification of the causing Aspergilli is not always clear-cut and some isolates have been

misidentified (Balajee et al., 2005a,b, 2006; Álvarez-Pérez et al., 2014; Howard, 2014). For example pathogenic isolates identified as *A. viridinutans* (Varga et al., 2000; Vinh et al., 2009a; Kano et al., 2013) proved to be *A. felis, A. pseudoviridinutans, A. parafelis,* or *A. pseudofelis* (Barrs et al., 2013; Novaková et al., 2014; Sugui et al., 2014). *Aspergillus* species in subgenus *Circumdati* have also been reported as pathogenic including *Aspergillus terreus* in section *Terrei*, *A. flavus* in section *Flavi* and *A. tubingensis* in section *Nigri*, *A. persii,* and *A. tanneri* in section *Circumdati*, *A. nidulans* in section *Nidulantes*, (Sugui et al., 2012, 2015; Howard, 2014; Visagie et al., 2014) and *Aspergillus* section *Phialosimplex* [*Ph. caninus* = *Aspergillus caninus* and *Ph. salinarum* = *Aspergillus salinarus* (Sigler et al., 2010; Greiner et al., 2014)]. Small molecule extrolites (secondary metabolites) have been shown to be involved in the infection process (Kamei and Watanabe, 2005; Abad et al., 2010), so it might be expected that the pathogenic Aspergilli produce the same extrolites. In this review we examine whether the closely related pathogenic species in *Aspergillus* section *Fumigati* produce the same extrolites.

ASPERGILLUS TAXONOMY

Since 2011, all ascomycetous species can only have one name (Hawksworth et al., 2011; Hawksworth, 2012; McNeill et al., 2012). All species formerly included in *Dichotomomyces*, *Cristaspora, Phialosimplex*, *Polypaecilum*, in addition to *Penicillium inflatum*, have been formally combined into *Aspergillus* (Houbraken et al., 2014; Samson et al., 2014). Furthermore, all species of *Eurotium, Emericella, Chaetosartorya, Fennellia, Neocarpenteles, Neopetromyces, Neosartorya, Petromyces, Saitoa,* and *Stilbothamnium* have also been transferred to *Aspergillus* (Samson et al., 2014). Ascoma producing species in section *Fumigati* were originally described under the name *Neosartorya* (Samson et al., 2006, 2007), but have now all been transferred to *Aspergillus* (Samson et al., 2014). Several of the species originally thought to produce only the asexual state have later been shown to be able to produce mature ascomata when crossed with the opposite mating type, for example *A. fumigatus* (O'Gorman et al., 2009) and *A. lentulus* (Swilaiman et al., 2013). Other opportunistically pathogenic species such as *A. flavus* (Horn et al., 2009), *A. tubingensis* (Horn et al., 2013), and *A. terreus* (Samson et al., 2011; Arabatsis and Velegraki, 2013) can also produce mature ascomata when crossed with the opposite mating type. All species in *Aspergillus* and *Penicillium* have now been placed in the family *Aspergillaceae* (Houbraken and Samson, 2011). Species in *Aspergillus* section *Fumigati* are both phenotypically and genotypically distinct (Raper and Fennell, 1965; Geiser et al., 1998; Hong et al., 2005, 2006, 2008; Katz et al., 2005; Geiser et al., 2007; Samson et al., 2007; Yaguchi et al., 2007). *Aspergillus lentulus* was originally claimed to be a sibling species of *A. fumigatus*, but was later shown to be phenotypically very different from *A. fumigatus*, especially concerning extrolite profiles (Larsen et al., 2007; Tamiya et al., 2015). The species *A. pseudofelis, A. parafelis,* and *A. pseudoviridinutans* have not been examined chemically, but they are very close phylogenetically and morphologically to *A. felis* and may be real sibling species with no phenotypic differences (Sugui et al., 2014). The 63 species listed in **Table 1** are all those that have been described in *Aspergillus* section *Fumigati* and *Neosartorya*, but some of them are not yet available for the scientific community, so their identity and probably synonymy with other species is unknown. Samson et al. (2007) indicated that several species were synonyms of already known species in *Aspergillus* section *Fumigati* and *Neosartorya*. Thus the total number of species in *Fumigati* may be less than 63.

CHEMOTAXONOMY OF *ASPERGILLUS* SUBGENUS *FUMIGATI*

Species in subgenus *Fumigati* can produce many different extrolites (Frisvad and Samson, 1990; Samson et al., 2007; Stack et al., 2007; Varga et al., 2007; Frisvad et al., 2009; Sanchez et al., 2012; Kang et al., 2013; Frisvad and Larsen, 2015) of which some are specific to section *Fumigati*, while others are shared with the closely related section *Clavati* and the *Dichotomomyces* clade. *Aspergillus cejpii* in the *Dichotomomyces* clade produces gliotoxin, acetylgliotoxin, acetylgliotoxin G, bis(dethio)bis(methylthio)gliotoxin, fiscalin B, xanthocillin X monomethylether, tryptoquivalones, emindole SB, emindole SB β-mannoside, and 27-O-methylasporyzin (Varga et al., 2007; Harms et al., 2014; Rodrigues et al., 2015) possibly in addition to asporyzin A-C, emeniveol, JBIR-03, and asporyergosterol and other sterols (Qiao et al., 2010a,b). The producing strain of the latter exometabolites was probably misidentified as *A. oryzae*, since none of these exometabolites have ever been found in *A. oryzae* (Rank et al., 2012). Apart from some few other shared extrolites with *Aspergillus* species in other sections, most extrolites are unique to section *Fumigati*.

Aspergillus section *Clavati* contains species mostly associated to dung, and have not been reported to cause infections of vertebrate lungs (Varga et al., 2007). Species in *Aspergillus* section *Clavati* produce several bioactive extrolites, but few of these are found in *Aspergillus* section *Fumigati*. Examples of such *Aspergillus* section *Clavati* specific extrolites include patulin, cytochalasin E and K, antafumicins, expansolides, and clavatols, and these extrolites may be important for competition in a dung habitat, rather than in the compost habitats in which species of *Aspergillus* section *Fumigati* thrives. Some similar extrolites are in common between species in *Aspergillus* sections *Fumigati* and *Clavati*, however. Ribotoxins like the sarcins in *Aspergillus* section *Clavati* (Varga and Samson, 2008) are closely related to mitogillin and restrictocin in *Aspergillus* section *Fumigati* (Kao et al., 2001; Schwienbacher et al., 2005; Virágh et al., 2014). Furthermore, some tryptoquivalins are produced by species in both *Aspergillus* sections.

Like other filamentous fungi, *A. fumigatus* isolates produce extrolites in a species specific manner (Larsen et al., 2005; Frisvad et al., 2008), but some strains do not produce all the extrolites expected. This weaker exometabolic vigor is most pronounced in isolates directly isolated from patients (Frisvad and Samson, 1990; Tamiya et al., 2015). These isolates are often floccose and less strongly sporulating. However, isolates from natural habitats, such as compost, always sporulate heavily and

TABLE 1 | Species in *Aspergillus* section *Fumigati* and their extrolite production (species written in bold are known to be pathogenic to humans and/or other mammals).

Aspergillus arcoverdensis: N.E. (Matsusawa et al., 2015)

Aspergillus assulatus: aszonapyrone A, indole alkaloids and apolar metabolites (Samson et al., 2007)

Aspergillus auratus: helvolic acid (Samson et al., 2007)

Aspergillus aureolus: fiscalins, fumagillin, fumiquinazolines, helvolic acid, pseurotin A, tryptoquivalines, tryptoquivalones, viriditoxin (Samson et al., 2007; Kaur et al., 2013)

Aspergillus australensis: aszonalenins, wortmannins (Samson et al., 2007)

Aspergillus beijingensis: N.E. (Li et al., 1998)

Aspergillus botucatensis (= ***A. spinosus***) (Horie et al., 1995; Samson et al., 2007)

Aspergillus brevipes: roquefortine C, cf. meleagrin, viriditoxin (trace) (Lillehoj and Milburn, 1973; Samson et al., 2007)

Aspergillus brevistipitatus: N.E. (Novaková et al., 2014)

Aspergillus caatingaensis: N.E. (Matsusawa et al., 2014b)

Aspergillus conversis: N.E. (Novaková et al., 2014)

Aspergillus "coreanus" (*Neosartorya coreana*): aszonalenins (Samson et al., 2007)

Aspergillus delicatus (= *A. tatenoi*) (Samson et al., 2007)

Aspergillus denticulatus: gliotoxin, viriditoxin (Samson et al., 2007)

Aspergillus duricaulis: asperdurin, asperpentyn, cyclopaldic acid, duricaulic acid, fumagillin, 3-O-methylcyclopolic acid, furochromanols and phthalides, pseurotin A (Brillinger et al., 1978; Achenbach et al., 1982a,b, 1985a,b; Mühlenfeld and Achenbach, 1988a,b; Samson et al., 2007)

Aspergillus felis: fumagillin, fumigaclavine C, fumitremorgin A and C, helvolic acid, monomethylsulochrin, pyripyropen A, E, O, S, trypacidin (reported as "*A. viridinutans*", Tamiya et al., 2015, but *A. viridinutans* has a very different profile of extrolites, and many isolates reported as *A. viridinutans* have been shown to be *A. felis*; Barrs et al., 2013)

Aspergillus fennelliae: asperfuran, aszonalenins, fumigaclavines, viridicatumtoxin (Samson et al., 2007)

Aspergillus ferenczii: asperfuran, aszonalenins, fumigaclavine, fumigatins, cf. gliotoxin, viridicatumtoxin (Samson et al., 2007)

Aspergillus fischeri: 5-N-acetylardeemin, 5-N-acetyl-15b-didehydroardeemin, 5-N-acetyl-16-hydroxyardeemin, acetylaszonalenin, ardeemin, aszonalenin, aszonapyrone A, B, cottoquinazolin E & F, cyclotryprostatin B, 12α,13α-dihydroxyfumitremorgin C, *rel*-(8S)-19,20-dihydroxy-8-methoxy-9,18-*epi*fumitremorgin C, fiscalin A, B, C, fischerin, 1-formyl-5-hydroxyaszonalenin, fumitremorgin A, B, C, helvolic acid, 6-hydroxyaszonalenin, 15b-β-hydroxy-5-N-ardeemin, isoterrein, neofipiperazine A, B, C, neosartorin, nortryptoquivalone, 13-oxofumitremorgin B, pyripyropene A, pyripyrone S, sarcins, sartorypyrone B & D, sesterfischeric acid, sesterfischerol, terrein, TR-2, trypacidin, tryptoquivalines, verruculogen (Samson et al., 1990; Wong et al., 1993; Wakana et al., 2006; Samson et al., 2007; Yin et al., 2009; Eamvijarn et al., 2013a; Lee et al., 2013; Gomes et al., 2014; Shan et al., 2014; Sodngam et al., 2014) (as "*Xylaria humosa*") (Zhang et al., 2014; Zheng et al., 2014; Kaifuchi et al., 2015; Shan et al., 2015; Ye et al., 2015). There are indications that *A. fischeri* can also produce fumagillin (Lin et al., 2013; Wiemann et al., 2013)

[fiscalin B, helvolic acid, helvolinic acid, 27-*epi*-nortryptoquivaline, setosusin, 2-(1-oxo-2-hydroxyethyl)furan, 27-*epi*-tryptoquivaline, was found in "*Corynascus setosus*," which is probably an *Aspergillus fischeri* or alternatively the *Corynascus* culture was overgrown by *A. fischeri*; Fujimoto et al., 1996]

[cladoquinazoline, *epi*-cladoquinazoline, CS-C, deoxynortryptoquivaline, deoxytryptoquivaline, glyantrypine, 3-hydroxyglyantrypine, norquinadoline A, oxoglyantrypine, prelapatin B, quinadoline A, B, tryptoquivaline was reported from a *Cladosporium* sp., but the culture may have been overgrown with an *Aspergillus fischeri*; Peng et al., 2013]

Aspergillus fumigatiaffinis: auranthine, cycloechinuline, fumigaclavines, helvolic acid, neosartorin, palitantin, pyripyropen A, E, O, S, tryptoquivalins (Samson et al., 2007; Ola et al., 2014)

Aspergillus fumigatus: Wang compound 1,2, 3, Zhao compound 1, 2, 3, Zuck compound 1,2,3, N-acetyltyramine, asperfumigatin, asperfumin, asperfumoid, azaspirene, bisdechlorogeodin, chaetominine, bisdethio(bismethylthio)gliotoxin, brevianamide F, 4-carboxy-5,5′-dihydroxy-3,3′-dimethyldiphenylether, cephalimycin A, B, C, D, 2-chloro-1,3,8-trihydroxy-6-methyl-9-anthrone, cyclo-(Ala-Val), cyclotryprostatin A, B, C, D, cyclo(L-4-hydroxyproline-L-leucine), cyclo(L-4-hydroxyproline-L-phenylalanine), cyclo(L-Pro-L-Pro), cyclo(L-Pro-L-Gly), cyclo(L-Pro-L-Leu), cyclo(L-Pro-L-Pro), cyclo(L-Pro-L-Val), cyclo(L-Val-L-Leu), cyclotrypostatin C, 9-deacetoxyfumigaclavine C, 9-deacetylfumigaclavine C, 13-dehydroxycyclotryprostatin C, demethoxyfumitremorgin C, (4S,5S,6S,8S,9S,10R,13R,14S,16S,17Z)-6,16-diacetoxy-25-hydroxy-3,7-dioxy-29-nordammara-1,17(20)-dien-21-oic acid, didehydrobisdethiobis(methylthio)gliotoxin, difructosedianhydride, 1,2-dihydrohelvolic acid, 12,13-dihydroxyfumitremorgin C = TR-3, 2,3-dihydroxy-5-methyl-1,4-benzoquinone, 5,8-dihydroxy-9,12-octadecadienoic acid, 2,6-dihydroxyphenylacetic acid, dimethoxyfumitremorgin C, emodin, emodin 1,6-dimethylether, epoxysuccinic acid, ferrichrome C, festuclavine, FD-889, FK-463, fumagillin, fumagiringillin, fumifungin, fumigaclavine A, B, C,D, E, F, G, H (fumigaclavine A reported also from *A. tamarii*, but this was an *A. fumigatus*, Janardhanan et al., 1984), fumigatin, fumigatin chlorohydrin, fumigatin oxide, fumigatin quinol, fumigatonin (identity of producer not verified), fumigatoside B, C, and D, fumipyrrole, fumiquinazolin A, B, C, D, E, F, G, J, and K, fumiquinone A and B, fumitremorgin A, B, C, and D, (GERI-BP002-A), fusarinine C, glionitrin A and B, gliotoxin, gliotoxin E and G, helvolic acid, helvolinic acid, hexahydropolyprenol-18, 19, 20, 21, 22, 23, 24, 3-β-hydroxy-cyclo-L-tryptophyl-L-proline, 2-hydroxy-3-methoxy-5-methyl-1,4-benzoquinone, N-(2-(4-hydroxyphenyl)ethenyl)formamide, 14-hydroxyterezine D, 3-hydroxytoluquinone, 20-hydroxytryrpostatin B, isochaetominin, isorhodoptilometrin, LL-S490β, 6-methoxyspirotryprostatin B, 8′-O-methylasterric ac id, 11-O-methylpseurotin, monomethylsulochrin, orsellinic acid, 13-oxofumitremorgin B, 18-oxotryprostatin A, 13-oxo-verruculogen, 14-norpseurotin A, N-prenyl-cyclo-L-tryptophyl-L-proline, pseurotin A, A1, A2, D, F1, F2, pyripyropen A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, and S, questin, RK-95113, *cis* and *trans*-ruakuric acid (Cutler et al., 1996, identity of fungus could not be checked), Sch-528647, sphingofungin A, B, C, D, spinulosin, spinulosin hydrate, spinulosin quinol hydrate, spirotryprostatin A, B, C, D, and E, synerazol, terezine D, TR-2, 1,2,3,4-tetrahydroxy-5-methylbenzene, 4,8,10,14-tetramethyl-6-acetoxy-14-[16-acetoxy-19-(20,21-dimethyl)-18-ene]phenanthrene-1-ene-3,7-dione, 3-thiomethyl-cyclo(Ser, Phe), triacetylfusarinine, tryptostatin A, B, trypacidin, 1,2-*seco*-tryprostatin, tryptoquivaline F, G, H, I, J, L, M, and N, tryptoquivalin R?, S?, verruculogen (Samson et al., 1990; Land et al., 1993; Cui et al., 1996; Tepsic et al., 1997; Furtado et al., 2005; Han et al., 2007a,b; Samson et al., 2007; Wang et al., 2008; Zhang et al., 2008 (as "*A. sydowi*"); Frisvad et al., 2009; Zhao et al., 2010; Afiyatullov et al., 2012; Zhang et al., 2012; Cano et al., 2013; Ding et al., 2013; Zhou et al., 2013; Alcazar-Fuoli et al., 2014; Haas, 2014; Kim et al., 2014; Owens et al., 2014; Wiekmann et al., 2014; Xu et al., 2014; Liang et al., 2015; Liu et al., 2015; MacHeleidt et al., 2015; Tamiya et al., 2015; Xie et al., 2015)

(Continued)

TABLE 1 | Continued

[*Aspergillus fumigatus* (fungus misidentified): antafumicin A and B, cytochalasin E, expansolide A and B Macías et al., 2003, the strain used was misidentified and was an *Aspergillus clavatus*; isosclerone Li et al., 2014]

Aspergillus fumisynnematus: cyclopiazonic acid, fumimycin, neosartorin, pyripyropens (Kwon et al., 2007; Samson et al., 2007)

Aspergillus galapagensis: gregatins (Samson et al., 2007)

Aspergillus hiratsukae: avenaciolide (Samson et al., 2007)

Aspergillus huiyaniae: N.E. (Matsusawa et al., 2014a)

Aspergillus indohii: N.E. (Horie et al., 2003)

Aspergillus laciniosus: aszonalenins, aszonapyrone A and B, 3′-(4-oxoquinazolin-3-yl)spiro[1H-indol-3,5′-oxolane]2,2′-dione, 4(3H)-quinazoline, tryptoquivaline L & T (Samson et al., 2007; Eamvijarn et al., 2013a; Gomes et al., 2014)

Aspergillus lentulus: auranthine, cyclopiazonic acid, fumifungin, fumigaclavine A, B, C, fumiquinazoline F or G, monomethylsulochrin, neosartorin, pyripyropen A, E, O, S, sphingofungin A, B, C, D, terrein, trypacidin (Larsen et al., 2007; Samson et al., 2007,: Frisvad et al., 2009; Tamiya et al., 2015)

Aspergillus marvanovae: apolar indoloterpenes (Hubka et al., 2013)

Aspergillus multiplicatus: aszonapyrone A, helvolic acid (Samson et al., 2007)

Aspergillus neoglaber: asperpentyn, avenaciolide, glabramycin A, B, C, Mer-NF8054A, Mer-NF8054X, NK-372135A, B, C, sartoryglabrin A, B, C, wortmannins (Ellis et al., 1964; Morino et al., 1994; Samson et al., 2007; Jayasuriya et al., 2009; Kijjoa et al., 2011)

Aspergillus nishimurae: Anishidiol, 4-hydroxybenzaldehyde, 4-methylbenzylalcohol, monochaetin (Hosoe et al., 2011)

Aspergillus novofumigatus: Dihydroterrein, epi-aszonalenin A, B, C, ent-cycloechinulin, dihydroterrein, fiscalins, helvolic acid, neosartorin, novoamauromin, novobenzomalvin A, B, C, novofumigatamide, novofumigatonin, palitantin, terrein, territrem B (Rank et al., 2006; Samson et al., 2007; Rank et al., 2008; Hosoe et al., 2009; Ishikawa et al., 2010a,b, 2011)

Aspergillus otanii = A. fennelliae (Takeda et al., 2001; Samson et al., 2007)

Aspergillus papuensis: wortmannins (Samson et al., 2007)

Aspergillus parafelis: N.E. (Sugui et al., 2014)

Aspergillus paulistensis [= *A. spinosus* according to Samson et al. (2007)]: 3′-(4-oxoquinazolin-3-yl)spiro[1H-indol-3,5′-oxolane]2,2′-dione, 4(3H)-quinazoline, sartorypyrone C, tryptoquivaline L (Horie et al., 1995; Gomes et al., 2014)

Aspergillus pernambucoensis: N.E. (Matsusawa et al., 2014b)

Aspergillus primulinus = A. quadricinctus (Samson et al., 2007)

Aspergillus pseudofelis: N.E. (Sugui et al., 2014)

Aspergillus pseudoviridinutans: N.E. (Sugui et al., 2014)

Aspergillus quadricinctus: aszonalenins, PF1223, quinolactacin (Ozoe et al., 2004; Samson et al., 2007)

Aspergillus qizutongii: N.E. (Li et al., 1998)

Aspergillus shendawei: N.E. (Yaguchi et al., 2010)

Aspergillus siamensis: chevalone B, C, 4-dihydroxy-3-methylacetophenone, fiscalin A, C, *epi*-fiscalin A, C, neofiscalin A, *epi*-neofiscalin A, 3′-(4-oxoquinazolin-3-yl)spiro[1H-indole-3,5′-oxolane]-2,2′-dione, sartorymensin, tryptoquivaline, tryptoquivaline F, H, L, O (Buttachon et al., 2012; Eamvijarn et al., 2013b)

Aspergillus similanensis: chevalone, B, C, E, 6,8-dihydroxy-3,7-dimethylisocoumarin, 6,8-dihydroxy3-methylisocoumarin, p-hydroxybenzaldehyde, 5-hydroxy-8-methyl-2H,6H-pyrano[3,4-g]chromene-2,6-dione, pyripyropen E, S, and T, reticulol, S14-95, similanamide, similanpyrone C (Prompanya et al., 2014, 2015)

Aspergillus solicola: aszonalenins, chromanols, tryptoquivalines, tryptoquivalones, wortmannins (Samson et al., 2007, 2014)

Aspergillus spathulatus: aszonalenins, xanthocillins (Samson et al., 2007)

Aspergillus spinosus: aszonalenins, pseurotins, 2-pyrovoylaminobenzamide, fumigachlorin (Atsumi et al., 1970; Samson et al., 2007)

Aspergillus stramenius: avenaciolide, quinolactacin (Samson et al., 2007)

Aspergillus sublevisporus: N.E. (Someya et al., 1999)

Aspergillus takakii = A. spinosus (?) (Samson et al., 2007): acetylaszonalenin, aszonalenin, aszonapyrone A, chevalone B, 6-hydroxymellein, 3′-(4-oxoquinazolin-3-yl) spiro[1H-indole-3,5′-oxalone]-2-2′-dione, tryptoquivaline F, H, L, U (Zin et al., 2015)

Aspergillus tatenoi: aszonalenin, aszonapyrone A, B, tatenoic acid (Samson et al., 2007; Yim et al., 2014)

Aspergillus thermomutatus: 6-acetylbis(methylthio)gliotoxin, acetylgliotoxin, asperfuran, bisdethiobis(methylthio)gliotoxin, bis-N-norgliovictin, brasilianamide B, cadinene, CJ-12662?, 3,8-diacetyl-4-(3-methoxy-4,5-methylenedioxy)benzyl-7-phenyl-6-oxa-3,8-diazabicyclo[3.2.1]octane, didehydrobisdethiobis(methylthio)gliotoxin, euchevalierine, fiscalins (?), fischerindoline, gliotoxin, helvolic acid, 3-hydroxy-5-methylphenyl-2,4-dihydroxy-6-methylbenzoate, N-methyl-1H-indole-2-carboxamide, neosartorin A, B, C, pseudofischerine, pyripyropen A, E, O, S, 1,2,3,4-tetrahydro-2,3-dimethyl-1,4-dioxopyrazino[1,2-a]indole, 1,2,3,4-tetrahydro-2-methyl-3-methylene-1,4-dioxopyrazino[1,2-a]indole, 1,2,3,4-tetrahydro-2-methyl-1,3,4-trioxopyrazino[1,2-a]indole, (tryptoquivalin R, S ?) [maybe: misidentified as "*Eurotium chevalieri*": cadinene, chevalone A, B, C, D, aszonapyrone A, B, euchevalerine, CJ-12662 Kanokmedhakul et al., 2011] (Samson et al., 2007; Eamvijarn et al., 2012; Masi et al., 2013; Xu et al., 2013; Liang et al., 2014)

Aspergillus tsunodae: helvolic acid, sartorypyrone A and B (Yaguchi et al., 2010; Eamvijarn et al., 2013a; Gomes et al., 2014)

Aspergillus tsurutae: N.E. (Horie et al., 2003)

Aspergillus turcosus: aszonalenins, gliotoxin, kotanins (Samson et al., 2007; Hubka et al., 2013)

(Continued)

TABLE 1 | Continued

Aspergillus udagawae: fumagillin, fumigaclavine A and C, fumigatins, fumiquinazolin F or G, helvolic acid, monomethylsulochrin, pyripyropene A, E, trypacidin, tryptoquivalines, tryptoquivalones (Samson et al., 2007; Tamiya et al., 2015)

Aspergillus unilateralis: aszonapyrones, mycophenolic acid (Samson et al., 2007; Hubka et al., 2013)

Aspergillus viridinutans: 4-acetyl-6,8-dihydroxy-5-methyl-2-benzopyran-1-1 A, 13-O-methylviriditin, phomaligin A, SC-28763, SC-30532, semiviriditoxin, viriditoxin, viritin, viriditin, wasabidienone B0 and B1 (Omolo et al., 2000; Samson et al., 2007)

Aspergillus waksmanii: apolar indoloterpenes (Hubka et al., 2013)

Aspergillus wangduanglii: N.E. (Li et al., 1998)

Aspergillus wyomingensis: N.E. (Novaková et al., 2014)

N.E: Not Examined.

produce most of the expected species specific extrolites (Frisvad and Samson, 1990; Tepsic et al., 1997; Hong et al., 2010a,b). Production of small molecule extrolites is depending on the growth conditions and the growth media (Nielsen et al., 2011; Frisvad, 2012; Brakhage, 2013), and some of these extrolites may need biological / chemical stimulants of the producing fungus to be expressed (Brakhage and Schroeckh, 2011; Zuck et al., 2011; Netzker et al., 2015).

Being species specific, the difference between the extrolites profiles of different species of *Aspergillus* section *Fumigati* can be used in identification of the species in *Aspergillus* section *Fumigati* as an alternative to sequence—based or MALDI-TOF based identification (Panda et al., 2015), or used together with morphology and physiology in a polyphasic identification approach (Samson et al., 2007). For example *A. fumigatus* can be distinguished from *A. lentulus* by exometabolite profiling (Larsen et al., 2007), MALDI-TOF (Verwer et al., 2014), and sequencing (Balajee et al., 2005a; Samson et al., 2007), but only partially by morphology and Raman spectroscopy (Verwer et al., 2014).

EXTROLITES PRODUCED BY *ASPERGILLUS FUMIGATUS* AND OTHER PATHOGENIC SPECIES IN *FUMIGATI*

A. fumigatus has been reported to produce many different extrolites that are bioactive and may contribute to infection in humans and other animals (Amitani et al., 1995; Tomee and Kauffman, 2000; Reeves et al., 2006; Cramer et al., 2009; Abad et al., 2010; Coleman et al., 2011). Melanins are polyketide derived conidium pigments that may have an influence on the infection process (Tsai et al., 1998; Jahn et al., 2000; Tsai et al., 2001; Langfelder et al., 2003). Since all species in *Aspergillus* section *Fumigati* produce green conidia, it is expected that they all contain melanin (Perrin et al., 2007). Another more general small molecule pathogenicity factor is siderophores, of which *A. fumigatus* produces fusarinine C and triacetylfusarinine C extracellularly (Haas, 2014; Petrik et al., 2014). Furthermore hydrophobins are also present in all species of *Aspergillus* section *Fumigati* (Geiser et al., 1998; Pedersen et al., 2011). These proteins will protect conidia from being recognized by the immune system in mammals (Aimanianda et al., 2009). Other proteins, especially proteases also play a role in the infection process and may be expected to be produced by many pathogenic species (Tomee and Kauffman, 2000; Abad et al., 2010; Dhingra et al., 2012). Small molecule siderophores are also considered to be important pathogenicity factors, and given the general importance for fungi they can be expected to be produced by all pathogenic species of *Aspergillus* (Fedorova et al., 2008; Abad et al., 2010; Haas, 2014), but probably also by non-pathogenic species.

However, other extrolites are not produced by all species in *Aspergillus* section *Fumigati*. Gliotoxin has long been known to be important for the infection process by inhibiting the immune response, phagocytosis and angiogenesis (Watanabe et al., 2003, 2004; Tsunawaki et al., 2004; Bok et al., 2005; Lewis et al., 2005; Stanzani et al., 2005; Coméra et al., 2007; Sugui et al., 2007; Ben-Ami et al., 2009; Abad et al., 2010). Gliotoxin has been reported from the pathogenic species *A. fumigatus* and *A. thermomutatus*, but also from *A. denticulatus*, *A. ferenczii* and *A. turcosus* (**Table 1**) the latter three not yet known to be pathogenic. Annotation of the genomes of *A. fumigatus* and *A. fischeri* indicates that the latter species can also produce gliotoxin given the right conditions (Inglis et al., 2013). However, many other *Aspergillus* section *Fumigati* extrolites appear to be involved in pathogenesis. Verruculogen, produced by *A. fumigatus* and *A. fischeri*, modifies electrophysical properties of the human nasal epithelial cells (Khoufache et al., 2007) but is also a potent tremorgen (Land et al., 1993; Kelman et al., 2004). Verruculogen and fumitremorgin C (Rabindran et al., 2000) are produced by *A. fumigatus* and *A. fischeri* (**Table 1**) in section *Fumigati*. Fumagillin suppresses the immune response, neutrophil function and angiogenesis (Fallon et al., 2010, 2011) and is produced by the pathogenic species *A. felis*, *A. fumigatus*, and *A. udagawae*, but also by species in *Aspergillus* section *Fumigati,* such as *A. aureolus* and *A. viridinutans* that have not been reported as yet to be pathogenic (**Table 1**). Pseurotin A is an inhibitor of immunoglobulin E and is responding to hypoxia (Schmeda-Hirschmann et al., 2008; Ishikawa et al., 2009; Vödisch et al., 2011). Pseurotins are produced by the pathogenic *A. fumigatus* and *A. spinosus*, but are also produced by *A. duricalis* and *A. aureolus* (**Table 1**). Sulochrin inhibits eosinophil activation (Ohashi et al., 1997, 1998) and is produced by four pathogenic species in section *Fumigati*: *A. felis, A. fumigatus, A. lentulus,* and *A. udagawae* (**Table 1**). The related asterric acid is produced by the same species and this extrolite inhibits vascular endothelial growth factor induced tube formation (Lee et al., 2013). Another related extrolite is trypacidin, which is cytotoxic (Gauthier

et al., 2012), but was originally isolated as an antiprotozoan metabolite (Balan et al., 1963). The fumiquinazolins are also cytotoxic (Lim et al., 2014), and are produced consistently by *A. fumigatus* (Frisvad et al., 2009). The fumiquinazolines (Takahashi et al., 1995) are produced by the pathogenic *A. fumigatus* and *A. lentulus*, but are also produced by *A. aureolus* (**Table 1**). The chemically similar fiscalins and cottoquinazolins (norfumiquinazolins; Ames and Walsh, 2010; Shan et al., 2015) are produced by *A. fischeri*, indicating that these metabolites are of importance for the competitiveness of these fungi. The pyripyropenes have antiangiogenic activity (Hayashi et al., 2009) and are produced by nearly all the known pathogenic species in section *Fumigati*: *A. fumigatus*, *A. fumigatiaffinis, A. fumisynnematus, A. lentulus, A. thermomutatus,* and *A. udagawae* (**Table 1**). In addition pyripyropens are produced by *A. similanensis*, a species that has not yet been tested for pathogenicity or isolated from any animal tissues.

Helvolic acid has been reported as an antibiotic and antifungal extrolite (Rementeria et al., 2005), but it also has been reported to affect human respiratory epithelium (Amitani et al., 1995) and the metabolism of macrophages (Shinohara et al., 1992). Helvolic acid has been reported from *Aspergillus auratus, A. aureolus, A. felis, A. fischeri, A. fumigatiaffinis, A. fumigatus, A. multiplicatus, A. novofumigatus, A. thermomutatus, A. tsunodae,* and *A. udagawae*. It is upregulated with gliotoxin in *A. fumigatus* (O'Keeffe et al., 2014). Thus helvolic acid may also be a pathogenicity factor, but of the species listed above *A. auratus, A. aureolus*, *A. multiplicatus,* and *A. tsunodae* have not been reported as pathogenic. Among bioactive proteins it seems that mitogillin is playing a role in the infection process (Schwienbacher et al., 2005; Abad et al., 2010), but these ribotoxins have not been screened in the other 62 species in *Aspergillus* section *Fumigati*.

Several small molecule extrolites have not yet been claimed to be involved in pathogenesis. The fumigaclavines are produced by the pathogenic species *A. felis, A. fumigatus, A. fumigatiaffinis,* and *A. lentulus*, but are also produced by *A. fennelliae* and *A. ferenczii* (**Table 1**). Even though these ergot alkaloids are associated with conidiation in *A. fumigatus* (Coyle et al., 1981), their role in animal pathogenesis is unknown. The fumigatins have mostly been found in soil-borne strains of *A. fumigatus* (Frisvad and Samson, 1990), and may rather have a role in competitiveness in compost and soil, than in animal pathogenesis.

PREDICTION OF OTHER POTENTIAL OPPORTUNISTIC PATHOGENIC SPECIES IN *ASPERGILLUS* SECTION *FUMIGATI* BASED ON EXTROLITES

Among the 63 species described in *Aspergillus* section *Fumigati*, 17 have until now been reported to be opportunistic pathogens of vertebrate animals (in bold, **Table 1**). Several extrolites have been shown to have a certain role in the infection process, but these extrolites may have a different role in the natural habitats of these fungi, of which plant compost may be the primary habitat (Latgé, 1999; Abad et al., 2010). It appears that when growing on plant compost these fungi need a certain profile of extrolites (small molecule extrolites and exoproteins), while as vertebrate opportunistic pathogens they may need quite a different profile of extrolites (Abad et al., 2010). For example cellulases would be important in the compost situation (Srivastava et al., 2014; Miao et al., 2015), while hemolysins are probably only important for the vertebrate infection process (Abad et al., 2010). The same would be the case for antifungals and antibiotics, especially anti-streptomycete metabolites, as *A. fumigatus* and other members of *Aspergillus* section *Fumigati* are thermotolerant / thermophilic species competing with other thermotolerant and thermophilic species of fungi and bacteria (Langarica-Fuentes et al., 2014). Several species, such as *A. assulatus, A. australensis, A. brevipes*, *A.* "*coreanus,*" *A. duricaulis, A. fennelliae*, *A. galapagensis*, *A. neoglaber, A. marvanovae, A. nishimurae, A. papuensis, A. quadricinctus, A. solicola*, *A. spathulatus, A. tatenoi, A. unilateralis, A. viridinutans*, and *A. waksmanii* produce few if any of the extrolites suspected to play a role in the infection process, and so would not be predicted to be potential opportunistic pathogens of vertebrates. Some species, such as *A. auratus, A. denticulatus, A. similanensis, A. tsunodae,* and *A. turcosus* only produce one of the extrolites believed to play a role in pathogenesis, and may or may not be prospective vertebrate pathogens. Finally *A. aureolus, A. ferenczii,* and *A. siamensis* produce several of the extrolites potentially involved in pathogenesis, and thus may be predicted to be potential opportunistic vertebrate pathogens.

The many extrolites that have been suspected to be pathogenicity factors and are produced by species in *Aspergillus* section *Fumigati* are biosynthetically derived from polyketides, amino acids, terpenes, shikimic acid or are of mixed biosynthetic origin. The formula of some of the most important extrolites common in *Aspergillus* section *Fumigati* are shown in **Figure 1**. Some of the extrolites are not produced in the same patterns in different species in *Aspergillus* section *Fumigati*. While *A. fumigatus* produces fumiquinazolins A–G, J, and K, *A. fischeri* produces the related norfumiquinazolins (Shan et al., 2015). These extrolites may have the same function, even though they are chemically somewhat different. Whether such proxy-extrolites have the same function for pathogenicity in vertebrates is unknown. It is known, however, that other opportunistic pathogenic aspergilli in other sections of *Aspergillus* produce secondary metabolites that are biosynthetically and functionally closely related. While *A. fumigatus*, *A. thermomutatus,* and other species in section *Fumigati* produce gliotoxin, *A. flavus* in *Aspergillus* section *Flavi* can produce aspirochlorine and *A. terreus* in *Aspergillus* section *Terrei* can produce acetylaranotin (Frisvad and Larsen, 2015). While not identical to gliotoxin, these epidithiodioxopiperazines could be predicted to play a role in pathogenicity of *A. flavus* and *A. terreus*. The reports that *A. niger*, *A. flavus,* and *A. terreus* could produce gliotoxin (Lewis et al., 2005; Kupfahl et al., 2008) have not been confirmed (Samson et al., 2011; Varga et al., 2011a,b).

Close phylogenetic relationships seem to be less suited pathogenicity predictors. For example *A. viridinutans* seems to be non-pathogenic, while the closely related *A. felis* is pathogenic

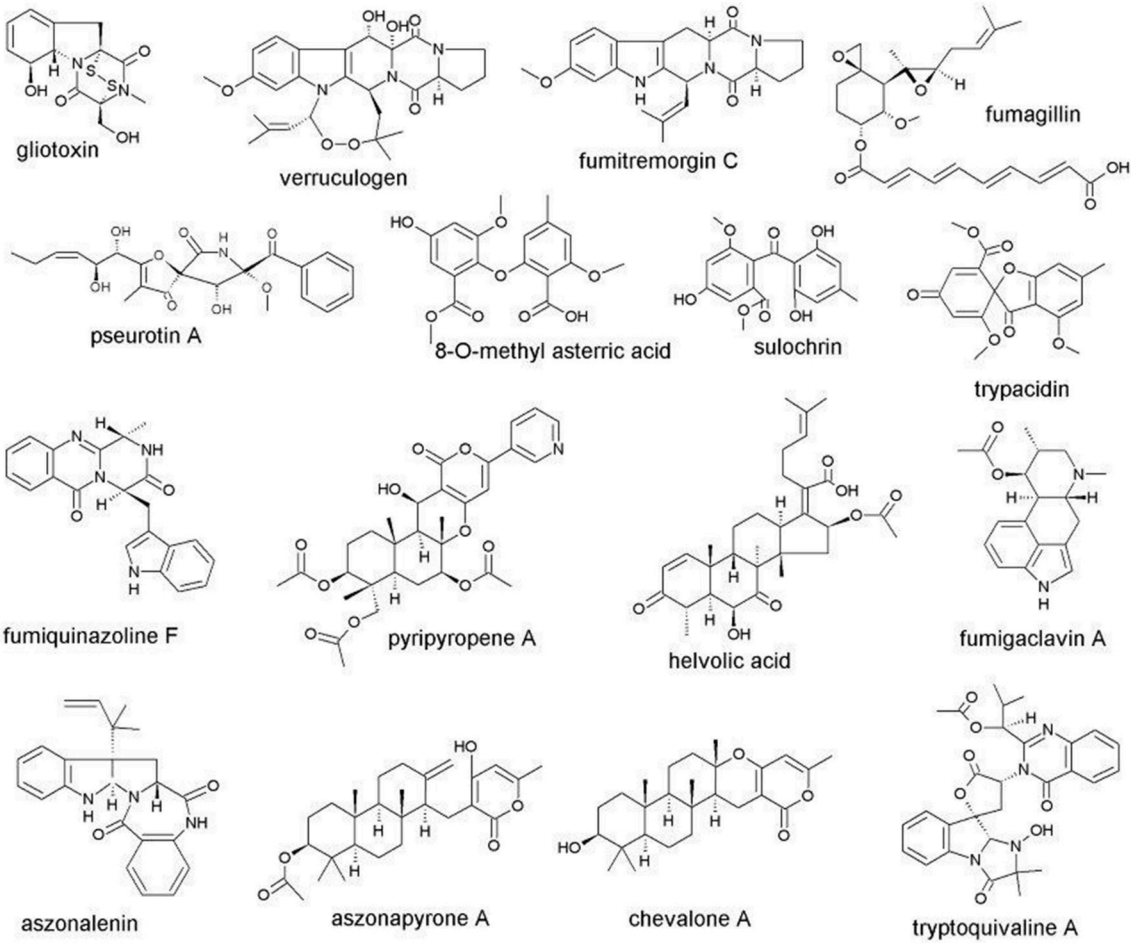


FIGURE 1 | Structures of the most important extrolites from *Aspergillus* section *Fumigati* potentially involved in pathogenesis.

(Barrs et al., 2013; Novaková et al., 2014; Sugui et al., 2014). Good growth at 37°C also seems to be a contributing factor to pathogenicity, and for example *A. brevipes*, *A. duricaulis* and *A. viridinutans* grow relatively poorly at 37°C, and in addition are not considered potentially pathogenic *Aspergillus* species in section *Fumigati*, based on extrolite evidence and absence of reports of pathogenicity. However, while there a many data on the involvement of exometabolites for *A. fumigatus* (Abad et al., 2010), there are few data on production of exoproteins for other opportunistic pathogenic species such as *A. thermomutatus*.

Genome sequencing and systematic comparison of the genomes and transcriptomes of other members of *Aspergillus* section *Fumigati* may help in predicting which pathogenicity factors are especially important (Galaghan et al., 2005; Nierman et al., 2005; Wortman et al., 2006; Fedorova et al., 2008; McDonagh et al., 2008; Chooi et al., 2013; Inglis et al., 2013; Cerqueira et al., 2014; Kusuya et al., 2015; Lind et al., 2015). These data should be compared to phenotypic data such as profiles of large and small molecule extrolites, growth temperatures, carbon dioxide tolerance etc.

Altogether, approximately one third of the species in *Aspergillus* section *Fumigati* are common pathogenic species, one third are rare species of unknown pathogenicity and one third are predicted to be non-pathogenic, based on their production of relatively few exometabolites. Exometabolite pathogenicity factors found in the successful opportunistic pathogenic fungus *A. fumigatus* may have proxy-exometabolites with the same function in other species in that section, but also in less closely related pathogenic Aspergilli, especially species from sections *Nigri*, *Terrei*, *Circumdati,* and *Flavi*.

ACKNOWLEDGMENTS

We thank Agilent technologies for Agilent Thought Leader Award # 2871 and the Novo Nordisk Foundation for grant NNF 13OC0005201.

REFERENCES

Abad, A., Fernández-Molina, J. V., Bikandi, J., Ramírez, A., Margareto, J., Sendino, J., et al. (2010). What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. *Rev. Iberoam. Micol.* 27, 155–182. doi: 10.1016/j.riam.2010.10.003

Achenbach, H., Mühlenfeld, A., and Brillinger, G. U. (1985a). Stoffwechselprodukte von mikroorganismen. XXX. phthalide und chromanole aus *Aspergillus duricaulis*. *Liebigs Annalen der Chemie* 1985, 1596–1628.

Achenbach, H., Mühlenfeld, A., Kohl, W., and Brillinger, G. U. (1985b). Stoffwechselprodukte von mikroorganismen, XXXI [1]. Duricaulinsäure, ein neuer Naturstoff vom phthalimidin-Typ aus *Aspergillus duricaulis*. *Z. Naturfors.* 40b, 1219–1225.

Achenbach, H., Mühlenfeld, A., Weber, B., and Brillinger, G. U. (1982b). Highly substituted chromanols of *Aspergillus duricaulis*. *Tetrahedron Lett.* 23, 4659–4660.

Achenbach, H., Mühlenfeld, A., Weber, B., Kohl, W., and Brillinger, G. U. (1982a). Stoffwechselprodukte von mikroorganismen. XXVII [1]. Cyclopaldsäure und 3-O-methylcyclopolsäure, zwei antibiotisch worksame Substanzen aus *Aspergillus duricaulis*. *Z. Naturfors.* 37b, 1091–1097.

Afiyatullov, S. S., Zhuravleva, O. I., Antonov, A. S., Kalinovsky, A. I., Pivkin, M. V., Mentinskaya, E. S., et al. (2012). New metabolites from the marine-derived fungus *Aspergillus fumigatus*. *Nat. Prod. Commun.* 7, 497–500.

Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perrucio, K., Elluru, S. R., et al. (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 460, 1117–1121. doi: 10.1038/nature08264

Alcazar-Fuoli, L., Cairns, T., Lopen, J. F., Zonja, B., Pérez, J., Barceló, D., et al. (2014). A modified recombineering protocol for the genetic manipulation of gene clusters in *Aspergillus fumigatus*. *PLoS ONE* 9:e111875. doi: 10.1371/journal.pone.0111875

Alhambra, A., Catalán, M., Moragues, M. D., Brena, S., Pontón, J., Montejo, J. C., et al. (2008). Isolation of *Aspergillus lentulus* in Spain from a critically ill patient with chronic obstructive pulmonary disease. *Rev. Iberoam. Micol.* 25, 246–249. doi: 10.1016/S1130-1406(08)70058-5

Álvarez-Pérez, S., Mellado, E., Serrano, P., Blanco, J. L., Garcia, M. E., Kwon, M., et al. (2014). Polyphasic characterization of fungal isolates from a published case of invasive aspergillosis reveals misidentification of *Aspergillus felis* as *Aspergillus viridinutans*. *J. Med. Microbiol.* 63, 617–619. doi: 10.1099/jmm.0.068502-0

Ames, B. D., and Walsh, C. T. (2010). Anthranilate-activating modules from fungal non-ribosomal peptide assembly lines. *Biochemistry* 49, 3351–3365. doi: 10.1021/bi100198y

Amitani, R., Taylor, G., Elezis, E. N., Llwellyn-Jones, C., Mitchell, J., Kuza, F., et al. (1995). Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. *Infect. Immun.* 63, 3266–3271.

Arabatsis, M., and Velegraki, A. (2013). Sexual reproduction cycle in the opportunistic human pathogen *Aspergillus terreus*. *Mycologia* 105, 71–79. doi: 10.3852/11-426

Atsumi, K., Takada, M., Mizuno, K., and Ando, T. (1970). Fumigachlorin, a new antifungal antibiotic. *J. Antibiot.* 23, 223–224. doi: 10.7164/antibiotics.23.223

Balajee, S. A., Gribskov, J., Brandt, M., Ito, J., Fothergill, A., and Marr, K. A. (2005b). Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. *J. Clin. Microbiol.* 43, 5996–5999. doi: 10.1128/JCM.43.12.5996-5999.2005

Balajee, S. A., Gibskov, J. L., Hanley, E., Nickle, C., and Marr, K. A. (2005a). *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryotic Cell* 4, 625–632. doi: 10.1128/EC.4.3.625-632.2005

Balajee, S. A., Nickle, D., Varga, J., and Marr, K. A. (2006). Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. *Eukaryotic Cell* 5, 1705–1712. doi: 10.1128/EC.00162-06

Balan, J., Ebringer, L., Nemec, P., Kovac, S., and Dobias, J. (1963). Antiprotozoal antibiotics. II. Isolation and characterization of trypacidin, a new antibiotic, active against *Trypanosoma cruzi* and *Toxoplasma gondii*. *J. Antibiot.* 16, 157–160.

Barrs, V. R., van Doorn, T. M., Houbraken, J., Kidd, S. E., Martin, P., Pinheiro, M. D., et al. (2013). *Aspergillus felis* sp. nov., an emerging agent of *aspergillosis* in humans, cats, and dogs. *PLOS ONE* 8:e64871. doi: 10.1371/journal.pone.0064871

Ben-Ami, R., Lewis, R. E., Leventakos, K., and Kontoyiannis, D. P. (2009). *Aspergillus fumigatus* inhibits angiogenesis through the production of gliotoxin and other secondary metabolites. *Blood* 114, 5393–5399. doi: 10.1182/blood-2009-07-231209

Bok, J. W., Chung, D., Balajee, S. A., Marr, K. A., Andes, D., Nielsen, K. F., et al. (2005). GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. *Infect. Immun.* 74, 6761–6768. doi: 10.1128/IAI.00780-06

Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. *Nat. Rev. Microbiol.* 11, 21–32. doi: 10.1038/nrmicro2916

Brakhage, A. A., and Schroeckh, V. (2011). Fungal secondary metabolites – strategies to activate silent gene clusters. *Fungal Genet. Biol.* 48, 15–22. doi: 10.1016/j.fgb.2010.04.004

Brillinger, G. U., Heberle, W., Weber, B., and Achenbach, H. (1978). Metabolic products of microorganisms. 167. Cyclopaldic acid from *Aspergillus duricaulis*. 1. Production, isolation and biological properties. *Arch. Microbiol.* 116, 245–252. doi: 10.1007/BF00417847

Buttachon, S., Chandrapatya, A., Manoch, L., Silva, A., Gales, L., Bruyère, C., et al. (2012). Sartorymensin, a new indole alkaloid, and new analogues of tryptoquivaline and fiscalins produced by *Neosartorya siamensis* (KUFC 6349). *Tetrahedron* 68, 3253–3262. doi: 10.1016/j.tet.2012.02-024

Cano, P. M., Jamin, E. L., Tadrist, S., Bourdau'hui, P., Pean, M., Debrauwer, L., et al. (2013). New untargeted metabolite profiling combining mass spectrometry and isotopic labelling: application to *Aspergillus fumigatus* grown on wheat. *Anal. Chem.* 85, 8412–8420. doi: 10.1021/ac401872f

Cerqueira, G. C., Arnaud, M. B., Inglis, D. O., Skrzypek, M. S., Binkley, G., Simison, M., et al. (2014). The *Aspergillus* genome database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations. *Nucleic Acids Res.* 42, D705–D710. doi: 10.1093/nar/gkt1029

Chooi, Y.-H., Fang, J., Liu, H., Filler, S. G., Wang, P., and Tang, Y. (2013). Genome mining of a prenylated and immunosuppressive polyketide from pathogenic fungi. *Org. Lett.* 15, 780–783. doi: 10.1021/ol303435y

Coelho, D., Silva, S., Vale-Silva, L., Gomes, H., Pinto, E., Sarmento, A., et al. (2011). *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. *Med. Mycol.* 49, 755–759. doi: 10.3109/13693786.2011.556672

Coleman, J. J., Ghosh, S., Okoli, I., and Mylonakis, E. (2011). Antifungal activity of microbial secondary metabolites. *PLoS ONE* 6:e25321. doi: 10.1371/journal.pone.0025321

Coméra, C., André, K., Lafitte, J., Collet, X., Galtier, P., and Maridonneau-Parini, I. (2007). Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and the organization of the actin cytoskeleton by distinct signaling pathways in human neutrophils. *Microb. Infect.* 9, 47–54. doi: 10.1016/j.micinf.2006.10.009

Coyle, C. M., Kenaley, S. C., Rittenour, W. R., and Panaccione, D. G. (1981). Association of ergot alkaloids with conidiation in *Aspergillus fumigatus*. *Mycologia* 99, 804–811. doi: 10.3852/mycologia.99.6.804

Cramer, R. A., Keats Shwab, E. Jr., and Keller, N. P. (2009). "Genetic regulation of *Aspergillus* secondary metabolites and their role in pathogenesis," in *Aspergillus fumigatus and Aspergillosis* eds J. P. Latgé and W. J. Steinbach (Washington, DC: ASM Press), 185–199. ISBN 978-1-55581-438-0

Cui, C. B., Kakeya, H., Okada, G., Onose, R., and Osada, H. (1996). Novel mammalian cell cycle inhibitor, tryprostatins A, B and other diketopiperazines produced by *Aspergillus fumigatus*. II. Physico-chemical properties and structures. *J. Antibiot.* 49, 527–533. doi: 10.7164/antibiotics.49.527

Cutler, H. G., Lauren, D. R., Wilkins, A. L., Holland, P. T., Hill, R. A., and Dugan, F. M. (1996). Ruakuric acid: a natural product from *Aspergillus fumigatus*. *Phytochemistry* 43, 209–214. doi: 10.1016/0031-9422(96)00224-5

Dhingra, S., Andes, D., and Calvo, A. M. (2012). VeA regulated conidiation, Gliotoxin production, and protease activity in the opportunistic human pathogen *Aspergillus fumigatus*. *Eukaryotic Cell* 11, 1531–1543. doi: 10.1128/EC.00222-12

Ding, G.-Z., Liu, J., Wang, J.-M., Faing, L., and Yi, S.-S. (2013). Secondary metabolites from the endophytic fungi *Penicillium polonicum* and *Aspergillus fumigatus*. *J. Asian Nat. Prod. Res.* 15, 446–452. doi: 10.1080/10286020.2013.780349

Eamvijarn, A., Gomes, N. M., Dethoup, T., Buaruang, J., Manoch, L., Manoch, L., et al. (2013a). Bioactive meroditerpenes and indole alkaloids from the

soil fungus *Neosartorya fischeri* (KUFC 6344), and the marine-derived fungi *Neosartorya laciniosa* (KUFC 7896) and *Neosartorya tsunodae* (KUFC 9213). *Tetrahedron* 69, 8583–8591. doi: 10.1016/j.tet.2013.07.078

Eamvijarn, A., Kijjoa, A., Bruyère, C., Mathieu, V., Manoch, L., Lefranc, F., et al. (2012). Secondary metabolites from a culture of the fungus *Neosartorya pseudofischeri* and their *in vitro* cytostatic activity in human cancer cells. *Planta Med.* 78, 1767–1776. doi: 10.1055/s-0032-1315301

Eamvijarn, A., Manoch, L., Chamswarng, C., Piasai, O., Visarathanonth, N., Langsa-ard, J. J., et al. (2013b). *Aspergillus siamensis* sp. nov. from soil in Thailand. *Mycoscience* 54, 401–405. doi: 10.1016/j.myc.2013.01.005

Ellis, J. J., Stodola, F. H., Vesonder, R. F., and Glass, C. A. (1964). $C_{15}H_{22}O_4$ compound produced by the fungus *Aspergillus fischeri* var. *glaber*. *Nature* 203:1382. doi: 10.1038/2031382a0

Fallon, J. P., Reeves, E. P., and Kavanagh, K. (2010). Inhibition of neutrophil function following exposure to the *Aspergillus fumigatus* toxin fumagillin. *J. Med. Microbiol.* 59, 625–633. doi: 10.1099/jmm.0.018192-0

Fallon, J. P., Reeves, E. P., and Kavanagh, K. (2011). The *Aspergillus fumigatus* toxin fumagillin suppresses the immune response of *Galleria mellonella* larvae by inhibiting the action of haemocytes. *Microbiology* 157, 1481–1488. doi: 10.1099/mic.0.043786-0

Fedorova, N. D., Khaldi, N., Joarder, V. S., Maiti, R., Amedeo, P., Anderson, M. J., et al. (2008). Genomic islands in in the pathogenic filamentous fungus *Aspergillus fumigatus*. *PLoS Genet.* 4:e1000046. doi: 10.1371/journal.pgen.1000046

Frisvad, J. C. (2012). "Media and growth conditions for induction of secondary metabolites," in *Fungal Secondary Metabolism: Methods and Protocols* eds N. P. Keller and G. Turner (New York, NY: Humana Press), 47–58.

Frisvad, J. C., Andersen, B., and Thrane, U. (2008). The use of secondary metabolite profiling in fungal taxonomy. *Mycol. Res.* 112, 231–240. doi: 10.1016/j.mycres.2007.08.018

Frisvad, J. C., and Larsen, T. O. (2015). Chemodiversity in the genus *Aspergillus*. *Appl. Microbiol. Biotechnol.* 99, 7859–7877. doi: 10.1007/s00253-015-6839-z

Frisvad, J. C., Rank, C., Nielsen, K. F., and Larsen, T. O. (2009). Metabolomics of *Aspergillus fumigatus*. *Med. Mycol.* 47, S53–S71. doi: 10.1080/13693780802307720

Frisvad, J. C., and Samson, R. A. (1990). "Chemotaxonomy and morphology of *Aspergillus fumigatus* and related species," in *Modern Concepts in Penicillium and Aspergillus Classification* eds R. A. Samson and J. I. Pitt (New York, NY: Plenum Press), 201-208.

Fujimoto, H., Negishi, E., Yamaguchi, K., Nishi, N., and Yamazaki, M. (1996). Isolation of new tremorgenic metabolites from an Ascomycete, *Corynascus setosus*. *Chem. Pharm. Bull.* 44, 1843–1848. doi: 10.1248/cpb.44.1843

Furtado, N. A. J. C., Pupo, M. T., Carvalho, I., Campo, V. L., Duarte, M. C. T., and Bastos, J. K. (2005). Diketopiperazines produced by an *Aspergillus fumigatus* Brazilian strain. *J. Braz. Chem. Soc.* 16, 1448–1453. doi: 10.1590/S0103-50532005000800026

Galaghan, J. E., Calvo, S. E., Cuomo, C., Ma, L. J., Wortman, J. R., Batzoglou, S., et al. (2005). Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* 438, 1105–1115. doi: 10.1038/nature04341

Gauthier, T., Wang, X., Dos Santos, J. S., Fysikopoulos, A., Tadrist, S., Canlet, C., et al. (2012). Trypacidin, a spore-borne toxin from *Aspergillus fumigatus*, is cytotoxic to lung cells. *PLoS ONE* 7:e29906. doi: 10.1371/journal.pone.0029906

Geiser, D. M., Frisvad, J. C., and Taylor, J. W. (1998). Evolutionary relationships in *Aspergillus* section *Fumigati* inferred from partial beta-tubulin and hydrophobin DNA sequences. *Mycologia* 90, 832–846. doi: 10.2307/3761325

Geiser, D. M., Klich, M. A., Frisvad, J. C., Peterson, S. W., Varga, J., and Samson, R. A. (2007). The current status of species recognition and identification in *Aspergillus*. *Stud. Mycol.* 59, 1–10. doi: 10.3114/sim.2007.59.01

Gomes, N. M., Bessa, L. J., Buttachon, S., Costa, P. M., Buaruang, J., Dethoup, T., et al. (2014). Antibacterial and antibiofilm activities of tryptoquivalines and meroditerpenes isolated from the marine-derived fungi *Neosartorya paulistensis, N. laciniosa, N. tsunodae,* and the soil fungi *N. fischeri* and *N. siamensis*. *Mar. Drugs* 12, 822–839. doi: 10.3390/md12020822

Greiner, K., Peršoh, D., Weig, A., and Rambold, G. (2014). *Phialosimplex salinarum*, a new species of *Eurotiomycetes* from a hypersaline habitat. *IMA Fungus* 5, 161–172. doi: 10.5598/imafungus.2014.05.02.01

Guarro, J., Kallas, E. G., Godoy, P., Karenina, A., Gene, J., Stchigel, A., et al. (2002). Cerebral aspergillosis caused by *Neosartorya hiratsukae* infection. *Emerging Infect. Dis.* 8, 989–991. doi: 10.3201/eid0809.020073

Gyotoku, H., Izumikawa, K., Ikeda, H., Takazono, T., Morinaga, Y., Nakamura, S., et al. (2012). A case of bronchial aspergillosis caused by *Aspergillus udagawae* and its mycological features. *Med. Mycol.* 50, 631–636. doi: 10.3109/13693786.2011.639036

Haas, H. (2014). Fungal siderophore metabolism with a focus on *Aspergillus fumigatus*. *Nat. Prod. Rep.* 31, 1266–1276. doi: 10.1039/C4NP00071D

Han, X., Xu, X., Cui, C., and Gu, Q. (2007a). Alkaloidal compounds produced by a marine-derived fungus, *Aspergillus fumigatus* H1-04, and their antitumor activities. *Chin. J. Med. Chem.* 17, 232–237.

Han, X., Xu, X., Cui, C., and Gu, Q. (2007b). Diketopiperazines produced by a marine-derived fungus, *Aspergillus fumigatus* H1-04, and their antitumor activities. *Chin. J. Med. Chem.* 17, 155–159.

Harms, H., Rempel, V., Kehraus, S., Kaiser, M., Hufendick, P., Müller, C. E., et al. (2014). Indoloterpenes from a marine-derived fungal strain of *Dichotomomyces cejpii* with an antagonistic activity at GPR18 and cannabinoid receptors. *J. Nat. Prod.* 77, 673–677. doi: 10.1021/np400850g

Hawksworth, D. L. (2012). Managing and coping with names of pleomorphic fungi in a period of transition. *IMA Fungus* 3, 15–24. doi: 10.5598/imafungus.2012.03.01.03

Hawksworth, D. L., Crous, P. W., Redhead, S. A., Reynolds, D. R., Samson, R. A., Seifert, K. A., et al. (2011). The Amsterdam declaration on fungal nomenclature. *IMA Fungus* 2, 105–112. doi: 10.5598/imafungus.2011.02.01.14

Hayashi, A., Artai, M., Fujita, M., and Kobayashi, M. (2009). Pyripyropens, fungal sesquiterpenes conjugated with alpha-pyrone and pyridine moieties, exhibits anti-angiogenicitivity against human umbilical vein endothelial cells. *Biol. Pharm. Bull.* 32, 1261–1265. doi: 10.1248/bpb.32.1261

Hong, S. B., Cho, H. S., Shin, H. D., Frisvad, J. C., and Samson, R. A. (2006). New *Neosartorya* species isolated from soil in Korea. *Int. J. Syst. Evol. Microbiol.* 56, 439–442. doi: 10.1099/ijs.0.63980-0

Hong, S. B., Go, S. J., Shin, H. D., Frisvad, J. C., and Samson, R. A. (2005). Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* 97, 1316–1329. doi: 10.3852/mycologia.97.6.1316

Hong, S. B., Kim, D. H., Park, I. C., Choi, Y. J., Shin, H. D., and Samson, R. A. (2010a). Re-identification of *Aspergillus fumigatus sensu lato* based on a new concept of species delimitation. *J. Microbiol.* 48, 607–615. doi: 10.1007/s12275-010-0084-z

Hong, S. B., Kim, D.-H., Park, I. C., Samson, R. A., and Shin, H. D. (2010b). Isolation and identification of *Aspergillus* section *Fumigati* strains from arable soil in Korea. *Mycobiology* 38, 1–6. doi: 10.4489/MYCO.2010.38.1.001

Hong, S. B., Shin, H. D., Hong, J., Frisvad, J. C., Nielsen, P. V., et al. (2008). New taxa of *Neosartorya* and *Aspergillus* in *Aspergillus* section *Fumigati*. *Antonie van Leeuwenhoek* 93, 87–98. doi: 10.1007/s10482-007-9183-1

Horie, Y., Abliz, P., Fukushima, K., Okada, K., and Takaki, G. M. C. (2003). Two new species of *Neosartorya* from Amazonian soil, Brazil. *Mycoscience* 44, 397–402. doi: 10.1007/S10267-003-0132-1

Horie, Y., Miyaji, M., Nishimura, K., Franco, M. F., and Colho, K. I. R. (1995). Two new species of *Neosartorya* from Brazilian soil. *Mycoscience* 36, 159–165. doi: 10.1007/BF02268552

Horn, B. W., Moore, G. G., and Carbone, I. (2009). Sexual reproduction in *Aspergillus flavus*. *Mycologia* 103, 174–183. doi: 10.3852/10-115

Horn, B. W., Olarte, R. A., Peterson, S. W., and Carbone, I. (2013). Sexual reproduction in *Aspergillus tubingensis* from section Nigri. *Mycologia* 105, 1153–1163. doi: 10.3852/13-101

Hosoe, T., Mori, N., Kamano, K., Itabashi, T., Yaguchi, T., and Kawai, K. (2011). A new antifungal yellow pigment from *Aspergillus nishimurae*. *J. Antibiot.* 64, 211–212. doi: 10.1038/ja.2010.132

Hosoe, T., Moriyama, H., Wakana, D., Itabashi, T., Kawai, K., Yaguchi, T., et al. (2009). Inhibitory effects of dihydroterrein and terrain isolated from *Aspergillus novofumigatus* on platelet aggregation. *Mycotoxins* 59, 75–82. doi: 10.2520/myco.59.75

Houbraken, J., de Vries, R. P., and Samson, R. A. (2014). Modern taxonomy of biotechnologically important *Aspergillus* and *Penicillium* species. *Adv. Appl. Microbiol.* 86, 199–249. doi: 10.1016/B978-0-12-800262-9.00004-4

Houbraken, J., and Samson, R. A. (2011). Phylogeny of *Penicillium* and the segregation of *Trichocomaceae* into three families. *Stud. Mycol.* 70, 1–51. doi: 10.3114/sim.2011.70.01

Howard, S. J. (2014). Multiresistant aspergillosis due to cryptic species. *Mycopathologia* 178, 427–433. doi: 10.1007/s11046-014-9774-0

Hubka, V., Peterson, S. W., Frisvad, J. C., Yaguchi, T., Kubátová, A., Kolaøik, M., et al. (2013). *Aspergillus* waksmanii sp. nov. and *Aspergillus* marvanovae sp. nov., two new closely related species in section *Fumigati*. *Int. J. Syst. Evol. Microbiol.* 63, 763–789. doi: 10.1099/ijs.0.047076-0

Inglis, D. O., Binkley, J., Skrzypek, M. S., Arnaud, M. B., Cerqueira, G. C., Shah, P., et al. (2013). Comprehensive annotation of secondary biosynthetic genes and gene clusters of *Aspergillus nidulans*, *A. fumigatus*, *A. niger* and *A. oryzae*. *BMC Microbiol.* 13:91. doi: 10.1186/1471-2180-13-91

Ishikawa, K., Hosoe, T., Itabashi, T., Sato, F., Wachi, H., Nagase, H., et al. (2011). Quinazolinobenzodiazepine derivatives, novobenzomalvins A-C: Fibronectin expression regulators from *Aspergillus novofumigatus*. *Sci. Pharm.* 79, 937–950. doi: 10.3797/scipharm.1106-21

Ishikawa, K., Hosoe, T., Itabashi, T., Takizawa, K., Yaguchi, T., and Kawai, K. (2010b). Novofumigatamide, new cyclic tripeptide from *Aspergillus novofumigatus*. *Heterocycles* 81, 2143–2148. doi: 10.3987/COM-10-12005

Ishikawa, K., Hosoe, T., Itabashi, T., Wakana, D., Takizawa, K., Yuguchi, T., et al. (2010a). Novoamauromine and *ent*-cycloechinulin: two new diketopiperazine derivatives from *Aspergillus novofumigatus*. *Chem. Pharm. Bull.* 58, 717–719. doi: 10.1248/cpb.58.717

Ishikawa, M., Nionomiya, T., Akabane, H., Kushida, N., Tsujiuchi, G., Ohyama, M., et al. (2009). Pseurotin A and its analogues as inhibitors of immunoglobulin E production. *Bioorg. Med. Chem. Lett.* 19, 1457–1460. doi: 10.1016/j.bmcl.2009.01.029

Jahn, B., Boukhallouk, F., Lotz, J., Langfelder, K., Wanner, G., and Brakhage, A. A. (2000). Interaction of human phagocytes with pigmentless *Aspergillus* conidia. *Infect. Immun.* 68, 3736–3739. doi: 10.1128/IAI.68.6.3736-3739.2000

Janardhanan, K. K., Satta, A., and Husain, A. (1984). Production of fumigaclavine by *Aspergillus tamarii* Kita. *Can. J. Microbiol.* 30, 247–250. doi: 10.1139/m84-036

Jayasuriya, H., Zink, D., Basilio, A., Vicente, F., Collado, J., Bills, G., et al. (2009). Discovery and antibacterial activity of glabramycin A-C from *Neosartorya glabra* by an antisense strategy. *J. Antibiot.* 62, 265–269. doi: 10.1038/ja.2009.26

Kaifuchi, S., Mori, M., Nonaka, K., Musauma, R., Omura, S., Shiomi, K., et al. (2015). Sartorypyrone D: a new NADH-fumarate reductase inhibitor produced by *Neosartorya fischeri* FO-5897. *J. Antibiot.* 68, 403–405. doi: 10.1038/ja.2014.167

Kamei, K., and Watanabe, A. (2005). *Aspergillus* mycotoxins and their effects on the host. *Med. Mycol.* 43, S95–S99. doi: 10.1080/13693780500051547

Kang, D., Son, G. H., Park, H. M., Kim, J., Choi, J. N., Kim, H. Y., et al. (2013). Culture condition-dependant metabolite profiling of *Aspergillus fumigatus* with antifungal activity. *Fungal Biol.* 117, 211–219. doi: 10.1016/j.funbio.2013.01.009

Kano, R., Itamoto, K., Okuda, M., Inokuma, H., Hasegawa, A., and Balajee, S. A. (2008). Isolation of *Aspergillus udagawae* from a fatal case of feline orbital aspergillosis. *Mycoses* 51, 360–361. doi: 10.1111/j.1439-0507.2008.01493.x

Kano, R., Shinahashi, A., Fujino, Y., Sakai, H., Mori, T., Tsujimoto, H., et al. (2013). Two cases of feline orbital aspergillosis due to *Aspergillus udagawae* and *A. viridinutans*. *J. Vet. Med. Sci.* 75, 7–10. doi: 10.1292/jvms.12-0119

Kano, R., Takahashi, T., Hayawaka, T., Yamayaka, Y., Hasegawa, A., and Kamata, H. (2015). The first case of feline sinonasal aspergillosis due to *Aspergillus fischeri* in Japan. *J. Vet. Med. Sci.* 77, 1183–1185. doi: 10.1292/jvms.14-0454

Kanokmedhakul, K., Kanokmedhakul, S., Suwannatrai, R., Soytong, K., Prabpai, S., and Kongsaeree, P. (2011). Bioactive meroterpenoids and alkaloids from the fungus *Eurotium chevalieri*. *Tetrahedron* 67, 5461–5468. doi: 10.1016/j.tet.2011.05.066

Kao, R., Martinez-Ruiz, A., del Poozo, A. M., Crameri, R., and Davies, J. (2001). Mitogillin and related fungal ribotoxins. *Meth. Enzymol.* 34, 324–335. doi: 10.1016/S0076-6879(01)41161-X

Katz, M. E., Dougall, A. M., Weeks, K., and Cheetham, B. F. (2005). Multiple genetically distinct groups revealed among clinical isolates identified as atypical *Aspergillus fumigatus*. *J. Clin. Microbiol.* 43, 551–555. doi: 10.1128/JCM.43.2.551-555.2005

Kaur, A., Swenson, D. C., Wicklow, D. T., and Gloer, J. B. (2013). New fiscalin, tryptoquivaline, and fumiquinazoline analogues from an endophytic isolate of *Neosartorya aureola*. *Planta Med.* 79, 852. doi: 10.1055/s-0033-1348666

Kelman, B. J., Robbins, C. A., Swenson, L. J., and Hardin, B. D. (2004). Risk from inhaled mycotoxins in indoor office and residential environments. *Int. J. Toxicol.* 23, 3–10. doi: 10.1080/10915810490265423

Khare, R., Gupta, S., Arif, S., Jentoft, M. E., Denziel, P. H., Roden, A. C., et al. (2014). Misidentification of *Neosartorya pseudofischeri* as *Aspergillus fumigatus* in a lung transplant patient. *J. Clin. Microbiol.* 52, 2722–2725. doi: 10.1128/JCM.00216-14

Khoufache, K., Puel, O., Loiseau, N., Delaforge, M., Rivollet, D., Coste, A., et al. (2007). Verruculogen associated with *Aspergillus fumigatus* hyphae and conidia modifies the electrophysiological properties of human nasal epithelial cells. *BMC Microbiol.* 25:5. doi: 10.1186/1471-2180-7-5

Kijjoa, A., Santos, S., Dethoup, T., Manoch, L., Almeida, A. P., Vasconselos, M. H., et al. (2011). Sartoryglabrins, analogs of ardeemins, from *Neosartorya glabra*. *Nat. Prod. Commun.* 6, 807–812.

Kim, Y.-J., Park, H. B., Yoo, J.-H., Kwon, H. C., Kim, J., and Yang, H. O. (2014). Glionitrin A, a new diketopiperazine disulphide, asctivates ATM-ATR-Chk1/2 via 53BP1 phosphorylation in DU145 cells and shows antitumor effect in xenograft model. *Biol. Pharm. Bull.* 37, 378–386. doi: 10.1248/bpb.b13-00719

Kupfahl, C., Michalka, A., Lass-Flörl, C., Fischer, G., Haase, G., Ruppert, T., et al. (2008). Gliotoxin production by clinical and environmental *Aspergillus fumigatus* strains. *Int. J. Med. Microbiol.* 298, 319–327. doi: 10.1016/j.ijmm.2007.04.006

Kusuya, Y., Takahashi-Nakaguchi, A., Takahashi, H., and Yaguchi, T. (2015). Draft genome sequence of the pathogenic filamentous fungus *Aspergillus udagawae* strain IFM 46973^T. *Genome Announc.* 3, e00834–e00815. doi: 10.1128/genomeA.00834-15

Kwon, Y.-J., Sohn, M.-J., Zheng, C.-J., and Kim, W.-G. (2007). Fumimycin: a peptide deformylase inhibitor with an unusual skeleton produced by *Aspergillus fumisynnematus*. *Org. Lett.* 9, 2449–2451. doi: 10.1021/ol0703231

Land, C. J., Lundström, H., and Werner, S. (1993). Production of tremorgenic mycotoxins by isolates of *Aspergillus fumigatus* from sawmills in Sweden. *Mycopathologia* 124, 87–93. doi: 10.1007/BF01103107

Langarica-Fuentes, A., Handley, P. S., Houlden, A., Fox, G., and Robson, G. D. (2014). An investigation of the biodiversity of thermophilic and thermotolerant fungal species in composts using culture-based and molecular techniques. *Fungal Ecol.* 11, 132–144. doi: 10.1016/j.funeco.2014.05.007

Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhaage, A. A. (2003). Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genet. Biol.* 38, 143–158. doi: 10.1016/S1087-1845(02)00526-1

Larsen, T. O., Smedsgaard, J., Nielsen, K. F., Hansen, M. E., and Frisvad, J. C. (2005). Phenotypic taxonomy and metabolite profiling in microbial drug discovery. *Nat. Prod. Rep.* 22, 672–695. doi: 10.1039/b404943h

Larsen, T. O., Smedsgaard, J., Nielsen, K. F., Hansen, M. E., Samson, R. A., and Frisvad, J. C. (2007). Production of mycotoxins by *Aspergillus lentulus* and other medically important and closely related species in section *Fumigati*. *Med. Mycol.* 45, 225–232. doi: 10.1080/13693780601185939

Latgé, J. P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12, 310–350.

Lee, M. Y., Park, H. M., Son, G. H., and Lee, C. H. (2013). Liquid chromatography-mass spectrometry based chemotaxonomic classification of *Aspergillus* species and evaluation of the biological activity of its unique metabolite neosartorin. *J. Microbiol. Biotechnol.* 23, 932–941. doi: 10.4014/jmb.1212.12068

Lewis, R. E., Wiederhold, N. P., Lionakis, M. S., Prince, R. A., and Kontoyiannis, D. P. (2005). Frequency and species distribution of gliotoxin-producing *Aspergillus* isolates recovered from patients at a tertiary-care cancer center. *J. Clin. Microbiol.* 43, 6120–6122. doi: 10.1128/JCM.43.12.6120-6122.2005

Li, D. M., Horie, Y., Wang, Y., and Li, R. (1998). Three new *Aspergillus* species isolated from clinical sources as a causal agent of human aspergillosis. *Mycoscience* 39, 299–305. doi: 10.1007/BF02464012

Li, Y.-X., Himaya, S. W. A., Dewapriya, P., Kim, H. J., and Kim, S. K. (2014). Anti-proliferative effects of isosclerone from marine fungus *Aspergillus fumigatus* in MCF-7 human breast cancer cells. *Process Biochem.* 49, 2292–2298. doi: 10.1016/j.procbio.2014.08.016

Liang, W.-L., Le, X., Li, H.-J., Yang, X.-L., Chen, J.-X., Xu, J., et al. (2014). Exploring the chemodiversity and biological activities of the secondary metabolites from

the marine fungus *Neosartorya pseudofischeri*. *Mar. Drugs* 12, 5657–5676. doi: 10.3390/md12115657

Liang, Z., Zhang, T., Zhang, X., Zhang, J., and Zhao, C. (2015). An alkaloid and a steroid from the endophytic fungus *Aspergillus fumigatus*. *Molecules* 20, 1424–1433. doi: 10.3390/molecules20011424

Lillehoj, E. B., and Milburn, M. S. (1973). Viriditoxin production by *Aspergillus viridi-nutans* and related species. *Appl. Microbiol.* 26, 202–205.

Lim, F. Y., Ames, B., Walsch, C. T., and Keller, N. P. (2014). Co-ordination between BrlA regulation and secretion of the oxidoreductase FmqD directs selective accumulation of fumiquinazoline C to conidial tissues in *Aspergillus fumigatus*. *Cell. Microbiol.* 16, 1267–1283. doi: 10.1111/cmi.12284

Lin, H.-C., Chooi, Y.-H., Dhingra, S., Xu, W., Calvo, A. M., and Tang, Y. (2013). The fumagillin biosynthetic gene cluster in *Aspergillus fumigatus* encodes a cryptic terpene cyclase involved in the formation of β-trans-bergamotene. *J. Am. Chem. Soc.* 135, 4616–4619. doi: 10.1021/ja312503y

Lind, A. L., Wisecaver, J. H., Smith, T. D., Feng, X., Calvo, A. M., and Rokas, A. (2015). Examining the evolution of the regulatory circuit controlling secondary metabolism and development in the fungal genus *Aspergillus*. *PLoS Genet.* 11:e1005096. doi: 10.1371/journal.pgen.1005096

Liu, J., Wei, X., Kim, E. L., Lin, X., Yang, X.-W., Zhou, X., et al. (2015). New glucosidated pyrazinoquinazoline indole alkaloids from the fungus *Aspergillus fumigatus* derived from a jellyfish. *Tetrahedron* 71, 271–275. doi: 10.1016/j.tet.2014.11.063

MacHeleidt, J., Scherlach, K., Neuwirth, T., Schmidt-Heck, W., Strassburger, M., Spraker, J., et al. (2015). Transcriptome analysis of cyclic AMP-dependent protein kinase A-regulated genes reveals the production of the novel natural compound fumipyrrole by *Aspergillus fumigatus*. *Mol. Microbiol.* 96, 148–162. doi: 10.1111/mmi.12926

Macías, F. A., Varela, R. M., Simonet, A. M., Cutler, H. G., Cutler, S. J., and Hill, R. A. (2003). Absolute configuration of bioactive expansolides A and B from *Aspergillus fumigatus* Fresenius. *Tetrahedron Lett.* 44, 941–943. doi: 10.1016/S0040-4039(02)02778-8

Malejczyk, K., Sigler, L., Gibas, G. F. C., and Smith, S. W. (2013). Invasive sino-orbital mycosis in an aplastic anaemia patient caused by *Neosartorya laciniosa*. *J. Clin. Microbiol.* 51, 1316–1319. doi: 10.1128/JCM.02919-12

Masi, M., Andolfi, A., Mathieu, V., Boari, A., Cimmino, A., Y., Banuls, L. M. et al. (2013). Fischerindoline, a pyrrolindole sesquiterpenoid isolated from *Neosartorya pseudofischeri*, with *in vitro* growth inhibitory activity in human cancer cell lines. *Tetrahedron* 69, 7466–7470. doi: 10.1016/j.tet.2013.06.031

Matsusawa, T., Horie, Y., Abliz, P., Gonoi, T., and Yaguchi, T. (2014a). *Aspergillus huiyaniae* sp. nov., a teleomorphic species in sect. *Fumigati* isolated from desert soil in China. *Mycoscience* 55, 213–220. doi: 10.1016/j.myc.2013.08.007

Matsusawa, T., Takaki, G. M. C., Yaguchi, T., Okada, K., Abliz, P., Gonoi, T., et al. (2015). *Aspergillus arcoverdensis*, a new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in the state of Pernambuco, Brazil. *Mycoscience* 56, 123–131. doi: 10.1016/j.myc.2014.04.006

Matsusawa, T., Takaki, G. M. C., Yaguchi, T., Okada, K., Gonoi, T., and Horie, Y. (2014b). Two new species of *Aspergillus* section *Fumigati* isolated from caatinga soil in the state of Pernambuco, Brazil. *Mycoscience* 55, 79–88. doi: 10.1016/j.myc.2013.04.001

McNeill, J., Barrie, F. R., Buck, W. R., Demoulin, V., Greuter, W., Gams, W., et al. (2012). *International Code of Nomenclature for Algae, Fungi and Plants (Melbourne Code) Adopted by the 18th International Botanical Congress, Melbourne, Australia, Jul 2011. Regnum Vegetabile 154*. Koenigstein: Koeltz Scientific Books.

McDonagh, A., Fedorova, N. D., Crabtree, J., Yu, Y., Kim, S., Chen, D., et al. (2008). Sub-telomere directed gene expression during initiation of invasive aspergillosis. *PLoS Pathog.* 4:e1000154. doi: 10.1371/journal.ppat.1000154

Miao, Y.-Z., Liu, D.-Y., Li, G.-Q., Li, P., Xu, Y.-C., Shen, Q.-R., et al. (2015). Genome-wide transcriptomic analysis of a superior biomass-degrading strain of *Aspergillus fumigatus* releated active lignocellulose-degrading genes. *BMC Genomics* 16:459. doi: 10.1186/s12864-015-1658-2

Morino, T., Nishimoto, M., Itou, N., and Nishikiori, T. (1994). NK372135s, novel antifungal agents produced by *Neosartorya fischeri*. *J. Antibiot.* 47, 1546–1548. doi: 10.7164/antibiotics.47.1546

Mühlenfeld, A., and Achenbach, H. (1988a). Asperpentyn, a novel acetylenic cyclohexene epoxide from *Aspergillus duricaulis*. *Phytochemistry* 27, 3853–3855.

Mühlenfeld, A., and Achenbach, H. (1988b). Stofwechselprodukte von mikroorganismen. 35 mitt. Asperdurin, ein neues antimykotisches phthalide aus *Aspergillus duricaulis*. *Archiv der Pharmazie (Weinheim)* 321, 803–805.

Netzker, T., Fischer, J., Weber, J., Mattem, D. J., Konig, C. C., Valiante, V., et al. (2015). Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Front. Microbiol.* 6:299. doi: 10.3389/fmicb.2015.00299

Nielsen, M. L., Nielsen, J. B., Rank, C., Klejnstrup,. M. L., Holm, D. M. K., Brogaard, K. H., et al. (2011). A genome-wide polyketide synthase deletion library uncovers novel genetic links to polyketides and meroterpenoids in *Aspergillus nidulans*. *FEMS Microbiol. Lett.* 321, 157–166. doi: 10.1111/j.1574-6968.2011.02327.x

Nierman, W. C., Pain, A., Anderson, M. J., Wortman, J. R., Kim, H. S., Arroyo, J., et al. (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* 438, 1151–1156. doi: 10.1038/nature04332

Novaková, A., Hubka, V., Dudova, Z., Matsuzawa, T., Kubatová, A., Yaguchi, T., et al. (2014). New species in *Aspergillus* section *Fumigati* from reclamation sites in Wyoming (USA) and revision of *A. viridinutans complex*. *Fungal Divers.* 64, 253–274. doi: 10.1007/s13225-013-0262-5

O'Gorman, C. M., Fuller, H. T., and Dyer, P. S. (2009). Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* 457, 471–474. doi: 10.1038/nature07528

Ohashi, H., Ishikawa, M., Ito, J., Ueno, A., Gleich, G. J., Kita, H., et al. (1997). Sulochrin inhibits eosinophil degranulation. *J. Antibiot.* 50, 972–974. doi: 10.7164/antibiotics.50.972

Ohashi, H., Motegi, Y., Kitas, H., Gleich, G. J., Miura, T., Ishikawa, M., et al. (1998). Sulochrin inhibits eosinophil activation and chemotaxis. *Inflamm. Res.* 47, 409–415. doi: 10.1007/s000110050352

O'Keeffe, G., Hammel, S., Owens, R. A., Keane, T. M., FitzPatrick, O. A., Jones, G. W., et al. (2014). RNA-sequencing reveals the pan-transcriptomic impact of attenuating the gliotoxin self-protection mechanism in *Aspergillus fumigatus*. *BMC Genomics* 15:894. doi: 10.1186/1471-2164-15-894

Ola, A. R. B., Debbab, A., Aly, A. H., Mandi, A., Zerfass, I., Hamacher, A., et al. (2014). Absolute configuration and antibiotic activity of neosartorin from the endophytic fungus *Aspergillus fumigatiaffinis*. *Tetrahedron Lett.* 55, 1020–1023. doi: 10.1016/j.tetlet.2013.12.070

Omolo, J. O., Anke, H., Chhabara, S., and Sterner, O. (2000). New variotin analogues from *Aspergillus viridi-nutans*. *J. Nat. Prod.* 63, 975–977. doi: 10.1021/np990509b

Owens, K. A., Hammel, S., Sheridan, K. J., Jones, G. W., and Doyle, S. (2014). A proteomic approach to investigating gene cluster expression and secondary metabolite functionality in *Aspergillus fumigatus*. *PLoS ONE* 9:e106942. doi: 10.1371/journal.pone.0106942

Ozoe, Y., Kuriyama, T., Tachibana, Y., Hariyama, K., Takahashi, N., Yuguchi, T., et al. (2004). Isocoumarin derivative as a novel GABA receptor ligand from *Neosartorya quadricincta*. *J. Pestic. Sci.* 29, 328–333. doi: 10.1584/jpestics.29.328

Panda, A., Ghosh, A. K., Mirdha, B. R., Xess, I., Paul, S., Samanaray, J. C., et al. (2015). MALDI-TOF mass spectrometry for rapid identification of clinical fungal isolates based on ribosomal protein markers. *J. Microbiol. Methods* 109, 93–105. doi: 10.1016/j.mimet.2014.12.014

Pedersen, M. H., Borodina, I., Moresco, J. L., Svendsen, W. E., Frisvad, J. C., and Søndergaard, I. (2011). High-yield production of hydrophobins RodA and RodB from *Aspergillus fumigatus* in *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* 90, 1923–1932. doi: 10.1007/s00253-011-3235-1

Peláez, T., Álvarez-Perez, S., Mellado, E., Serrano, D., Valerio, M., Blanco, J. L., et al. (2013). Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukemia. *J. Med. Mycol.* 62, 474–478. doi: 10.1099/jmm.0.044867-0

Peng, J., Lin, T., Wang, W., Xin, Z., Zhu, T., Gu, Q., et al. (2013). Antiviral alkaloids produced by the mangrove-derived fungus *Cladosporium* sp. PJX-41. *J. Nat. Prod.* 76, 1133–1140. doi: 10.1021/np400200k

Perrin, R. M., Fedorova, N. D., Bok, J. W., Cramer, R. A. Jr., Wortman, J. R., Kim, H. S. et al. (2007). Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. *PLoS Pathog.* 3:e50. doi: 10.1371/journal.ppat.0030050

Petrik, M., Haas, J. H., Laverman, P., Schrettl, M., Franssen, G. M., Blatzer, M., et al. (2014). ^{68}Ga-triacetylfusarinine C and ^{68}Ga-ferrioxamine E for *Aspergillus* infection imaging: Uptake specificity in various microorganisms. *Mol. Imag. Biol.* 16, 102–108. doi: 10.1007/s11307-013-0654-7

Posteraro, B., Mattei, R., Trivella, F., Maffei, A., Torre, A., De Carolis, E., et al. (2011). Uncommon *Neosartorya udagawae* fungus as a causative agent of severe corneal infection. *J. Clin. Microbiol.* 49, 2357–2360. doi: 10.1128/JCM.00134-11

Prompanya, C., Dethoup, T., Bessa, L. J., Pinto, M. M. M., Gales, L., Costa, P. M., et al. (2014). New isocoumarin derivatives and meroterpenoids from the marine sponge-associated fungus *Aspergillus similanensis*. *Mar. Drugs* 12, 5160–5173. doi: 10.3390/md12105160

Prompanya, C., Fernandes, C., Cravo, S., Pinto, M. M. M., Dethoup, T., Silva, A. M. S., et al. (2015). A new cyclic hexapeptide and a new isocoumarin derivative from the marine sponge-associated fungus *Aspergillus similanensis* KUFA 0013. *Mar. Drugs* 13, 1432–1450. doi: 10.3390/md13031432

Qiao, M.-F., Ji, N.-Y., Liu, X.-H., Li, F., and Xue, Q.-Z. (2010b). Asporyergosterol, a new steroid from an algiocolous isolate of *Aspergillus oryzae*. *Nat. Prod. Commun.* 5, 1575–1578.

Qiao, M.-F., Ji, N.-Y., Liu, X.-H., Li, K., Zhu, Q.-M., Xue, Q.-Z., et al. (2010a). Indoloterpenes from an algicolous isolate of *Aspergillus oryzae*. *Biorg. Med. Chem. Lett.* 20, 5677–5680. doi: 10.1016/j.bmcl.2010.08.024

Rabindran, S. K., Ross, D. D., Doyle, L. A., Yang, W., and Greenberger, L. M. (2000). Fumitremorgin C reverses multidrug resistance in cells transfected with the cancer resistance protein. *Cancer Res.* 60, 47–50.

Rank, C., Phipps, R. K., Harris, P., Frisvad, J. C., Gotfredsen, C. H., and Larsen, T. O. (2006). *Epi*-aszonalenin A, B, and C from *Aspergillus novofumigatus*. *Tetrahedron Lett.* 47, 6099–6102. doi: 10.1016/j.tetlet.2006.06.086

Rank, C., Phipps, R. K., Harris, P., Fristrup, P., Larsen, T. O., and Gotfredsen, C. H. (2008). Novofumigatonin, a new orthoester meroterpenoid from *Aspergillus novofumigatus*. *Org. Lett.* 10, 401–404. doi: 10.1021/ol7026834

Rank, C., Klejnstrup, M. L., Petersen, L. M., Kildegaard, S., Frisvad, J. C., Gotfredsen, C. H., et al. (2012). Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357). *Metabolites* 2, 39–56. doi: 10.3390/metabo2010039

Raper, K. B., and Fennell, D. I. (1965). *The Genus Aspergillus.* Baltimore, MD: Williams and Wilkins.

Reeves, E. P., Reiber, K., Neville, C., Schreibner, O., Kavanagh, K., and Doyle, S. (2006). A nonribosomal peptide synthetase (Pes1) confers protection against oxidative stress in *Aspergillus fumigatus*. *FEBS J.* 273, 3038–3053. doi: 10.1111/j.1742-4658.2006.05315.x

Rementeria, A., Lopez-Molina, N., Ludwig, A., Vivanco, A. B., Belen, A., Bikandi, J., et al. (2005). Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev. Iberoam. Micol.* 22, 1–23. doi: 10.1016/S1130-1406(05)70001-2

Rodrigues, B. S. F., Sahm, B. D. B., Jimenez, P. C., Pinto, F. C. L., Mafezoli, J., Mattos, M. C., et al. (2015). Bioprospection of cytotoxic compounds in fungal strains recovered from sediments of the Brazilian coast. *Chem. Biodivers.* 12, 432–442. doi: 10.1002/cbdv.201400193

Samson, R. A., Hong, S.-B., and Frisvad, J. C. (2006). Old and new concepts of species differentiation in *Aspergillus*. *Med. Mycol.* 44, S133–S144. doi: 10.1080/13693780600913224

Samson, R. A., Hong, S.-B., Peterson, S. W., Frisvad, J. C., and Varga, J. (2007). Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Stud. Mycol.* 59, 147–203. doi: 10.3114/sim.2007.59.14

Samson, R. A., Nielsen, P. V., and Frisvad, J. C. (1990). "The genus *Neosartorya*: differentiation by scanning electron microscopy and mycotoxin profiles," in *Modern Concepts in Penicillium and Aspergillus Classification,* R. A. Samson and J. I. Pitt (New York, NY: Plenum Press), 455-467. doi: 10.1007/978-1-4899-3579-3_40

Samson, R. A., Peterson, S. W., Frisvad, J. C., and Varga, J. (2011). New species in *Aspergillus* section *Terrei*. *Stud. Mycol.* 69, 39–55. doi: 10.3114/sim.2011.69.04

Samson, R. A., Visagie, C. M., Houbraken, J., Hong, S.-B., Hubka, V., Klaasen, C. H. W., et al. (2014). Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud. Mycol.* 78, 141–173. doi: 10.1016/j.simyco.2014.07.004

Sanchez, J. F., Somoza, A. D., Keller, N. P., and Wang, C. C. (2012). Advances in *Aspergillus* secondary metabolite research in the post-genomic era. *Nat. Prod. Rep.* 29, 351–371. doi: 10.1039/c2np00084a

Schmeda-Hirschmann, G., Hormazabal, E., Rodriguez, J. A., and Theoduloz, C. (2008). Cycloaspeptide A and pseurotin A from the endophytic fungus Penicillium janczewskii. *Z. Naturforsch. C* 63, 383–388. doi: 10.1515/znc-2008-5-612

Schwienbacher, M., Weig, M., Thies, S., Regula, J. T., Heeseman, J., and Ebel, F. (2005). Analysis of the major proteins secreted by the human opportunistic pathogen *Aspergillus fumigatus* under *in vitro* conditions. *Med. Mycol.* 43, 623–630. doi: 10.1080/13693780500089216

Shan, W., Wang, S., Ying, Y., Ma, L., and Zhan, Z. (2014). Indole-benzodiazepine-2,5-dione derivatives from *Neosartorya fischeri*. *J. Chem. Res.* 38, 692–694. doi: 10.3184/174751914X14140034695581

Shan, W.-G., Wang, S.-L., Lang, H. Y., Chen, S. M., Ying, Y.-M., and Zhan, Z.-J. (2015). Cottoquinazolins E and F from *Neosartorya fischeri* NRRL 181. *Helv. Chim. Acta* 98, 552–556. doi: 10.1002/hlca.201400270

Shinohara, C., Hasumi, K., and Endo, A. (1992). Inhibition of oxidized low-density lipoprotein metabolism in macrophage J774 by helvolic acid. *Biochim. Biophys. Acta* 1167, 303–306. doi: 10.1016/0005-2760(93)90233-Y

Sigler, L., Sutton, D. A., Gibas, C. F. C., Summerbell, R. C., Noel, R. K., and Iwen, P. C. (2010). *Phialosimplex*, a new anamorphic genus associated with infections in dogs and having phylogenetic affinity to the *Trichomaceae*. *Med. Mycol.* 48, 335–345. doi: 10.3109/13693780903225805

Sodngam, S., Sawadsitang, S., Suwannasai, N., and Mongkoltharanuk, W. (2014). Chemical constituents, and their cytotoxicity, of the rare wood decaying fungus *Xylaria humosa*. *Nat. Prod. Commun.* 9, 157–158.

Someya, A., Yaguchi, T., and Udagawa, S. (1999). *Neosartorya sublevispora*, a new species of soil-borne Eurotiales. *Mycoscience* 40, 405–409. doi: 10.1007/BF02464395

Srivastava, N., Rawal, R., Sharma, R., Oberoi, H. S., Srivastava, M., and Singh, J. (2014). Effect of nickel-cobaltite nanoparticles on production and thermostability of cellulases from newly isolated thermotolerant *Aspergillus fumigatus* NS (Class: Eurotiomycetes). *Appl. Biochem. Biotechnol.* 174, 1092–1103. doi: 10.1007/s12010-014-0940-0

Stack, D., Neville, C., and Doyle, S. (2007). Nonribosomal peptide synthesis in *Aspergillus fumigatus* and other fungi. *Microbiology* 153, 1297–1306. doi: 10.1099/mic.0.2006/006908-0

Stanzani, M., Orciuolo, E., Lewis, R., Kontoyiannis, D. P., Martins, S. L. R., St. John, L. S., et al. (2005). *Aspergillus fumigatus* suppresses the human cellular immune response via gliotoxin-mediated apoptosis of monocytes. *Blood* 105, 2258–2265. doi: 10.1182/blood-2004-09-3421

Sugui, J. A., Pardo, J., Chang, Y. C., Zarember, K. A., Nardone, G., Galvez, E. M., et al. (2007). Gliotoxin is a virulence factor of *Aspergillus fumigatus*; *gliP* deletion attenuates virulence in mice immunocompromised with hydrocortisone. *Eukaryotic Cell* 6, 1562–1569. doi: 10.1128/EC.00141-07

Sugui, J. A., Kwon-Chung, K. J., Juvvadi, P. R., Latg,é, J. P., and Steinbach, W. J. (2015). *Aspergillus fumigatus* and related species. *Cold Spring Harb. Perspect. Med.* 5: a019786. doi: 10.1101/cshperspect.a019786

Sugui, J. A., Peterson, S. W., Clark, L. P., Nardone, G., Folio, L., Riedlinger, G., et al. (2012). Aspergillus tanneri sp. nov., a new pathogen that causes invasive disease refractory to antifungal therapy. *J. Clin. Microbiol.* 50, 3309–3317. doi: 10.1128/JCM.01509-12

Sugui, J. A., Peterson, S. W., Figat, A. B., Hansen, B., Samson, R. A., Mellado, E., et al. (2014). Genetic relatedness versus biological compatibility between *Aspergillus fumigatus* and related species. *J. Clin. Microbiol.* 52, 3707–3721. doi: 10.1128/JCM.01704-14

Sugui, J. A., Vinh, D. C., Nardone, G., Sheat, Y. R., Chang, Y. C., Zelazny, A. M., et al. (2010). *Neosartorya udagawae* (*Aspergillus udagawae*), an emerging agent of aspergillosis: how different is it from *Aspergillus fumigatus*? *J. Clin. Microbiol.* 48, 220–228. doi: 10.1128/JCM.01556-09

Sutton, D. A., Summerbell, R. C., Samson, R., and Rinalde, M. F. (2002). "First report of *Neosartorya spinosa* (Raper & Fennell) Kozakiewicz inciting human disease," in *Abstracts of the General Meeting of ASM*, Vol. 102 (Salt Lake City, UT), 212.

Swilaiman, S. S., O'Gorman, C. M., Balajee, S. A., and Dyer, P. S. (2013). Discovery of a sexual cycle in *Aspergillus lentulus*, a close relative of *A. fumigatus*. *Eukaryotic Cell* 12, 962–969. doi: 10.1128/EC.00040-13

Takahashi, C., Matsushida, T., Doi, M., Minoura, K., Shingu, T., Komeda, Y., et al. (1995). Fumiquinazolions A-G, novel metabolites of a fungus separated from a *Pseudolabrus* marine fish. *J. Chem. Soc. Perkin Trans. I* 1995, 2345–2353. doi: 10.1039/p19950002345

Takeda, M., Horie, Y., and Abliz, P. (2001). Two new heterothallic *Neosartorya* from African soil. *Mycoscience* 42, 361–367. doi: 10.1007/BF02461219

Tamiya, H., Ochiai, E., Kikuchi, K., Yahiro, M., Toyotome, T., Watanabe, A., et al. (2015). Secondary metabolite profiles and antifungal drug susceptibility of *Aspergillus fumigatus* and closely related species, *A. lentulus*, *A. udagawae*,

and *A. viridinutans*. *Journal of Infection and Chemotherapy*. 21, 385–391. doi: 10.1016/j.jiac.2015.01.005

Tepsic, K., Gunde-Cimerman, N., and Frisvad, J. C. (1997). Growth and mycotoxin production by *Aspergillus fumigatus* strains isolated from a saltern. *FEMS Microbiol. Lett.* 157, 9–12. doi: 10.1111/j.1574-6968.1997.tb12745.x

Tomee, J. F., and Kauffman, H. F. (2000). Putative virulence factors of *Aspergillus fumigatus*. *Clin. Exp. Allergy* 30, 476–484. doi: 10.1046/j.1365-2222.2000.00796.x

Toskova, M., Palousova, D., Kocmanova, I., Pavlovsky, Z., Timilsinas, S., Langerova, M., et al. (2013). Invasive mould disease involving the gastro-intestinal tract caused by *Neosartorya pseudofischeri* in a haematological patient. *Mycoses* 56, 385–388. doi: 10.1111/myc.12038

Tsai, H.-F., Chang, Y. C., Washburn, R. G., Wheeler, M. H., and Kwon-Chung, K. J. (1998). The developmentally regulated *alb1* gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. *J. Bacteriol.* 180, 3031–3038.

Tsai, H.-F., Fujii, I., Watanabe, A., Wheeler, M. H., Chang, Y. C., Yasuaka, Y., et al. (2001). Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. *J. Biol. Chem.* 276, 29292–29298. doi: 10.1074/jbc.M101998200

Tsunawaki, S., Yoshida, L. S., Nishida, S., Kobayashi, T., and Shimoyama, T. (2004). Fungal metabolite gliotoxin inhibits assembly of the human respiratory burst NADPH oxidase. *Infect. Immun.* 72, 3373–3382. doi: 10.1128/IAI.72.6.3373-3382.2004

Varga, J., Due, M., Frisvad, J. C., and Samson, R. A. (2007). Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data. *Stud. Mycol.* 59, 89–106. doi: 10.3114/sim.2007.59.11

Varga, J., Frisvad, J. C., Kocsubé, S., Brancovics, B., Szigeti, G., and Samson, R. A. (2011a). New and revisited species in *Aspergillus* section *Nigri*. *Stud. Mycol.* 69, 1–17. doi: 10.3114/sim.2011.69.01

Varga, J., Frisvad, J. C., and Samson, R. A. (2011b). Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Stud. Mycol.* 69, 57–80. doi: 10.3114/sim.2011.69.05

Varga, J., and Samson, R. A. (2008). Ribotoxin genes in *Aspergillus* section *Clavati*. *Antonie van Leeuwenhoek* 94, 481–485. doi: 10.1007/s10482-008-9266-7

Varga, J., Tóth, B., Rigó, K., Debets, F., and Kozakiewicz, Z. (2000). Genetic variability within the *Aspergillus viridinutans* species. *Folia Microbiol.* 45, 423–428. doi: 10.1007/BF02817615

Verwer, P. E. B., van Leuwen, W. B., Girard, V., van Belkum, A., Staab, J. F., Verbrugh, H. A., et al. (2014). Diuscrimination of *Aspergillus lentulus* from *Aspergillus fumigatus* by Raman spectroscopy and MALDI-TOF MS. *Eur. J. Clin. Microbiol.* 33, 245–251. doi: 10.1007/s10096-013-1951-4

Vinh, D. C., Shea, Y. R., Jones, P. A., Freeman, A. F., Zelazny, A., and Holland, S. M. (2009a). Chronic invasive aspergillosis caused by *Aspergillus viridinutans*. *Emerging Infect. Dis.* 15, 1292–1294. doi: 10.3201/eid1508.090251

Vinh, D. C., Shea, Y. R., Sugui, J. A., Parrilla-Castellar, E. R., Freeman, A. F., Campbell, J. W., et al. (2009b). Invasive aspergillosis due to *Neosartorya udagawae*. *Clin. Infect. Dis.* 49, 102–111. doi: 10.1086/599345

Virágh, M., Vörös, D., Kele, Z., Kovács, L., Fizilo, Á., Lakatos, G., et al. (2014). Production of a defensin-like antifungal protein NFAP from *Neosartorya fischeri* in *Pichia pastoris* and its antifungal activity against filamentous fungal isolates from human infections. *Protein Expr. Purif.* 94, 78–84. doi: 10.1016/j.pep.2013.11.003

Visagie, C., Varga, J., Houbraken, J., Meijer, M., Yilmaz, N., Fotedar, R., et al. (2014). Ochratoxin production and taxonomy of the yellow aspergilli (*Aspergillus* section *Circumdati*). *Stud. Mycol.* 78, 1–61. doi: 10.1016/j.simyco.2014.07.001

Vödisch, M., Scherlach, K., Winkler, R., Hertweck, C., Braun, H. P., Roth, M., et al. (2011). Analysis of the *Aspergillus fumigatus* proteome reveals metabolic changes and the activation of the pseurotin A biosynthesis gene cluster in response to hypoxia. *J. Proteome Res.* 10, 2508–2524. doi: 10.1021/pr1012812

Wakana, D., Hosoe, T., Itabashi, T., Nozawa, K., Okada, K., Campos Takaki, G. M., et al. (2006). Isolation of isoterrein from *Neosartorya fischeri*. *Mycotoxins* 56, 3–6. doi: 10.2520/myco.56.3

Wang, F., Fang, Y., Zhu, T., Zhang, M., Lin, A., Gu, Q., et al. (2008). Seven new prenylated indole diketopiperazine alkaloids from holothurian-derived fungus *Aspergillus fumigatus*. *Tetrahedron* 64, 7986–7991. doi: 10.1016/j.tet.2008.06.013

Watanabe, A., Kamei, K., Sekine, T., Hiraguchi, H., Ochiai, E., Hashimoto, Y., et al. (2004). Cytotoxic substances from *Aspergillus fumigatus* in oxygenated or poorly oxygenated environment. *Mycopathologia* 158, 1–7. doi: 10.1023/B:MYCO.0000038439.56108.3c

Watanabe, A., Kamei, K., Sekine, T., Waku, M., Nishimura, K., Miyaji, M., et al. (2003). Immunosuppressive substances in *Aspergillus fumigatus* culture filtrate. *J. Infect. Chemother.* 9, 114–121. doi: 10.1007/s10156-002-0227-1

Wiekmann, I., Lechner, B. E., Baccile, J. A., Velk, T. A., Yin, W.-B., Bok, J. B., et al. (2014). Pertubations in small molecule synthesis uncovers an iron-responsive secondary metabolite network in *Aspergillus fumigatus*. *Front. Microbiol.* 5:530. doi: 10.3389/fmicb.2014.00530

Wiemann, P., Guo, C. J., Palmer, J. M., Sekonyela, R. R., Wang, C. C. C., and Keller, N. P. (2013). Prototype of an intertwined secondary-metabolite supercluster. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17065–17070. doi: 10.1073/pnas.1313258110

Wong, S. M., Musza, L. L., Kydd, G. C., Kullnig, R., Gillum, A. M., and Cooper, R. (1993). Fiscalins: new substance P inhibitors produced by the fungus *Neosartorya fischeri*. Taxonomy, fermentation, structures, and biological properties. *J. Antibiot.* 46, 545–553. doi: 10.7164/antibiotics.46.545

Wortman, J. R., Fedorova, N., Crabtree, J., Joardar, V., Maiti, R., Haas, B. J., et al. (2006). Whole genome comparison of the *Aspergillus fumigatus* family. *Med. Mycol.* 44, S3–S7. doi: 10.1080/13693780600835799

Xie, F., Li, X.-B., Zhou, J.-C., Xu, Q.-Q., Wang, X.-N., Yuan, H.-Q., et al. (2015). Secondary metabolites from *Aspergillus fumigatus*, an endophytic fungus from the liverwort *Heteroscyphus tener* (STEPH.) SCHIFFN. *Chem. Biodivers.* 12, 1313–1321. doi: 10.1002/cbdv.201400317

Xu, N., Cao, Y., Wang, L., Chen, G., and Pei, Y.-H. (2013). New alkaloids from a marine-derived fungus *Neosartorya* sp. HN-M-3. *J. Asian Nat. Prod. Res.* 15, 731–736. doi: 10.1080/10286020.2013.797895

Xu, J., Song, Y. C., Guo, Y., Mei, Y. N., and Tan, R. X. (2014). Fumigaclavine D-H, new ergot alkaloids from endophytic *Aspergillus fumigatus*. *Planta Med.* 80, 1131–1137. doi: 10.1055/s-0034-1382958

Yaguchi, T., Horie, Y., Tanaka, R., Matsuzawa, T., Ito, J., and Nishimura, K. (2007). Molecular phylogenetics of multiple genes on *Aspergillus* section *Fumigati* isolated from clinical specimens in Japan. *Jpn. J. Med. Mycol.* 48, 37–46. doi: 10.3314/jjmm.48.37

Yaguchi, T., Matsuzawa, T., Tanaka, R., Abliz, P., Hui, Y., and Horie, Y. (2010). Two new species of *Neosartorya* isolated from soil in Xinjiang, China. *Mycoscience* 51, 253–262. doi: 10.1007/S10267-010-0037-8

Ye, Y., Minami, A., Mandi, A., Liu, C., Taniguchi, T., Kuzuyama, T., et al. (2015). Genome mining for sesterpenes using bifunctional terpene synthases reveals a unified intermediate of di/sesterpenes. *J. Am. Chem. Soc.* 137, 11846–11853. doi: 10.1021/jacs.5b08319

Yim, T., Kanokmedhakul, K., Kanokmedhakul, S., Sanmanoch, W., and Boonlue, S. (2014). A new meroterpenoid tatenoic acid from the fungus *Neosartorya tatenoi*. *Nat. Prod. Res.* 28, 1847–1852. doi: 10.1080/14786419.2014.951353

Yin, W.-B., Grundmann, A., Cheng, Y., and Li, S.-M. (2009). Acetylaszonalenin biosynthesis in Neosartorya fischeri. Identification of the biosynthetic gene cluster by genomic mining and functional proof of the genes by biochemical characterization. *J. Biol. Chem.* 284, 100–109. doi: 10.1074/jbc.M807606200

Zhang, H.-W., Ying, C., and Tang, Y.-F. (2014). Four ardeemin analogs from endophytic *Aspergillus fumigatus* SPS-02 and their reversal effects on multi-drug resistant tumor cells. *Chem. Biodivers.* 11, 85–91. doi: 10.1002/cbdv.201300220

Zhang, M., Wang, W.-L., Fang, Y.-C., Zhu, T.-J., Gu, Q.-Q., and Zhu, W.-M. (2008). Cytotoxic alkaloids and antibiotic nordammarane triterpenoids from the marine-derived fungus *Aspergillus sydowi*. *J. Nat. Prod.* 71, 985–989. doi: 10.1021/np700737g

Zhang, H.-C., Ma, Y.-M., Liu, R., and Zhou, F. (2012). Endophytic fungus *Aspergillus tamarii* from *Ficus carica* L., a new source of indolyl diketopiperazines. *Biochem. Syst. Ecol.* 45, 31–33. doi: 10.1016/j.bse.2012.07.020

Zhao, W. Y., Zhu, T. J., Fan, G. T., Liu, H. B., Fang, Y. C., Gu, Q. Q., et al. (2010). Three new dioxopiperazine metabolites from a marine-derived fungus *Aspergillus fumigatus* Fres. *Nat. Prod. Res.* 24, 953–957. doi: 10.1080/14786410902726134

Zheng, Z. Z., Shan, W. C., Wang, S.-L., Ying, Y.-M., Ma, L.-F., and Zhan, Z.-J. (2014). Three new prenylated diketopiperazines from *Neosartorya fischeri*. *Helv. Chim. Acta* 97, 1020–1026. doi: 10.1002/hlca.201300416

Zhou, F., Zhang, H., Liu, R., and Zhang, D. (2013). Isolation and biological evaluation of secondary metabolites from the endophytic fungus *Aspergillus*

fumigatus from *Astralagus membranaceus*. *Chem. Nat. Comp.* 49, 568–570. doi: 10.1007/s10600-013-0675-0

Zin, W. W. M., Buttachon, S., Buaruang, J., Gales, L., Pereira, J. A., Pinto, M. M. M., et al. (2015). A new meroditerpene and a new tryptoquivaline analog from the algicolous fungus *Neosartorya takakii* KUFC 7898. *Mar. Drugs* 13, 3776–3790. doi: 10.3390/md13063776

Zuck, K. M., Shipley, S., and Newman, D. J. (2011). Induced production of N-formyl alkaloids from *Aspergillus fumigatus* by co-culture with *Streptomyces peucetius*. *J. Nat. Prod.* 74, 1653–1657. doi: 10.1021/np200255f

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Frisvad and Larsen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 May 2015
doi: 10.3389/fmicb.2015.00428

Prevalence and mechanism of triazole resistance in *Aspergillus fumigatus* in a referral chest hospital in Delhi, India and an update of the situation in Asia

Anuradha Chowdhary[1], Cheshta Sharma[1], Shallu Kathuria[1], Ferry Hagen[2] and Jacques F. Meis[2,3]*

[1] Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India, [2] Department of Medical Microbiology and Infectious Diseases, Canisius-Wilhelmina Hospital, Nijmegen, Netherlands, [3] Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, Netherlands

OPEN ACCESS

Edited by:
Frederic Lamoth, Lausanne University Hospital, Switzerland

Reviewed by:
Dominique Sanglard, University of Lausanne and University Hospital Center, Switzerland
Jesus Guinea, Hospital Gregorio Marañón, Spain

***Correspondence:**
Anuradha Chowdhary, Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110 007, India
dranuradha@hotmail.com

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *04 March 2015*
Accepted: *22 April 2015*
Published: *08 May 2015*

Citation:
Chowdhary A, Sharma C, Kathuria S, Hagen F and Meis JF (2015) Prevalence and mechanism of triazole resistance in Aspergillus fumigatus in a referral chest hospital in Delhi, India and an update of the situation in Asia. Front. Microbiol. 6:428. doi: 10.3389/fmicb.2015.00428

Aspergillus fumigatus causes varied clinical syndromes ranging from colonization to deep infections. The mainstay of therapy of *Aspergillus* diseases is triazoles but several studies globally highlighted variable prevalence of triazole resistance, which hampers the management of aspergillosis. We studied the prevalence of resistance in clinical *A. fumigatus* isolates during 4 years in a referral Chest Hospital in Delhi, India and reviewed the scenario in Asia and the Middle East. *Aspergillus* species ($n = 2117$) were screened with selective plates for azole resistance. The isolates included 45.4% *A. flavus*, followed by 32.4% *A. fumigatus*, 15.6% *Aspergillus* species and 6.6% *A. terreus.* Azole resistance was found in only 12 (1.7%) *A. fumigatus* isolates. These triazole resistant *A. fumigatus* (TRAF) isolates were subjected to (a) *calmodulin* and β *tubulin* gene sequencing (b) *in vitro* antifungal susceptibility testing against triazoles using CLSI M38-A2 (c) sequencing of *cyp51A* gene and real-time PCR assay for detection of mutations and (d) microsatellite typing of the resistant isolates. TRAF harbored TR_{34}/L98H mutation in 10 (83.3%) isolates with a pan-azole resistant phenotype. Among the remaining two TRAF isolates, one had G54E and the other had three non-synonymous point mutations. The majority of patients were diagnosed as invasive aspergillosis followed by allergic bronchopulmonary aspergillosis and chronic pulmonary aspergillosis. The Indian TR_{34}/L98H isolates had a unique genotype and were distinct from the Chinese, Middle East, and European TR_{34}/L98H strains. This resistance mechanism has been linked to the use of fungicide azoles in agricultural practices in Europe as it has been mainly reported from azole naïve patients. Reports published from Asia demonstrate the same environmental resistance mechanism in *A. fumigatus* isolates from two highly populated countries in Asia, i.e., China and India and also from the neighboring Middle East.

Keywords: triazole resistant *A. fumigatus*, TR_{34}/L98H, G54E, microsatellite typing, India, Asia

Introduction

Among the *Aspergillus* species, *Aspergillus fumigatus* is the leading etiologic agent of all forms of aspergillosis, which could be attributed to the ubiquitous presence of its thermo-tolerant spores that are refractory to adverse environmental conditions (Kwon-Chung and Sugui, 2013). *A. fumigatus*, in contrast to *Candida albicans*, has no reservoir in the immunocompetent population; thus, infections with *A. fumigatus* are generally environmentally acquired (Verweij et al., 2009; Chowdhary et al., 2013b). Furthermore, aspergillosis is associated with high morbidity and mortality in both immunocompetent and immunosuppressed populations primarily due to difficulties in early diagnosis or delay in recovery of the immune system (Kosmidis and Denning, 2015). Triazole antifungals, the competitive inhibitors of *cyp51A*, are preferred for prophylaxis and treatment of aspergillosis. However, failure of treatment with azoles and a steady increase in the occurrence of triazole resistant *A. fumigatus* (TRAF) isolates from environment as well as clinical settings has been reported (Denning et al., 1997; Mellado et al., 2007; Verweij et al., 2007; Rodriguez-Tudela et al., 2008; Snelders et al., 2008, 2009; Baddley et al., 2009; Howard et al., 2009; Arendrup et al., 2010; Mortensen et al., 2010, 2011; van der Linden et al., 2011, 2013; Burgel et al., 2012; Chowdhary et al., 2012a,b, 2013b, 2014a,b; Alastruey-Izquierdo et al., 2013; Badali et al., 2013; Bader et al., 2013; Escribano et al., 2013; Seyedmousavi et al., 2013; Prigitano et al., 2014; Spiess et al., 2014; Lavergne et al., 2015; Steinmann et al., 2015). The most common mechanism of triazole resistance has been linked to theTR_{34}/L98H mutation with tandem repeat in the *cyp*51A promoter region combined with a single amino acid exchange of leucine 98 to histidine (Chowdhary et al., 2014c). Apparently, this mutated allele has spread throughout the *A. fumigatus* population and thus has been reported worldwide from patients as well as the environment (Mellado et al., 2007; Verweij et al., 2007; Rodriguez-Tudela et al., 2008; Snelders et al., 2008, 2009; Baddley et al., 2009; Howard et al., 2009; Mortensen et al., 2010, 2011; Lockhart et al., 2011; van der Linden et al., 2011; Burgel et al., 2012; Chowdhary et al., 2012a,b; Hamprecht et al., 2012; Jeurissen et al., 2012; Morio et al., 2012; Rath et al., 2012; Alastruey-Izquierdo et al., 2013; Bader et al., 2013; Escribano et al., 2013; Rocchi et al., 2014; Kidd et al., 2015; Steinmann et al., 2015). In addition several point mutations such as G54, G138, or M220 lead to disturbances in the docking of azole drugs to *cyp*51A protein rendering azole resistant *A. fumigatus* phenotype (Diaz-Guerra et al., 2003; Mann et al., 2003; Mellado et al., 2004; Chen et al., 2005; Howard et al., 2006, 2009, 2013; Rodriguez-Tudela et al., 2008; Snelders et al., 2008; Albarrag et al., 2011; van der Linden et al., 2011). These mutations have been previously reported to occur *de novo* due to prolonged exposure of *A. fumigatus* isolates to azole antifungal drugs in clinical settings (Chen et al., 2005; Howard et al., 2009; Escribano et al., 2012). However, a recent study reports presence of *A. fumigatus* carrying G54 point mutation in the environment of Tanzania, Romania, and India suggesting that environment may predominately be the cause in acquisition of azole resistant isolates (Sharma et al., 2015). Also, non-*cyp51A* mediated mutations have been increasingly recognized in the development of azole resistance and are mainly reported from Manchester, UK (Bueid et al., 2010). We conducted a prospective study for the assessment of prevalence of TRAF and the underlying *cyp51A* mutations in clinical isolates of *Aspergillus* species collected during a 4-year (2011–2014) period in a referral Chest Hospital in Delhi, India and reviewed the reports on TRAF isolates from environmental and clinical sources from Asia and the neighboring Middle East.

Materials and Methods

Fungal Isolates and Their Phenotypic Characterization

During 2011–2014 a total of 8222 clinical samples were processed for fungal culture and microscopy collected from patients of our hospital and three neighboring referral hospitals included in **Table 1**. The clinical specimens included sputum, endotracheal aspirates, bronchial aspirates, bronchoalveolar lavages (BAL), bronchial tissues, fine needle aspiration biopsies, lung biopsies, nasal polyps, bone marrow aspirations, pleural fluid, bronchial plugs, and cerebrospinal fluid. The study was approved by the Institute's Ethics Committee. All *Aspergillus* species cultured from the specimens were preliminarily identified based on colony color and morphology of the isolates on Czapek dox agar plates incubated at 28°C for 7 days. In order to investigate azole resistance in all of the *Aspergillus* species, they were screened on itraconazole (ITC, 4 μg/ml) and voriconazole (VRC, 1 μg/ml) supplemented Sabouraud dextrose agar (SDA) plates. *Aspergillus* isolates that exhibited growth on either of the antifungal plate were confirmed as *Aspergillus* species by amplification and sequencing of β-*tubulin* and *calmodulin* genes.

Antifungal Susceptibility Testing (AFST)

All resistant *A. fumigatus* were subjected to AFST against four standard medical triazoles, amphotericin B (AMB), echinocandins, and 10 commonly used azole fungicides using CLSI M38-A2 broth microdilution Clinical and Laboratory Standards Institute [CLSI] (2008). The drugs tested included ITC (Lee Pharma, Hyderabad, India, and Janssen Research Foundation, Beerse, Belgium), VRC (Pfizer Central Research, Sandwich, Kent, UK), isavuconazole (ISA, Basilea Pharmaceutica International AG, Basel, Switzerland), posaconazole (POS, Merck, Whitehouse Station, NJ, USA), AMB (Sigma–Aldrich, Germany), caspofungin (CAS, Merck), micafungin (MFG, Astellas Toyama Co. Ltd., Japan), and anidulafungin (AFG, Pfizer). The tested azole fungicides were bromuconazole, cyproconazole, difenoconazole, epoxiconazole, penconazole, tebuconazole, triadimefon, metconazole, hexaconazole (Rallis India, Mumbai, India), and tricyclazole (Cheminova India, Mumbai, India). The AFST results were analyzed by using epidemiological cutoff values (ECVs) proposed by Espinel-Ingroff et al. (2011a,b, 2013) ITC, 1 μg/ml; VRC, 1 μg/ml; POS, 0.5 μg/ml; ISA,

TABLE 1 | Clinical characteristics of 12 patients with triazole resistant *Aspergillus fumigatus* (TRAF) isolates.

Patient no.	Age/sex/year of isolation	Specimen	Institution	MICs (μg/ml)[a,b]				Mutation	*Aspergillus* disease	Underlying condition	Treatment	Outcome
				ITC	VRC	ISA	POS					
1	60/M/2011	FNAB[c]/sputum	VPCI[d]	>16	≥8	≥8	≥8	TR_{34}/L98H	IPA[e]	COPD[f], Diabetes mellitus	VRC	Alive
2	65/M/2012	Lung biopsy	VPCI	>16	≥8	2	≥8	TR_{34}/L98H	IPA	Pulmonary adenocarcinoma	VRC	Died
3	38/F/2012	FNAB	Hospital 1[g]	>16	≥8	2	≥8	TR_{34}/L98H	IPA	Myelodysplastic syndrome	VRC	Died
4	19/F/2012	Endotracheal aspirate/tissue aspirate	VPCI	>16	8	8	1	TR_{34}/L98H	IPA	COPD, Tuberculosis	AMB	Died
5	26/F/2012	Resected sinus tissue	Hospital 2[h]	≥16	>16	8	2	TR_{34}/L98H	IA rhino-cerebral sinusitis	COPD	AMB	Died
6	52/M/2013	BAL[i]	Hospital 3[j]	>16	0.25	0.25	1	G54E	CPA[k]	Preexisting tubercular cavities	VRC	Died
7	76/M/2014	Sputum	VPCI	16	16	>8	4	TR_{34}/L98H	ABPA[l]		Systemic steroids	Died
8	55/M/2014	Bronchial aspirate	VPCI	16	≥8	≥8	1	TR_{34}/L98H	ABPA	–	Systemic steroids	No follow up
9	50/M/2014	BAL	VPCI	16	≥8	≥8	1	TR_{34}/L98H	CPA	Tuberculosis	VRC	Died
10	50/M/2014	BAL	VPCI	16	2	4	0.06	F46Y, D255E, M172V	CPA	–	AMB for 1 month, discharged on VRC	No follow up
11	57/M/2014	Sputum	VPCI	≥16	>16	>8	2	TR_{34}/L98H	ABPA	–	Systemic steroids	No follow up
12	60/M/2014	Sputum	VPCI	>16	>16	>8	>8	TR_{34}/L98H	ABPA	–	Systemic steroids	No follow up

[a]*MIC, minimum inhibitory concentration; ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POS, posaconazole; AMB, amphotericin B;* [b]*Geometric mean MICs of all isolates against azole fungicides was 32 μg/ml except metconazole (3.8 μg/ml);* [c]*FNAB, fine needle aspiration biopsy;* [d]*VPCI, Vallabhbhai Patel Chest Hospital, Delhi;* [e]*IPA, invasive pulmonary aspergillosis;* [f]*COPD, chronic obstructive pulmonary disease;* [g]*Hospital 1, Cancer Referral Hospital, Delhi;* [h]*Hospital 2, General Medical Referral Hospital, Delhi;* [i]*BAL, bronchoalveolar lavage;* [j]*Hospital 3, Central Tuberculosis Hospital, Delhi;* [k]*CPA, chronic pulmonary aspergillosis;* [l]*ABPA, allergic bronchopulmonary aspergillosis.*

1 μg/ml, AMB, 4 μg/ml, and CAS, 0.25 μg/ml (Pfaller et al., 2011).

Mutation Analysis

To gain insight into the mechanisms responsible for azole resistance in *A. fumigatus*, isolates were subjected to amplification and sequencing of *cyp51A* gene along with the promoter region (Sharma et al., 2015). The amplified product was purified followed by sequencing on an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye terminator kit (v3.1, RR-100; Applied Biosystems; Chowdhary et al., 2013a). DNA sequences were analyzed with Sequencing Analysis software version 5.3.1 (Applied Biosystems). Consensus sequences were made using BioEdit software (version 7.0.5.3; Hall, 1999). The sequences of the resistant *A. fumigatus* strains were compared with the wild type susceptible reference *A. fumigatus* strain (Af293). The mutations in the resistant strains were further confirmed by mixed format real time PCR analysis as described previously (Klaassen et al., 2010).

Microsatellite Typing

The genotypic relationship of the Indian resistant *A. fumigatus* isolates with the isolates from Asia, Middle East, and Europe harboring various mutations in *cyp51A* gene was determined by microsatellite typing using a panel of nine short tandem repeats (STR) as described previously (de Valk et al., 2005). The amplification of three di-, tri-, or tetranucleotide repeat markers was carried out using three multiplex PCRs, namely, M2, M3, and M4, respectively, (de Valk et al., 2005). Repeat numbers in each marker was assigned using *A. fumigatus* Af293 as reference. The STR data was imported to BioNumerics v5.1 software (Applied Maths, Sint-Martens-Latem, Belgium) for phylogenetic analysis. The dendrogram based on unweighted pair-group method with arithmetic mean (UPGMA) clustering using the Pearson correlation coefficient was generated. A total of 49 *A. fumigatus* isolates from Asia, Middle East, Europe, and Australia were included as controls to evaluate the genetic differences with the *A. fumigatus* isolates from India. The isolates from outside India were from following countries: China (clinical resistant, $n = 8$), Iran (environmental resistant, $n = 5$; environmental susceptible, $n = 4$), Kuwait (clinical resistant, $n = 2$; clinical susceptible, $n = 2$; environmental resistant, $n = 8$; environmental susceptible, $n = 2$), France (clinical resistant, $n = 1$), Germany (clinical resistant, $n = 7$; clinical susceptible, $n = 1$), the Netherlands (clinical resistant, $n = 2$; environmental resistant, $n = 3$), and Australia (clinical resistant, $n = 2$; clinical susceptible, $n = 2$). Also, Indian isolates (environmental resistant $n = 3$; environmental susceptible $n = 6$ and clinical susceptible $n = 3$) collected during the previous study on resistant *A. fumigatus* were included for comparison with the present clinical TRAF isolates from India (Chowdhary et al., 2012b).

Results

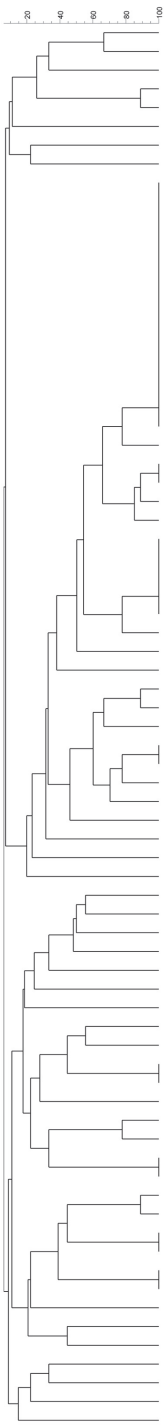
Overall, during a 4-year survey period, 25.7% of clinical samples harbored *Aspergillus* species ($n = 2117$). Out of these 2117 isolates, 45.4% ($n = 963$) were *A. flavus*, followed by 32.4% ($n = 685$) *A. fumigatus*, 15.6% ($n = 329$) *Aspergillus* species and 6.6% ($n = 140$) *A. terreus* (Kathuria et al., 2015). Barring 12 isolates of *A. fumigatus* none of the other *Aspergillus* species grew on SDA plates supplemented with ITC and/or VRC. Of these, 11 *A. fumigatus* grew on both ITC and VRC supplemented SDA plates while a solitary isolate grew only on ITC plate. All the 12 resistant isolates were identified as *A. fumigatus sensu stricto* by β-*tubulin* and *calmodulin* genes sequencing.

Of the 12 resistant *A. fumigatus*, 11 showed a pan-azole resistant phenotype exhibiting high MIC of all the triazoles, namely, ITC [geometric mean (GM) MIC, 16 μg/ml], VRC (GM MIC, 8 μg/ml), ISA (GM MIC, 6.34 μg/ml), and POS (GM MIC, 2.82 μg/ml). In contrast, a solitary *A. fumigatus* isolate exhibited high MIC of only ITC (>16 μg/ml) and POS (1 μg/ml; **Table 1**). However, AMB (GM MIC, 0.4 μg/ml) and three echinocandins, namely, CAS, MFG, and AFG were active against all the resistant *A. fumigatus* isolates with GM MICs of 0.13 μg/ml, 0.017 μg/ml, and 0.02 μg/ml, respectively. Further all TRAF uniformly revealed cross-resistance to all the azole fungicides (MICs,>32 μg/ml) tested excepting metconazole (MICs, 3.80 μg/ml).

Overall, 1.75% (12/685) of the clinical *A. fumigatus* isolates were resistant. The major resistance mechanism observed among the eleven pan-azole resistant phenotype was TR_{34}/L98H mutation ($n = 10$) and a solitary isolate exhibited three non-synonymous point mutations, namely, F46Y, D255E, and M172V. Another single point mutation, G54E, was observed in a solitary isolate that had high MICs to both ITC and POS.

The STR typing data revealed a single microsatellite complex (MC) among all the TR_{34}/L98H genotypes whereas solitary isolate each of G54E and non-synonymous mutant of *A. fumigatus* represented two distinct genotypes (**Figure 1**). The TR_{34}/L98H MC was homogenous and shared all the nine loci except one isolate that differed at two loci (4A and 4C). Further to determine the genetic relatedness among the present Indian TR_{34}/L98H clinical isolates ($n = 10$) comparison with Indian TR_{34}/L98H environmental isolates ($n = 3$) collected from the previous study was done (Chowdhary et al., 2012b). Both the environmental and clinical *A. fumigatus* isolates had an identical STR pattern. The genotypes of all Indian TR_{34}/L98H isolates were distinct from the TR_{34}/L98H strains of Chinese, Kuwait, Iran, and European isolates (Lockhart et al., 2011; Badali et al., 2013; Ahmad et al., 2014, 2015; Steinmann et al., 2015). However, environmental *A. fumigatus* isolates from Kuwait ($n = 4$) and Iran ($n = 5$) were more closely related with Indian TRAF isolates with similarity observed at 5–6 of the nine loci studied. Although the Chinese clinical TRAF isolates ($n = 8$) also formed a separate cluster but unlike Indian isolates they were markedly heterogeneous exhibiting variable STR patterns. The STR typing data of the clinical Kuwait TRAF isolates ($n = 6$) revealed homogeneity among them, however, when compared to Indian TRAF clinical isolates they showed differences at 6–7 loci (Ahmad et al., 2015).

The detailed clinical record of all patients whose clinical samples yielded TRAF was retrieved from the database. The majority of patients were diagnosed as invasive aspergillosis (IA, $n = 5$)



STR*Af* microsatellite panel

2A	2B	2C	3A	3B	3C	4A	4B	4C	Strain number	Source	Country	Genotype	Publication
39.0	16.0	13.0	13.0	19.0	8.0	5.0	7.0	11.0	IranSuscep6	Soil	Iran	Wildtype	Badali et al., 2013
39.0	16.0	13.0	11.0	17.0	5.0	5.0	7.0	11.0	IranSuscep7	Soil	Iran	Wildtype	Badali et al., 2013
25.0	10.0	13.0	45.0	10.0	9.0	10.0	7.0	11.0	1978	Clinical	Germany	TR34/L98H	Steinmann et al., 2015
23.0	22.0	13.0	68.0	9.0	8.0	8.0	9.0	11.0	2087	Clinical	Germany	TR34/L98H	Steinmann et al., 2015
23.0	22.0	13.0	98.0	9.0	8.0	8.0	9.0	11.0	1899	Clinical	Germany	TR34/L98H	Steinmann et al., 2015
10.0	16.0	10.0	25.0	12.0	8.0	7.0	5.0	6.0	1966	Clinical	Germany	Wildtype	Steinmann et al., 2015
46.0	11.0	11.0	8.0	21.0	5.0	7.0	9.0	9.0	IranSuscep3	Soil	Iran	Wildtype	Badali et al., 2013
45.0	11.0	10.0	11.0	28.0	10.0	10.0	7.0	9.0	IranSuscep5	Soil	Iran	Wildtype	Badali et al., 2013
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI325/14	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI235/14	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI1316/14	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI1320/14	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI271/E/11/3	Soil	India	TR34/L98H	Chowdhary et al., 2012b
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI343P11/1	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI343P11/2	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI373/E/12/3	Soil	India	TR34/L98H	Chowdhary et al., 2012b
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI591/12	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI598/12	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI124/W/11	Air	India	TR34/L98H	Chowdhary et al., 2012b
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI245/P/12-1	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI245/P/12-2	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	8.0	10.0	28.0	VPCI1166/12	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	31.0	9.0	10.0	12.0	10.0	8.0	VPCI236/14	Clinical	India	TR34/L98H	Present study
14.0	20.0	9.0	32.0	9.0	10.0	8.0	11.0	29.0	E218	Air	Kuwait	TR34/L98H	Ahmad et al., 2014
14.0	20.0	9.0	32.0	9.0	10.0	8.0	11.0	29.0	R015	Soil	Kuwait	TR34/L98H	Ahmad et al., 2014
14.0	20.0	9.0	31.0	9.0	10.0	8.0	11.0	29.0	E119	Dust	Kuwait	TR34/L98H	Ahmad et al., 2014
14.0	20.0	8.0	32.0	9.0	10.0	8.0	11.0	29.0	R018	Soil	Kuwait	TR34/L98H	Ahmad et al., 2014
14.0	20.0	8.0	32.0	9.0	6.0	8.0	10.0	20.0	Iran 02	Soil	Iran	TR34/L98H	Badali et al., 2013
14.0	20.0	8.0	32.0	9.0	6.0	8.0	10.0	20.0	Iran 04	Soil	Iran	TR34/L98H	Badali et al., 2013
14.0	20.0	8.0	32.0	9.0	6.0	8.0	10.0	20.0	Iran 08	Soil	Iran	TR34/L98H	Badali et al., 2013
14.0	20.0	8.0	32.0	9.0	6.0	8.0	10.0	20.0	Iran 11	Soil	Iran	TR34/L98H	Badali et al., 2013
14.0	20.0	8.0	32.0	9.0	6.0	8.0	10.0	20.0	Iran 13	Soil	Iran	TR34/L98H	Badali et al., 2013
14.0	21.0	8.0	31.0	9.0	6.0	8.0	10.0	20.0	04-202165	Clinical	Australia	TR34/L98H	Kidd et al., 2015
14.0	24.0	14.0	31.0	9.0	10.0	10.0	10.0	20.0	0817960/39	Clinical	The Netherlands	TR34/L98H	Chowdhary et al., 2012b
25.0	20.0	19.0	31.0	9.0	10.0	10.0	14.0	5.0	12-90032258	Clinical	Australia	TR34/L98H	Kidd et al., 2015
10.0	10.0	8.0	31.0	9.0	11.0	8.0	10.0	9.0	MYC-2008-002/46	Soil	The Netherlands	TR34/L98H	Chowdhary et al., 2012b
10.0	10.0	8.0	31.0	9.0	11.0	8.0	10.0	19.0	MYC-2008-002/60	Soil	The Netherlands	TR34/L98H	Chowdhary et al., 2012b
10.0	10.0	8.0	31.0	9.0	7.0	8.0	11.0	24.0	MYC-2008-002/42	Soil	The Netherlands	TR34/L98H	Chowdhary et al., 2012b
20.0	10.0	8.0	35.0	9.0	11.0	8.0	10.0	24.0	Kw2772-coln8	Clinical	Kuwait	TR34/L98H	Ahmad et al., 2015
20.0	10.0	8.0	35.0	9.0	11.0	8.0	10.0	24.0	Kw1431-coln9	Clinical	Kuwait	TR34/L98H	Ahmad et al., 2015
20.0	10.0	8.0	36.0	9.0	11.0	8.0	10.0	23.0	E454	Dust	Kuwait	TR34/L98H	Ahmad et al., 2014
20.0	10.0	8.0	36.0	9.0	11.0	8.0	11.0	25.0	E076	Air	Kuwait	TR34/L98H	Ahmad et al., 2014
13.0	10.0	8.0	72.0	9.0	9.0	8.0	9.0	9.0	094411/7/50	Clinical	The Netherlands	TR34/L98H	Chowdhary et al., 2012b
20.0	21.0	12.0	93.0	9.0	10.0	8.0	11.0	22.0	2091	Clinical	Germany	TR34/L98H	Steinmann et al., 2015
25.0	18.0	11.0	79.0	13.0	17.0	8.0	10.0	28.0	VPCI397/14	Clinical	India	F46Y/D255E/M172V	Present study
23.0	10.0	17.0	10.0	11.0	10.0	12.0	10.0	20.0	1976	Clinical	Germany	TR34/L98H	Steinmann et al., 2015
23.0	12.0	11.0	36.0	25.0	26.0	9.0	11.0	10.0	20643.023	Clinical	China	TR34/L98H	Lockhart et al., 2011
23.0	12.0	11.0	23.0	23.0	26.0	10.0	10.0	10.0	20677.079	Clinical	China	TR34/L98H	Lockhart et al., 2011
26.0	12.0	11.0	22.0	10.0	26.0	12.0	10.0	10.0	20684.022	Clinical	China	TR34/L98H	Lockhart et al., 2011
27.0	12.0	11.0	35.0	19.0	26.0	9.0	9.0	10.0	20684.007	Clinical	China	TR34/L98H	Lockhart et al., 2011
23.0	12.0	11.0	30.0	22.0	6.0	11.0	10.0	7.0	20677.086	Clinical	China	TR34/L98H	Lockhart et al., 2011
19.0	12.0	11.0	36.0	29.0	18.0	17.0	8.0	5.0	13-90057987	Clinical	Australia	Wildtype	Kidd et al., 2015
18.0	20.0	15.0	42.0	24.0	6.0	9.0	10.0	10.0	20643.017	Clinical	China	TR34/L98H	Lockhart et al., 2011
18.0	12.0	17.0	36.0	15.0	26.0	10.0	11.0	7.0	20677.089	Clinical	China	TR34/L98H	Lockhart et al., 2011
23.0	12.0	17.0	43.0	13.0	7.0	10.0	11.0	7.0	20684.002	Clinical	China	TR34/L98H	Lockhart et al., 2011
17.0	12.0	8.0	8.0	15.0	7.0	10.0	11.0	10.0	Kw2772-coln2	Clinical	Kuwait	Wildtype	Ahmad et al., 2015
17.0	12.0	8.0	8.0	15.0	7.0	10.0	11.0	10.0	Kw2772-coln5	Clinical	Kuwait	Wildtype	Ahmad et al., 2015
25.0	20.0	20.0	23.0	22.0	33.0	10.0	11.0	10.0	VPCI94/P/10	Clinical	India	Wildtype	Chowdhary et al., 2012b
23.0	22.0	16.0	97.0	12.0	7.0	16.3	9.0	10.0	1962	Clinical	Germany	TR34/L98H	Steinmann et al., 2015
23.0	19.0	16.0	96.0	12.0	7.0	16.3	9.0	10.0	A4813	Clinical	Germany	TR34/L98H	Steinmann et al., 2015
24.0	10.0	8.0	61.0	12.0	7.0	9.0	11.0	10.0	VPCI380/E/12/2	Soil	India	Wildtype	Chowdhary et al., 2012b
24.0	10.0	8.0	61.0	12.0	7.0	9.0	11.0	10.0	VPCI382/E/12/1	Soil	India	Wildtype	Chowdhary et al., 2012b
18.0	21.0	8.0	87.0	11.0	7.0	9.0	9.0	5.0	R044	Soil	Kuwait	TR34/L98H	Ahmad et al., 2014
18.0	21.0	8.0	88.0	11.0	7.0	9.0	9.0	5.0	R043	Soil	Kuwait	TR34/L98H	Ahmad et al., 2014
23.0	22.0	8.0	22.0	10.0	10.0	9.0	9.0	5.0	E142	Environmental	Kuwait	Wildtype	Ahmad et al., 2014
23.0	22.0	8.0	22.0	10.0	10.0	9.0	9.0	5.0	E149	Environmental	Kuwait	Wildtype	Ahmad et al., 2014
11.0	21.0	8.0	24.0	10.0	10.0	12.0	4.0	5.0	VPCI381/E/12/1	Soil	India	Wildtype	Chowdhary et al., 2012b
11.0	21.0	8.0	24.0	10.0	10.0	12.0	4.0	5.0	VPCI384/E/12/1	Soil	India	Wildtype	Chowdhary et al., 2012b
17.0	24.0	21.0	39.0	11.0	29.0	12.3	9.0	5.0	VPCI894/09	Clinical	India	Wildtype	Chowdhary et al., 2012b
20.0	12.0	15.0	37.0	21.0	22.0	23.0	9.0	5.0	VPCI1155/P/13	Clinical	India	G54E	Present study
18.0	12.0	15.0	52.0	25.0	19.0	15.0	9.0	5.0	VPCI388/E/12/1	Soil	India	Wildtype	Chowdhary et al., 2012b
19.0	23.0	28.0	44.0	12.0	26.0	24.0	9.0	8.0	10-90130864	Clinical	Australia	Wildtype	Kidd et al., 2015
18.0	23.0	15.0	38.0	11.0	48.0	10.0	9.0	8.0	18512R	Clinical	France	TR34/L98H	Chowdhary et al., 2012b
17.0	21.0	15.0	9.0	12.0	7.0	13.3	15.0	8.0	VPCI379/E/12/3	Soil	India	Wildtype	Chowdhary et al., 2012b
25.0	19.0	19.0	26.0	19.0	17.0	10.0	16.0	8.0	VPCI834/10	Clinical	India	Wildtype	Chowdhary et al., 2012b

FIGURE 1 | Genotypicrelationship of Indian *Aspergillus fumigatus* isolates (clinical resistant, *n* = 14; clinical susceptible, *n* = 3; environmental resistant, *n* = 3; environmental susceptible, *n* = 6) with isolates from Asia including China (clinical resistant, *n* = 8), Middle East including Iran (environmental resistant, *n* = 5; environmental susceptible, *n* = 4), and Kuwait (clinical resistant, *n* = 2; clinical susceptible, *n* = 2; environmental resistant, *n* = 8; environmental susceptible, *n* = 2), and Europe including France (clinical resistant, *n* = 1), Germany (clinical resistant, *n* = 7; clinical susceptible, *n* = 1), the Netherlands (clinical resistant, *n* = 2; environmental susceptible, *n* = 3), and Australia (clinical resistant, *n* = 2; clinical susceptible, *n* = 2). The dendrogram is based on a categorical analysis of nine microsatellite markers in combination with Unweighted Pair Group Method with arithmetic mean clustering. The scale bar indicates the percentage identity. The isolates VPCI 343/P/11/1, VPCI 343/P/11/2, and VPCI 245/P/12-1, VPCI 245/P/12-2 were serial isolates from two individual patients, respectively.

followed by allergic bronchopulmonary aspergillosis (ABPA, $n = 4$) and chronic pulmonary aspergillosis (CPA, $n = 3$). The most common underlying condition among the IA patients was chronic obstructive pulmonary disease (COPD) in three patients and myelodysplastic syndrome and pulmonary adenocarcinoma in the remaining two patients, respectively. IA was defined as probable or proven according to the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (Ascioglu et al., 2002; De Pauw et al., 2008). All cases of IA in the present study were proven cases and FNAB/lung biopsy or resected sinus tissue was positive for fungus and yielded *A. fumigatus* in culture. IPA ($n = 4$) was the most common manifestation and a solitary case had invasive rhino-cerebral sinusitis. All COPD patients who finally developed IA during the course of 5–10 years of illness had been admitted to the medical units or ICU repeatedly (4–9 episodes per year) for respiratory symptoms with severe airflow obstruction. All these patients were on systemic and inhaled steroids for a long period ranging from 10 to 12 years. Associated co-morbidities like diabetes mellitus and tuberculosis were observed in two of the three COPD patients. Allergic aspergillosis manifesting as ABPA was diagnosed in four patients by a combination of clinical, mycoserologic, and radiological features (Agarwal et al., 2013). None of the ABPA patients diagnosed in the present study were on azoles when their culture grew TRAF. The third clinical entity was CPA, which was diagnosed by chronic duration of clinical symptoms (>3 months), progressive pulmonary lesions with or without cavitation, precipitating antibodies to *A. fumigatus* in serum, and mycological evidence of fungal presence. Further, none of the patients harboring identical TR_{34}/L98H genotype ($n = 10$) admitted to the Chest hospital ($n = 8$) in the present study had an overlapping time frame during their admission. Also, two patients harboring the same genotype were from two different hospitals in Delhi (**Table 1**).

Overall, the mortality was highest in IA (four of five patients) and CPA (two of three patients) and a solitary case of ABPA also had a fatal outcome. Among IA patients, VRC (6 mg/kg intravenous 12 hourly followed by 4 mg/kg and switched to oral 200 mg twice a day when clinically stable) was administered in three patients and two were started on AmB deoxycholate (1 mg/kg/day). All CPA patients were given VRC for 7–10 days before the antifungal susceptibility results were conveyed. The two CPA patients had severe lung involvement and the disease was fatal in both of them within 1 week. However, in one case therapy was switched to amphotericin B for one month followed by repeat BAL culture that yielded negative results for *Aspergillus* and the patient was discharged on oral VRC on follow up. However, patient failed to report for the follow up visits. The patients with ABPA in the present study were managed symptomatically and systemic steroids were the mainstay of therapy.

Discussion

In the present study, we examined azole susceptibility and resistant mechanisms among *A. fumigatus* isolates from patients with bronchopulmonary aspergillosis in a referral Chest Institute, which caters to a vast population of Delhi and adjoining states of Uttar Pradesh, Haryana and also to far remote regions of India. The rate of azole resistance in *A. fumigatus* isolates in our study was 1.75% (12/685) during a 4-year period, which is remarkably low compared to the high prevalence in Europe including UK (6.6–27.8%), the Netherlands (3.1–4.6%), and Germany (3.2%; Snelders et al., 2008; Howard et al., 2009; Bueid et al., 2010; van der Linden et al., 2011; Bader et al., 2013). In contrast in Spain, a lower prevalence rate (2.5%) of azole resistance in *A. fumigatus* complex has been reported suggesting that azole resistance has not yet uniformly spread in Europe (Escribano et al., 2013). TR_{34}/L98H was the predominant resistance mechanism in 83.3% of the Indian TRAF isolates followed by G54E ($n = 1$) and a non-synonymous mutation ($n = 1$).

Short tandem repeats typing of the TRAF isolates demonstrated a single cluster, with a homogenous population. It is also worth mentioning here that the patients' positive for TRAF isolates in the present study were both from Delhi, and other states namely West Bengal, Haryana, and Uttar Pradesh. These states previously have been found to harbor *A. fumigatus* isolates carrying TR_{34}/L98H in soil samples (Chowdhary et al., 2012b). Notably the genotype detected in clinical isolates in this study was identical to the TR_{34}/L98H genotype reported earlier from environmental samples from India and shared the identical nine loci, suggesting environmental origin of this major resistance mechanism (Chowdhary et al., 2012b). This is the first study outside the Netherlands revealing the possibility of acquisition of clinical isolates linked with the environment. However, in contrast to the heterogeneity observed in environmental and clinical isolates in the Netherlands (Klaassen et al., 2012), the Indian TRAF in the present study had a homogenous population which was recently also confirmed with whole genome sequencing (Abdolrasoulia et al., 2015).

Table 2 summarizes the reports of TRAF isolation harboring mutations in the *cyp51A* gene from clinical and environmental sources in Asia and the neighboring Middle East. The initial reports describing azole resistance in clinical *A. fumigatus* isolates originated from Europe in the late 1990s followed by systematical investigations in several European countries which, reported prevalence, mechanism, and genomic aspects of TRAF (Mellado et al., 2007; Verweij et al., 2007; Rodriguez-Tudela et al., 2008; Howard et al., 2009; Mortensen et al., 2010; Burgel et al., 2012; Morio et al., 2012; Bader et al., 2013; Astvad et al., 2014; Fischer et al., 2014; Spiess et al., 2014; van der Linden et al., 2015). However, the first comprehensive report on the occurrence of TRAF isolates in Asia originated from China during 2008–2009 from the ARTEMIS global sentinel surveillance program demonstrating TR_{34}/L98H resistance mechanism in 27.5% (8/29) *A. fumigatus* isolates (Lockhart et al., 2011; **Table 2**). In contrast, more recently few studies from Japan, described TRAF in clinical isolates but interestingly none of them exhibited TR_{34}/L98H resistance mechanism, instead several SNPs and novel mutations, F332K and P216L were reported (Asano et al., 2011; Hagiwara et al., 2014). The single center study from Japan reported 5.2% (1/19) TRAF isolates harboring only G54E/R/W and I266N mutation (Tashiro et al., 2012a,b). Notably, a recent

TABLE 2 | Distribution of TRAF in clinical and environmental samples in Asia and Middle East harboring mutations in the *Cyp51A* gene.

Country	Resistance mechanism	Source	Resistance rate (No. of resistant *A. fumigatus* / No. of *A. fumigatus* tested)	Reference
China	M220I, G54R	Clinical	4/6 (66.6%)[a]	Chen et al. (2005); Xu et al. (2010)
	TR34/L98H	Clinical	8/29 (27.5%)	Lockhart et al. (2011)
	SNP[b]	Environmental, Poultry	11[c]/175	Wang et al. (2014)
India	TR34/L98H	Clinical	2/103 (1.9%)	Chowdhary et al. (2012a)
	TR34/L98H	Environmental	44/630 (7%)	Chowdhary et al. (2012b)
	TR34/L98H & TR46/Y121F/T289A	Environmental	8/126 (6.3%) 6/126 (4.8%)	Chowdhary et al. (2014a)
	TR34/L98H & G54E	Environmental	4/5 (80%) 1/5 (20%)	Sharma et al. (2015)
Iran	TR34/L98H	Clinical	4/124 (3.2%)	Seyedmousavi et al. (2013)
	TR34/L98H	Environmental	5/41 (12.1%)	Badali et al. (2013)
Japan	F332K	Clinical	1/19 (5.2%)	Asano et al. (2011)
	G54E/R/W and I266N	Clinical	12/196 (6.1%)	Tashiro et al. (2012a; 2012b)
	P216L	Clinical	1/8 (12.5%)	Hagiwara et al. (2014)
Kuwait	TR34/L98H	Environmental	8/115 (7%)	Ahmad et al. (2014)
	TR34/L98H	Environmental	1/50 (2%)	Ahmad et al. (2015)
	TR34/L98H	Clinical	2/16 (12.5%)	Ahmad et al. (2015)
Taiwan	Not mentioned[d]	Clinical	2/40 (5%)	Hsueh et al. (2005)

[a]*serial isolates from a patient suffering from lung aspergilloma;* [b]*SNP, single nucleotide polymorphisms such asF46Y, M172V, N248T, N248K, D255E, and E427K also present in susceptible A. fumigatus strains were detected;* [c]*total no. of A. fumigatus obtained from French and Chinese avian farms;* [d]*The mutation in cyp51A gene was not investigated.*

study on environmental sampling of air from a pumpkin farm sprayed with azole fungicides, at Nihon University, Japan was carried out by using an outdated and less efficacious settle plate method and reported no azole resistance in 50 *A. fumigatus* isolates (Kano et al., 2015). The lack of isolation of TRAF could be attributed to not adopting a highly efficacious sampling method such as using an air sampler and to the low number of isolates tested for resistance resulting in false rates of TRAF prevalence in the environment. The possibility of missing resistance was recently also highlighted in the SCARE study (van der Linden et al., 2015). Further, planned studies with wider coverage areas and different sources such as soil samples, air, wooden debris etc., should be undertaken for finding out the true prevalence of TRAF isolates. In India, a comprehensive wider environmental survey covering north, south, and eastern India analyzed 486 environmental samples comprising soil from flowerbeds of nurseries, surrounding parks of hospitals, cotton trees, tea gardens, paddy fields, soil containing bird excreta, and decayed wood of tree trunks revealed 7% TRAF isolates in the environment carrying TR_{34}/L98H mutation (Chowdhary et al., 2012b). Also, a recently described VRC resistant TR_{46}/Y121F/T289A mechanism was observed in environmental *A. fumigatus* isolates from agricultural fields in India (Chowdhary et al., 2014a). Barring the solitary environmental report on TR_{46}/Y121F/T289A mechanism from India none of the clinical or environmental samples from Asia has yet documented the occurrence of *A. fumigatus* isolates carrying this mechanism so far. However, considering the presence of this new resistant mechanism in the environment of India it may be anticipated that in future it may spread to the neighboring Asian countries. In addition to the above-discussed reports on triazole resistance in Asia, the neighboring Middle East countries, Iran, and Kuwait had reported 12.2 and 7% TRAF carrying TR_{34}/L98H resistance in the environment, respectively, (Badali et al., 2013; Ahmad et al., 2014). A similar range of resistance prevalence of 3.2 and 12.5% TR_{34}/L98H *A. fumigatus* isolates was observed in clinical samples from CPA and ABPA patients from Iran and Kuwait, respectively, (Seyedmousavi et al., 2013; Ahmad et al., 2015). Based on the fact that the Indian TRAF isolates exhibited a distinct cluster away from the Chinese TR_{34}/L98H isolates, it may be suggested that resistance among the *A. fumigatus* strains across Asia has evolved from separate strains and not from a common resistant ancestor, which may have spread worldwide.

It is also pertinent to mention that the high rates of triazole resistance in Europe as compared to Asia could be due to environmental factors or more so by frequent use of azoles in clinical settings and in the environment. Azole fungicides are extensively used in agriculture for crop protection to control mildews and rusts of grains, fruits, vegetables, and also for preservation of materials like wood etc. Geographically, Europe is the dominant market where fungicide usage is significantly high in production of fruits and vegetables along with wheat and vineyard (Stensvold et al., 2012). Thus, the reports of high resistance rates in environmental *A. fumigatus* isolates from the Netherlands, Belgium, UK, and Germany can be attributed to higher fungicide usage. Similarly, the absence or very low prevalence of triazole resistance in *A. fumigatus* in the USA could possibly be due to the low usage of fungicides in the USA as compared to Europe[1]. Notably, triazole resistance was screened among 1026 *A. fumigatus* isolates from

[1]http://ec.europa.eu/food/fs/sc/ssc/out278en.pdf

22 states of USA and none had TR_{34}/L98H mutation (Pham et al., 2014). In India, the crop protection chemicals account for ∼2% of the total chemicals market and currently India is the second largest manufacturer of pesticides in Asia, second only to Japan[2]. Among different classes of pesticides used in India the percent share of insecticides (60%) is highest followed by fungicides (19%), herbicides (16%), biopesticides (3%), and others (3%). Per capita consumption of crop protection products in India is 0.6 kg/ha compared to 16 kg/ha in Taiwan, 13 kg/ha in China, and 12 kg/ha in Japan[2]. Finally, the environmental resistance mechanism in *A. fumigatus* isolates have been reported from two highly populated countries in Asia, i.e., China and India. Further, the environment of agricultural fields in India has been harboring variable *cyp51A* mediated resistant patterns in *A. fumigatus* isolates, which are continuously exposed to agricultural azole fungicides. It is emphasized that in depth analysis of azole resistance in *A. fumigatus* isolates in both clinical laboratories and environmental settings is required to prevent its spread and emergence in neighboring Asian countries.

[2] http://www.ficci.com/spdocument/20292/petro1.pdf

Acknowledgments

This work was supported in part by a research grant from University Grants Commission Research Fellowship, India (F.2-15/2003 SA-I) and Indian Council of Medical Research (5/3/3/26/2010-ECD-I), Government of India, New Delhi, India. We are grateful to Paul E. Verweij (Radboud University Medical Centre, Nijmegen, the Netherlands) for providing several fungicides (bromuconazole, cyproconazole, difenoconazole, epoxiconazole, penconazole, tebuconazole, triadimefon, metconazole). We acknowledge Rallis India, India, and Cheminova India, for kindly providing us hexaconazole and tricyclazole fungicides.

References

Abdolrasoulia, A., Rhodes, J., Beale, M. A., Hagen, F., Rogers, T. R., Chowdhary, A., et al. (2015). Genomic context of azole-resistance mutations in *Aspergillus fumigatus* using whole-genome sequencing. *mBio* 6, (in press).

Agarwal, R., Chakrabarti, A., Shah, A., Gupta, D., Meis, J. F., Guleria, R., et al. (2013). Allergic bronchopulmonary aspergillosis: review of literature and proposal of new diagnostic and classification criteria. *Clin. Exp. Allergy* 43, 850–873. doi: 10.1111/cea.12141

Ahmad, S., Joseph, L., Hagen, F., Meis, J. F., and Khan, Z. (2015). Concomitant occurrence of itraconazole-resistant and -susceptible strains of *Aspergillus fumigatus* in routine cultures. *J. Antimicrob. Chemother.* 70, 412–415. doi: 10.1093/jac/dku410

Ahmad, S., Khan, Z., Hagen, F., and Meis, J. F. (2014). Occurrence of triazole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in outdoor and hospital environment in Kuwait. *Environ. Res.* 133, 20–26. doi: 10.1016/j.envres.2014.05.009

Alastruey-Izquierdo, A., Mellado, E., Peláez, T., Pemán, J., Zapico, S., Alvarez, M., et al. (2013). Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrob. Agents Chemother.* 57, 3380–3387. doi: 10.1128/AAC.00383-13

Albarrag, A. M., Anderson, M. J., Howard, S. J., Robson, G. D., Warn, P. A., Sanglard, D., et al. (2011). Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in cyp51A, upregulation of cyp51A, and integration and activation of transposon Atf1 in the cyp51A promoter. *Antimicrob. Agents Chemother.* 55, 5113–5121. doi: 10.1128/AAC.00517-11

Arendrup, M. C., Mavridou, E., Mortensen, K. L., Snelders, E., Frimodt-Møller, N., Khan, H., et al. (2010). Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS ONE* 5:e10080. doi: 10.1371/journal.pone.0010080

Asano, M., Kano, R., Makimura, K., Hasegawa, A., and Kamata, H. (2011). Molecular typing and in-vitro activity of azoles against clinical isolates of *Aspergillus fumigatus* and *A. niger* in Japan. *J. Infect. Chemother.* 17, 483–486. doi: 10.1007/s10156-010-0202-1

Ascioglu, S., Rex, J. H., de Pauw, B., Bennett, J. E., Bille, J., Crokaert, F., et al. (2002). Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin. Infect. Dis.* 34, 7–14. doi: 10.1086/323335

Astvad, K. M., Jensen, R. H., Hassan, T. M., Mathiasen, E. G., Thomsen, G. M., Pedersen, U. G., et al. (2014). First detection of TR46/Y121F/T289A and TR34/L98H alterations in *Aspergillus fumigatus* isolates from azole-naive patients in Denmark despite negative findings in the environment. *Antimicrob. Agents Chemother.* 58, 5096–5101. doi: 10.1128/AAC.02855-14

Badali, H., Vaezi, A., Haghani, I., Yazdanparast, S. A., Hedayati, M. T., Mousavi, B., et al. (2013). Environmental study of azole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in the cyp51A gene in Iran. *Mycoses* 56, 659–663. doi: 10.1111/myc.12089

Baddley, J. W., Marr, K. A., Andes, D. R., Walsh, T. J., Kauffman, C. A., Kontoyiannis, D. P., et al. (2009). Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the Transplant-Associated Infection Surveillance Network. *J. Clin. Microbiol.* 47, 3271–3275. doi: 10.1128/JCM.00854-09

Bader, O., Weig, M., Reichard, U., Lugert, R., Kuhns, M., Christner, M., et al. (2013). cyp51A-based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. *Antimicrob. Agents Chemother.* 57, 3513–3517. doi: 10.1128/AAC.00167-13

Bueid, A., Howard, S. J., Moore, C. B., Richardson, M. D., Harrison, E., Bowyer, P., et al. (2010). Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J. Antimicrob. Chemother.* 65, 2116–2118. doi: 10.1093/jac/dkq279

Burgel, P. R., Baixench, M. T., Amsellem, M., Audureau, E., Chapron, J., Kanaan, R., et al. (2012). High prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole. *Antimicrob. Agents Chemother.* 56, 869–874. doi: 10.1128/AAC.05077-11

Chen, J., Li, H., Li, R., Bu, D., and Wan, Z. (2005). Mutations in the cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J. Antimicrob. Chemother.* 55, 31–37. doi: 10.1093/jac/dkh507

Chowdhary, A., Agarwal, K., Kathuria, S., Singh, P. K., Roy, P., Gaur, S. N., et al. (2013a). Clinical significance of filamentous basidiomycetes illustrated by isolates of the novel opportunist *Ceriporia lacerata* from the human respiratory tract. *J. Clin. Microbiol.* 51, 585–590. doi: 10.1128/JCM.02943-12

Chowdhary, A., Kathuria, S., Xu, J., and Meis, J. F. (2013b). Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog.* 9:e1003633. doi: 10.1371/journal.ppat.1003633

Chowdhary, A., Kathuria, S., Randhawa, H. S., Gaur, S. N., Klaassen, C. H., and Meis, J. F. (2012a). Isolation of multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR/L98H mutations in the cyp51A gene in India. *J. Antimicrob. Chemother.* 67, 362–366. doi: 10.1093/jac/dkr443

Chowdhary, A., Kathuria, S., Xu, J., Sharma, C., Sundar, G., Singh, P. K., et al. (2012b). Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in the cyp51A gene in India. *PLoS ONE* 7:e52871. doi: 10.1371/journal.pone.0052871

Chowdhary, A., Sharma, C., Kathuria, S., Hagen, F., and Meis, J. F. (2014a). Azole-resistant *Aspergillus fumigatus* with the environmental TR46/Y121F/T289A mutation in India. *J. Antimicrob. Chemother.* 69, 555–557. doi: 10.1093/jac/dkt397

Chowdhary, A., Sharma, C., van den Boom, M., Yntema, J. B., Hagen, F., Verweij, P. E., et al. (2014b). Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania. *J. Antimicrob. Chemother.* 69, 2979–2983. doi: 10.1093/jac/dku259

Chowdhary, A., Sharma, C., Hagen, F., and Meis, J. F. (2014c). Exploring azole antifungal drug resistance in *Aspergillus fumigatus* with special reference to resistance mechanisms. *Future Microbiol.* 9, 697–711. doi: 10.2217/fmb.14.27

Clinical and laboratory Standards Institute [CLSI]. (2008). *Reference Method for Broth Dilution. (Antifungal) Susceptibility Testing of Filamentous Fungi; Approved Standard-Second Edition, M38- A2*. Wayne, PA: CLSI.

Denning, D. W., Venkateswarlu, K., Oakley, K. L., Anderson, M. J., Manning, N. J., Stevens, D. A., et al. (1997). Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 41, 1364–1368.

De Pauw, B., Walsh, T. J., Donnelly, J. P., Stevens, D. A., Edwards, J. E., Calandra, T., et al. (2008). Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin. Infect. Dis.* 46, 1813–1821. doi: 10.1086/588660

de Valk, H. A., Meis, J. F., Curfs, I. M., Muehlethaler, K., Mouton, J. W., and Klaassen, C. H. (2005). Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J. Clin. Microbiol.* 43, 4112–4120. doi: 10.1128/JCM.43.8.4112-4120.2005

Diaz-Guerra, T. M., Mellado, E.,Cuenca-Estrella, M., and Rodriguez-Tudela, J. L. (2003). A point mutation in the 14-alpha-sterol demethylase gene cyp51A contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 47, 1120–1124. doi: 10.1128/AAC.47.3.1120-1124.2003

Escribano, P., Peláez, T., Muñoz, P., Bouza, E., and Guinea, J. (2013). Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrob. Agents Chemother.* 57, 2815–2820. doi: 10.1128/AAC.02487-12

Escribano, P., Recio, S., Peláez, T., González-Rivera, M., Bouza, E., and Guinea, J. (2012). In vitro acquisition of secondary azole resistance in *Aspergillus fumigatus* isolates after prolonged exposure to itraconazole: presence of heteroresistant populations. *Antimicrob. Agents Chemother.* 56, 174–178. doi: 10.1128/AAC.00301-11

Espinel-Ingroff, A., Chowdhary, A., Gonzalez, G. M., Lass-Flörl, C., Martin-Mazuelos, E., Meis, J. F., et al. (2013). Multicenter study of isavuconazole MIC distributions and epidemiological cutoff values for *Aspergillus* spp. for the CLSI M38-A2 broth microdilution method. *Antimicrob. Agents Chemother.* 57, 3823–3828. doi: 10.1128/AAC.00636-13

Espinel-Ingroff, A., Fothergill, A., Fuller, J., Johnson, E., Pelaez, T., and Turnidge, J. (2011a). Wild-type MIC distributions and epidemiological cutoff values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Antimicrob. Agents Chemother.* 55, 2855–2859. doi: 10.1128/AAC.01730-10

Espinel-Ingroff, A., Cuenca-Estrella, M., Fothergill, A., Fuller, J., Ghannoum, M., Johnson, E., et al. (2011b). Wild-type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *Antimicrob. Agents Chemother.* 55, 5150–5154. doi: 10.1128/AAC.00686-11

Fischer, J., van Koningsbruggen-Rietschel, S., Rietschel, E., Vehreschild, M. J., Wisplinghoff, H., Krönke, M., et al. (2014). Prevalence and molecular characterization of azole resistance in *Aspergillus* spp. isolates from German cystic fibrosis patients. *J. Antimicrob. Chemother.* 69, 1533–1536. doi: 10.1093/jac/dku009

Hagiwara, D., Takahashi, H., Watanabe, A., Takahashi-Nakaguchi, A., Kawamoto, S., Kamei, K., et al. (2014). Whole-genome comparison of *Aspergillus fumigatus* strains serially isolated from patients with aspergillosis. *J. Clin. Microbiol.* 52, 4202–4209. doi: 10.1128/JCM.01105-14

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysisprogram for Windows 95/98/NT. *Nucleic Acids. Symp. Ser.* 41, 95–98. doi: 10.14601/Phytopathol_Mediterr-14998ů1.29

Hamprecht, A., Buchheidt, D., Vehreschild, J. J., Cornely, O. A., Spiess, B., Plum, G., et al. (2012). Azole-resistant invasive aspergillosis in a patient with acute myeloid leukaemia in Germany. *Euro. Surveill.* 17, 20262.

Howard, S. J., Cerar, D., Anderson, M. J., Albarrag, A., Fisher, M. C., Pasqualotto, A. C., et al. (2009). Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* 15, 1068–1076. doi: 10.3201/eid1507.090043

Howard, S. J., Lass-Flörl, C., Cuenca-Estrella, M., Gomez-Lopez, A., and Arendrup, M. C. (2013). Determination of isavuconazole susceptibility of *Aspergillus* and *Candida* species by the EUCAST method. *Antimicrob. Agents Chemother.* 57, 5426–5431. doi: 10.1128/AAC.01111-13

Howard, S. J., Webster, I., Moore, C. B., Gardiner, R. E., Park, S., Perlin, D. S., et al. (2006). Multi-azole resistance in *Aspergillus fumigatus*. *Int. J. Antimicrob. Agents* 28, 450–453. doi: 10.1016/j.ijantimicag.2006.08.017

Hsueh, P. R., Lau, Y. J., Chuang, Y. C., Wan, J. H., Huang, W. K., and Shyr, J. M. (2005). Antifungal susceptibilities of clinical isolates of *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species from Taiwan: surveillance of multicenter antimicrobial resistance in Taiwan program data from 2003. *Antimicrob. Agents Chemother.* 49, 512–517. doi: 10.1128/AAC.49.2.512-517.2005

Jeurissen, A., Cooreman, S., Van Kerckhoven, W., Van Leemput, J., Vanhove, P., Lagrou, K., et al. (2012). Invasive pulmonary aspergillosis due to a multi-azole resistant *Aspergillus fumigatus*. *Acta Clin. Belg.* 67, 46–48.

Kano, R., Kohata, E., Tateishi, A., Murayama, S. Y., Hirose, D., Shibata, Y., et al. (2015). Does farm fungicide use induce azole resistance in *Aspergillus fumigatus*? *Med. Mycol.* 53, 174–177. doi: 10.1093/mmy/myu076

Kathuria, S., Sharma, C., Singh, P. K., Agarwal, P., Agarwal, K., Hagen, F., et al. (2015). Molecular epidemiology and in-vitro antifungal susceptibility of *Aspergillus terreus* species complex isolates in Delhi, India: evidence of genetic diversity by amplified fragment length polymorphism and microsatellite typing. *PLoS ONE* 10:e0118997. doi: 10.1371/journal.pone.0118997

Kidd, S. E., Goeman, E., Meis, J. F., Slavin, M. A., and Verweij, P. E. (2015). Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses* 58, 350–355. doi: 10.1111/myc.12324

Klaassen, C. H., de Valk, H. A., Curfs-Breuker, I. M., and Meis, J. F. (2010). Novel mixed-format real-time PCR assay to detect mutations conferring resistance to triazoles in *Aspergillus fumigatus* and prevalence of multi-triazole resistance among clinical isolates in the Netherlands. *J. Antimicrob. Chemother.* 65, 901–905. doi: 10.1093/jac/dkq041

Klaassen, C. H., Gibbons, J. G., Fedorova, N. D., Meis, J. F., and Rokas, A. (2012). Evidence for genetic differentiation and variable recombination rates among Dutch populations of the opportunistic human pathogen *Aspergillus fumigatus*. *Mol. Ecol.* 21, 57–70. doi: 10.1111/j.1365-294X.2011.05364.x

Kosmidis, C., and Denning, D. W. (2015). The clinical spectrum of pulmonary aspergillosis. *Thorax* 70, 270–277. doi: 10.1136/thoraxjnl-2014-206291

Kwon-Chung, K. J., and Sugui, J. A. (2013). *Aspergillus fumigatus*-what makes the species a ubiquitous human fungal pathogen? *PLoS Pathog.* 9:e1003743. doi: 10.1371/journal.ppat.1003743

Lavergne, R. A., Morio, F., Favennec, L., Dominique, S., Meis, J. F., Gargala, G., et al. (2015). First description of azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation in France. *Antimicrob. Agents Chemother.* (Accepted). doi: 10.1128/AAC.00127-15

Lockhart, S. R., Frade, J. P., Etienne, K. A., Pfaller, M. A., Diekema, D. J., and Balajee, S. A. (2011). Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the cyp51A gene. *Antimicrob. Agents Chemother.* 55, 4465–4468. doi: 10.1128/AAC.00185-11

Mann, P. A., Parmegiani, R. M., Wei, S. Q., Mendrick, C. A., Li, X., Loebenberg, D., et al. (2003). Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14-alpha-demethylase. *Antimicrob. Agents Chemother.* 47, 577–581. doi: 10.1128/AAC.47.2.577-581.2003

Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Cuenca-Estrella, M., and Rodriguez-Tudela, J. L. (2004). Substitutions at methionine 220 in the 14-alpha-sterol demethylase (cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. *Antimicrob. Agents Chemother.* 48, 2747–2750. doi: 10.1128/AAC.48.7.2747-2750.2004

Mellado, E., Garcia-Effron, G., Alcázar-Fuoli, L., Melchers, W. J., Verweij, P. E., Cuenca-Estrella, M., et al. (2007). A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrob. Agents Chemother.* 51, 1897–1904. doi: 10.1128/AAC.01092-06

Morio, F., Aubin, G. G., Danner-Boucher, I., Haloun, A., Sacchetto, E., Garcia-Hermoso, D., et al. (2012). High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR/L98H, in a French cohort of patients with cystic fibrosis. *J. Antimicrob. Chemother.* 67, 1870–1873. doi: 10.1093/jac/dks160

Mortensen, K. L., Jensen, R. H., Johansen, H. K., Skov, M., Pressler, T., Howard, S. J., et al. (2011). *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J. Clin. Microbiol.* 49, 2243–2251. doi: 10.1128/JCM.00213-11

Mortensen, K. L., Mellado, E., Lass-Flörl, C., Rodriguez-Tudela, J. L., Johansen, H. K., and Arendrup, M. C. (2010). Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob. Agents Chemother.* 54, 4545–4549. doi: 10.1128/AAC.00692-10

Pfaller, M., Boyken, L., Hollis, R., Kroeger, J., Messer, S., Tendolkar, S., et al. (2011). Use of epidemiological cutoff values to examine 9-year trends in susceptibility of *Aspergillus* species to the triazoles. *J. Clin. Microbiol.* 49, 586–590. doi: 10.1128/JCM.02136-10

Pham, C. D., Reiss, E., Hagen, F., Meis, J. F., and Lockhart, S. R. (2014). Passivesurveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011–2013. *Emerg. Infect. Dis.* 20, 1498–1503. doi: 10.3201/eid2009.140142

Prigitano, A., Venier, V., Cogliati, M., De Lorenzis, G., Esposto, M. C., and Tortorano, A. M. (2014). Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. *Euro. Surveill.* 19, 20747. doi: 10.2807/1560-7917.ES2014.19.12.20747

Rath, P. M., Buchheidt, D., Spiess, B., Arfanis, E., Buer, J., and Steinmann, J. (2012). First reported case of azole-resistant *Aspergillus fumigatus* due to the TR/L98H mutation in Germany. *Antimicrob. Agents Chemother.* 56, 6060–6061. doi: 10.1128/AAC.01017-12

Rocchi, S., Daguindau, E., Grenouillet, F., Deconinck, E., Bellanger, A. P., Garcia-Hermoso, D., et al. (2014). Azole-resistant *Aspergillus fumigatus* isolate with the TR34/L98H mutation in both a fungicide-sprayed field and the lung of a hematopoietic stem cell transplant recipient with invasive aspergillosis. *J. Clin. Microbiol.* 52, 1724–1726. doi: 10.1128/JCM.03182-13

Rodriguez-Tudela, J. L., Alcazar-Fuoli, L., Mellado, E., Alastruey-Izquierdo, A., Monzon, A., and Cuenca-Estrella, M. (2008). Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 52, 2468–2472. doi: 10.1128/AAC.00156-08

Seyedmousavi, S., Hashemi, S. J., Zibafar, E., Zoll, J., Hedayati, M. T., Mouton, J. W., et al. (2013). Azole-resistant *Aspergillus fumigatus*, Iran. *Emerg. Infect. Dis.* 19, 832–834. doi: 10.3201/eid1905.130075

Sharma, C., Hagen, F., Moroti, R., Meis, J. F., and Chowdhary, A. (2015). Triazole resistant *Aspergillus fumigatus* harbouring G54 mutation: is it de novo or environmentally acquired? *J. Glob. Antimicrob. Resist.* 3, (in press). doi: 10.1016/j.jgar.2015.01.005

Snelders, E., Huis In 't Veld, R. A., Rijs, A. J., Kema, G. H., Melchers, W. J., and Verweij, P. E. (2009). Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl. Environ. Microbiol.* 75, 4053–4057. doi: 10.1128/AEM.00231-09

Snelders, E., van der Lee, H. A., Kuijpers, J., Rijs, A. J., Varga, J., Samson, R. A., et al. (2008). Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med.* 5:e219. doi: 10.1371/journal.pmed.0050219

Spiess, B., Postina, P., Reinwald, M., Cornely, O. A., Hamprecht, A., Hoenigl, M., et al. (2014). Incidence of Cyp51A key mutations in *Aspergillus fumigatus*-A study on primary clinical samples of immunocompromised patients in the period of 1995-2013. *PLoS ONE* 9:e103113. doi: 10.1371/journal.pone.0103113

Steinmann, J., Hamprecht, A., Vehreschild, M. J., Cornely, O. A., Buchheidt, D., Spiess, B., et al. (2015). Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *J. Antimicrob. Chemother.* 70, 1522–1526. doi: 10.1093/jac/dku566

Stensvold, C., Jørgensen, L., and Arendrup, M. (2012). Azole-resistant invasive aspergillosis: relationship to agriculture. *Curr. Fungal Infect. Rep.* 6, 178–191. doi: 10.1007/s12281-012-0097-7

Tashiro, M., Izumikawa, K., Hirano, K., Ide, S., Mihara, T., Hosogaya, N., et al. (2012a). Correlation between triazole treatment history and susceptibility in clinically isolated *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 56, 4870–4875. doi: 10.1128/AAC.00514-12

Tashiro, M., Izumikawa, K., Minematsu, A., Hirano, K., Iwanaga, N., Ide, S., et al. (2012b). Antifungal susceptibilities of *Aspergillus fumigatus* clinical isolates obtained in Nagasaki, Japan. *Antimicrob. Agents Chemother.* 56, 584–587. doi: 10.1128/AAC.05394-11

van der Linden, J. W., Arendrup, M. C., Warris, A., Lagrou, K., Pelloux, H., Hauser, P. M., et al. (2015). Prospective multicentre international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg. Infect. Dis.* (in press). doi: 10.3201/eid2106.140717

van der Linden, J. W., Camps, S. M., Kampinga, G. A., Arends, J. P., Debets-Ossenkopp, Y. J., Haas, P. J., et al. (2013). Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin. Infect. Dis.* 57, 513–520. doi: 10.1093/cid/cit320

van der Linden, J. W., Snelders, E., Kampinga, G. A., Rijnders, B. J., Mattsson, E., Debets-Ossenkopp, Y. J., et al. (2011). Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. *Emerg. Infect. Dis.* 17, 1846–1854. doi: 10.3201/eid1710.110226

Verweij, P. E., Mellado, E., and Melchers, W. J. (2007). Multiple-triazole-resistant aspergillosis. *N. Engl. J. Med.* 356, 1481–1483. doi: 10.1056/NEJMc061720

Verweij, P. E., Snelders, E., Kema, G. H., Mellado, E., and Melchers, W. J. (2009). Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect. Dis.* 9, 789–795. doi: 10.1016/S1473-3099(09)70265-8

Wang, D. Y., Gricourt, M., Arné, P., Thierry, S., Seguin, D., Chermette, R., et al. (2014). Mutations in the Cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* isolated from avian farms in France and China. *Poult. Sci.* 93, 12–15. doi: 10.3382/ps.2013-03541

Xu, H., Chen, W., Li, L., Wan, Z., Li, R., and Liu, W. (2010). Clinical itraconazole-resistant strains of *Aspergillus fumigatus*, isolated serially from a lung aspergilloma patient with pulmonary tuberculosis, can be detected with real-time PCR method. *Mycopathologia* 169, 193–199. doi: 10.1007/s11046-009-9249-x

Conflict of Interest Statement: Jacques F. Meis received grants from Astellas, Basilea, and Merck. He has been a consultant to Astellas, Basilea, and Merck and received speaker's fees from Merck and Gilead. All other authors: no potential conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Copyright © 2015 Chowdhary, Sharma, Kathuria, Hagen and Meis. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 10 February 2015
doi: 10.3389/fmicb.2015.00070

Multiple mechanisms contribute to the development of clinically significant azole resistance in *Aspergillus fumigatus*

W. S. Moye-Rowley*

Department of Molecular Physiology and Biophysics, Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Edited by:
Frederic Lamoth, Duke University, USA

Reviewed by:
Laura Alcazar-Fuoli, Instituto de Salud Carlos III, Spain
Alix Thérèse Coste, University Hospital of Lausanne, Switzerland

***Correspondence:**
W. S. Moye-Rowley, Department of Molecular Physiology and Biophysics, Carver College of Medicine, University of Iowa, 6-530 Bowen Science Building, Iowa City, IA 52242, USA
e-mail: scott-moye-rowley@uiowa.edu

Infections caused by the filamentous fungus *Aspergillus fumigatus* are a significant clinical issue and represent the second most-common form of fungal infection. Azole drugs are effective against this pathogen but resistant isolates are being found more frequently. Infections associated with azole resistant *A. fumigatus* have a significantly increased mortality making understanding drug resistance in this organism a priority. The target of azole drugs is the lanosterol α-14 demethylase enzyme encoded by the *cyp51A* gene in *A. fumigatus*. Mutations in *cyp51A* have been described that give rise to azole resistance and been argued to be the primary, if not sole, contributor to azole resistance. Here, I discuss recent developments that indicate multiple mechanisms, including increased expression of ATP-binding cassette (ABC) transporter proteins, contribute to azole resistance. ABC transporters are well-established determinants of drug resistance in other fungal pathogens and seem likely to play a similar role in *A. fumigatus*.

Keywords: *Aspergillus fumigatus*, ABC transporter, azole resistance, cyp51A, abcG1

INTRODUCTION

Antibiotics have been one of medical science's greatest advances but their continued efficacy is at risk. Over prescription and improper use has led to dramatic increases in resistant microorganisms with accompanying increased mortality. The problem of increased resistance is especially acute for fungal pathogens as the basic eukaryotic cell biology of these organisms limits the classes of antibiotics that avoid effects on the host.

Aspergillus fumigatus is the primary filamentous fungal pathogen of humans and an especially serious issue in any immunocompromised situation (Ruping et al., 2008). Azole drugs are most commonly deployed against *A. fumigatus* (and most fungi) as these drugs are well-tolerated and can be orally delivered. The success of azole antifungal therapy has naturally led to their extensive use both in the clinical setting and agriculturally, especially in Europe (discussed in Verweij et al., 2009). Strikingly, azole antifungals have been much more limited in their application in the United States (Pham et al., 2014) and this seems likely to help explain the differences between resistance mechanisms seen in these two regions. Increased azole resistance is a serious issue in treatment of aspergillosis. Mortality of azole-resistant aspergillosis can approach 90% (van der Linden et al., 2011).

Although the first azole resistant isolates of *A. fumigatus* were reported in the United States (Denning et al., 1997), understanding of the molecular basis of resistance was led by experiments from Europe. Early work on azole resistant isolates of *A. fumigatus* determined that these organisms contained mutations in one of the two genes (*cyp51A/B*) encoding the azole target enzyme: lanosterol 14α demethylase (Mellado et al., 2001). Focus on this gene (*cyp51A*) led to the surprising finding that the overwhelming majority of azole resistant strains contained two mutations and that both alterations were required for azole resistance (Verweij et al., 2007). This allele is referred to as TR34/L98H and consists of a 34 bp duplication in the promoter region linked to a replacement of leucine 98 with a histidine residue in the sequence of the enzyme (Mellado et al., 2007). This *cyp51A* allele confers multiazole resistance and has been found in resistant organisms ranging from Europe to India (Snelders et al., 2008; Chowdhary et al., 2012).

The origin of the TR34/L98H resistance allele as an environmentally selected variant is supported by several circumstantial lines of evidence. First, isolates from the soil led to the alarming discovery that 5–7% of these *A. fumigatus* strains contain this multiazole resistance lesion (reviewed in Chowdhary et al., 2013). Second, patients not previously exposed to azole drugs also exhibit aspergillosis with the TR34/L98H-containing fungus (Mellado et al., 2007). Third, genomic analyses of multiazole resistant *A. fumigatus* determined that strains containing the TR34/L98H allele were the most closely related compared to other azole-susceptible organisms, consistent with these drug resistant strains emerging from a single and recent alteration (Camps et al., 2012b). Finally, study of azole resistant organisms in the United States, where agricultural use of azoles is dramatically lower, has not uncovered the prevalence of the TR34/L98H *cyp51A* variant in drug resistant isolates (Pham et al., 2014).

While there is no question *cyp51A* mutations are important contributors to azole resistance, more recent studies have provided evidence that other resistance mechanisms are also at work in *A. fumigatus* (reviewed in Vermeulen et al., 2013). These other mechanisms have been observed in isolates derived from patients chronically exposed to azole drugs. This long term challenge with

azole drugs drives generation of mutant spores that are now drug resistant. Since *A. fumigatus* exists in a multicellular state, acquisition of a mutant allele in one nucleus is very unlikely to produce a resistant organism. However, if a mutant spore is generated, then a resistant isolate will be produced. Analyses of these mutant strains of *A. fumigatus* that have been recovered after azole exposure have uncovered multiple new mechanisms of resistance to these antifungal drugs. Here I will review recent experiments that implicate the participation of ATP-binding cassette (ABC) transporter proteins and other mechanisms in azole resistance of clinical isolates.

ABC TRANSPORTERS AND DRUG RESISTANCE IN PATHOGENIC FUNGI

The predominance of the TR34 L98H *cyp51A* allele in *A. fumigatus* represents an uncommon genetic distribution of azole resistance in other, better understood fungi. The pathogenic fungus for which we have the most detailed understanding of azole resistance mechanisms is the major human pathogen *Candida albicans*. Extensive analyses of the molecular basis for azole resistance in *C. albicans* led to the discovery of at least two different routes. The *C. albicans ERG11* gene encodes the lanosterol 14α demethylase in this fungus. Changes both in the sequence of the protein as well as alterations that increase *ERG11* transcription are associated with azole resistance (Perea et al., 2001; Morio et al., 2010). Increased expression of membrane transporter proteins is a second mechanism that synergizes with *ERG11* changes (reviewed in Morschhauser, 2010). Experiments in different clinical isolates (Selmecki et al., 2008; MacCallum et al., 2010) led to the finding that high level azole resistance required the cooperation of both the *ERG11* gene and a transcription factor encoded by the *TAC1* gene. The key role of Tac1 is to induce expression of ABC transporter proteins encoded by the *CDR1* gene along with other targets (Coste et al., 2004). These transporter proteins are of two different functional classes: the ABC transporters and major facilitator superfamily (MFS) transporters (reviewed in Prasad and Goffeau, 2012; Costa et al., 2014). Typically, these membrane proteins are found in the plasma membrane (PM) where they are thought to act as energy-dependent drug efflux pumps (Marger and Saier, 1993). Overproduction of the membrane transporters is usually due to amino acid substitutions in transcription factors that ultimately drive elevated mRNA level corresponding to these drug pumps.

This theme of elevated ABC transporter expression cooperating with Erg11 to confer azole resistance is seen in other pathogens including *Candida krusei* (Lamping et al., 2009) and *Candida glabrata* (Samaranayake et al., 2013). As is usual in instances of drug resistance, these multiple different mechanisms are engaged that work together to produce the full *in vivo* response to drug challenge. While the synergy of Erg11 and ABC transporters is well-described in *Candida* species, evidence implicating ABC transporters and other proteins in *A. fumigatus* has been less well-appreciated, likely owing in part to the elegant demonstrations tying *cyp51A* mutations to clinical azole resistance (Snelders et al., 2008). Recent experiments provide a rationale for reconsidering the importance of mechanisms beyond *cyp51A* in azole resistance in *A. fumigatus*.

AZOLE RESISTANCE IN *A. fumigatus* MAY UTILIZE NON-*cyp51A*-DEPENDENT MECHANISMS

As discussed above, the large environmental reservoir of *cyp51A* azole resistant organisms has focused much attention on changes in this key target gene giving rise to drug resistant isolates (Verweij et al., 2009). Analyses of *A. fumigatus* azole resistant isolates from patient populations undergoing chronic azole exposure have provided important new information implicating other resistance pathways in antifungal resistance (**Figure 1**).

The first detailed study of azole resistant *A. fumigatus* strains that also contained wild-type *cyp51A* genes came from analyses of fungal isolates submitted to a regional mycology center at Manchester Hospital in the UK (Howard et al., 2009; Bueid et al., 2010). These investigators discovered that, between the years of 2004–2009, azole resistant isolates increased from 5 to 20%. While another center reported a similar increase in azole resistant *A. fumigatus* isolates (Mortensen et al., 2011), the unique feature of the Manchester data was the presence of a wild-type *cyp51A* gene in 43% of these resistant strains. These findings are consistent with more recent data from the United States in which a broad survey of *A. fumigatus* isolates found an overall lower frequency of azole resistance (5%) but 98% of these resistant isolates had no *cyp51A* lesion known to be associated with azole resistance (Pham et al., 2014). The origin of the *A. fumigatus* isolates in the UK and US studies were very different with the Manchester patients corresponding to patients with chronic aspergillosis and the US isolates coming from a wide range of sources (Pham et al., 2014). The primary commonality for the resistant isolates from these two different studies was the lack of *cyp51A*-dependent changes, strongly indicating the existence of other avenues of azole resistance.

The first data implicating ABC transporters in clinically relevant drug resistance has emerged quite recently. Before discussing these data, I would like to mention the confusing state of current nomenclature for ABC transporters in *A. fumigatus*. There is no consensus for gene names in this (or really any) family of protein in *Aspergillus* and I propose to adopt a system suggested earlier by Kovalchuk and Driessen (2010). This system employs a Human Genome Organization-approved scheme for naming ABC transporters based on their structural organization. I will use this nomenclature to refer to ABC transporters from *A. fumigatus* discussed here and encourage others to do the same.

A number of laboratories have demonstrated correlation between increased ABC transporter expression with associated azole resistance (Tobin et al., 1997; Slaven et al., 2002; Nascimento et al., 2003; da Silva Ferreira et al., 2006) but a functional link between the mRNA levels of a given ABC transporter and azole resistance remained elusive. Follow-up work on *A. fumigatus* isolates with a wild-type *cyp51A* gene led to the finding that mRNA encoding a particular ABC transporter was elevated in 8/11 azole resistant strains (Fraczek et al., 2013). This gene, referred to here as *abcG1* (aka *cdr1B* aka *abcB* aka AFUA_1G14330) encodes a homolog of the well-studied ABC transporters *Saccharomyces cerevisiae* Pdr5 and the *Candida* species Cdr1. Importantly, disruption of *abcG1* from one of the clinical isolates that overproduced this transcript led to a reduction in itraconazole MIC from >8

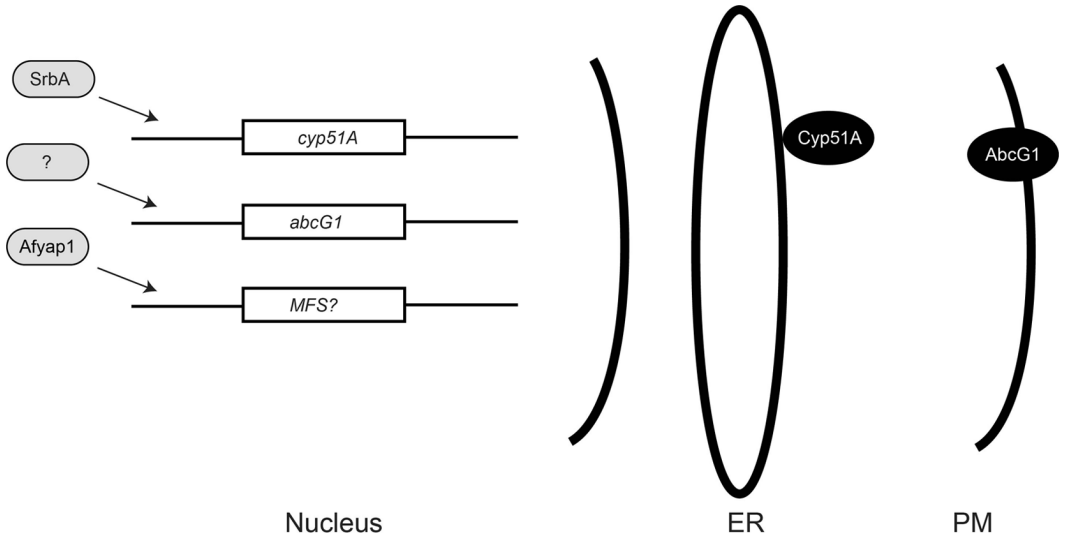


FIGURE 1 | Relationships of azole resistance determinants in *Aspergillus fumigatus*. Known or putative transcription regulators are indicated in light gray as positive regulators of gene expression. The nuclear genes encoding the lanosterol α-14 demethylase (*cyp51A*), a plasma membrane (PM) ABC transporter (*abcG1*), or a major facilitator superfamily (MFS) transporter protein are indicated. Activation of an MFS-encoding gene by Afyap1 is still speculative at this point but is known to occur in other fungi (Alarco et al., 1997; Coleman et al., 1997; Rognon et al., 2006). The Cyp51A protein is docked to the endoplasmic reticulum (ER) by an N-terminal membrane spanning domain (Monk et al., 2014) while the AbcG1 protein is localized to the PM.

to 2 mg/L, demonstrating the functional requirement for this in itraconazole resistance (Fraczek et al., 2013).

Along with this work in clinical isolates, loss of *abcG1* was demonstrated to cause profound azole hypersensitivity in four different laboratory strains (Fraczek et al., 2013; Paul et al., 2013). Overproduction of the *abcG4* (aka *cdr1A* aka *abcA* aka AFUA_2G15130) gene product, that shares the highest degree of sequence conservation to abcG1, also elevated azole resistance. Fusions of green fluorescent protein to the C-terminus of either abcG1 or abcG4 indicated that the resulting fusion proteins were localized to the PM, consistent with their activity as ATP-dependent drug efflux pumps (Paul et al., 2013). While functional data implicating both of these ABC transporter proteins has been provided, these types of demonstrations remained to be accomplished for the large number (15) of *A. fumigatus* ABC transporters in the ABCG class of transporter proteins (Kovalchuk and Driessen, 2010).

While data are accumulating for the role of ABC transporters in azole resistance in *A. fumigatus*, other organisms also exhibit drug resistance that is triggered by changes in transcriptional regulators (recently reviewed in Paul and Moye-Rowley, 2014). As mentioned above, *C. albicans* Tac1 can acquire gain-of-function mutations that lead to enhanced activation of genes under its control (Coste et al., 2004). These target genes include the important azole resistance determinant *CDR1*. The work of Fraczek et al. (2013) suggests that similar mutations may be present in *A. fumigatus* azole resistant isolates overproducing *abcG1* but these remain to be found.

However, at least three examples of transcription factors that are important in azole resistance have been described. The first is the sterol response element binding protein (SREBP) from *A. fumigatus* referred to as SrbA (Willger et al., 2008). This regulator was discovered via its sequence conservation with SREBP from *Schizosaccharomyces pombe* and mammals (reviewed in Bien and Espenshade, 2010). Disruption of the *srbA* gene led to pronounced azole hypersensitivity, and perhaps most interesting, increased fluconazole susceptibility. This seems likely to be due to the decreased expression of *cyp51A* in the absence of SrbA (Willger et al., 2008) as *cyp51A*Δ strains exhibit a similar increased fluconazole susceptibility (Mellado et al., 2005). The importance of the trans-acting factor SrbA in expression of *cyp51A* suggests the possibility that mutants that increase SrbA activity might elevate azole resistance via their effect on a wild-type *cyp51A* locus. These type of mutant alleles of *srbA* have not been described at present.

A recently discovered azole resistance mechanism involving a transcription factor was described in an isolate from an immunodeficient patient undergoing chronic pulmonary aspergillosis (Camps et al., 2012a). Isolates recovered from early in the infection exhibited an azole susceptible phenotype that ultimately transitioned to azole resistant in time. Clever genetic analyses coupled with whole genome sequencing approaches detected a substitution mutation in the *hapE* gene as being responsible for the increased azole resistance. *A. fumigatus hapE* encodes a DNA-binding subunit of the Hap-complex that is a relative of the mammalian CCAAT-binding protein (see Dolfini et al., 2012 for a review). While a mechanistic explanation of the increased azole resistance driven by the mutant HapE-containing complex is still unknown, a potential contributor to this effect is provided by the finding that *cyp51A* expression is elevated in isolates bearing this lesion (Camps et al., 2012a).

A common link between resistance supported by changes in SrbA or HapE is the potential involvement of *cyp51A*. Azole

resistant mutants with changes in these transcription factors could still possess a wild-type *cyp51A* allele yet exhibit elevated drug resistance. A final example that seems likely to be independent of *cyp51A* function is provided by the *A. fumigatus* homolog of *S. cerevisiae* Yap1. This protein is referred to as Afyap1 and is a basic region-leucine zipper transcription factor. ScYap1 is regulated by oxidative stress and normally resides in the cytoplasm due to rapid nuclear export (Kuge et al., 1997; Yan et al., 1998). Upon oxidative stress, nuclear export of ScYap1 is inhibited and the factor accumulates on target promoters, resulting in transcriptional induction of genes involved in redox regulation (reviewed in Morano et al., 2012). Afyap1 has been demonstrated to be regulated by oxidative stress at the level of nuclear localization and seems likely to be controlled by oxidants in a manner similar to that seen for ScYap1 (Lessing et al., 2007).

The relationship of Afyap1 to azole resistance was demonstrated by work using a truncation form of this protein (Qiao et al., 2010). The carboxy-terminal cysteine-rich domain (c-CRD) was deleted from Afyap1 and this mutant protein expressed from a multicopy plasmid in *A. fumigatus*. The c-CRD has been extensively studied in ScYap1 and serves as a negative regulatory site acting to exclude ScYap1 from the nucleus via interaction with the exportin protein Crm1 (reviewed in Guttler and Gorlich, 2011). The Afyap1 mutant lacking its c-CRD (referred to as TR Afyap1) was found to strongly elevate resistance to voriconazole but not influence itraconazole resistance (Qiao et al., 2010). A strain lacking Afyap1 was unaffected in terms of azole resistance but was highly sensitive to oxidants.

This behavior of Afyap1 is quite similar to that previously seen for both the *C. albicans* Yap1 homolog (Cap1) and ScYap1 (Alarco and Raymond, 1999). Loss of the Yap1-encoding genes from any of these organisms causes oxidative stress hypersensitivity but has no marked influence on azole sensitivity (Alarco et al., 1997; Alarco and Raymond, 1999). However, production of hypermorphic forms of Yap1 homologs does enhance azole resistance, typically through induction of expression of MFS-encoding gene expression (Alarco et al., 1997). Strikingly, in *S. cerevisiae*, while deletion of the gene encoding the major ABC transporter involved in azole resistance (Sc *PDR5*) led to a profound sensitivity to this drug (Sanglard et al., 1995), overproduction of ScYap1 fully suppressed this drug sensitivity (Alarco et al., 1997). This also seems likely to occur in *A. fumigatus* although further work will be required to confirm this possibility.

SUMMARY

While early analyses of azole resistance in *A. fumigatus* were consistent with changes in the *cyp51A* gene being the primary if not sole driver of drug resistance, recent findings and a more nuanced view of resistance mechanisms do not support this simple picture. The key differentiator between the nearly exclusive involvement of *cyp51A* mutants in azole resistance and contribution of other pathways comes from consideration of the origin of resistant *A. fumigatus* isolates. An environmental reservoir of resistant organisms, certainly impacted if not completely driven by extensive use of azole-based agricultural fungicides, produced a large number of highly azole resistant isolates that were routinely discovered in patient populations (Verweij et al., 2009). During screening for azole resistant *A. fumigatus* appearing during chronic drug exposure, frequent isolates were found that contained wild-type version of *cyp51A* (Bueid et al., 2010). These resistant isolates indicate the presence of other resistance pathways in this filamentous fungus as seen in more extensively studied pathogens like the *Candida* species (Morschhauser, 2002).

The involvement of ABC transporters in *A. fumigatus* is most clearly indicated by the studies of Fraczek et al. (2013). Overproduction of *abcG1* was linked to multiple clinical isolates and shown to be required for azole resistance in one. The increasing number of azole tolerant isolates recovered from patients that exhibit no changes in the *cyp51A* locus supports the view that other mechanisms, such as ABC transporter overproduction, will be found to play important roles in azole resistance in *A. fumigatus*.

ACKNOWLEDGMENTS

I thank Dr. Sanjoy Paul for a critical reading of this manuscript and Dr. Paul Bowyer for helpful discussions. Work on drug resistance in my laboratory is supported by NIH GM49825.

REFERENCES

Alarco, A. M., Balan, I., Talibi, D., Mainville, N., and Raymond, M. (1997). AP-1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires FLR1 encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* 272, 19304–19313. doi: 10.1074/jbc.272.31.19304

Alarco, A. M., and Raymond, M. (1999). The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J. Bacteriol.* 181, 700–708.

Bien, C. M., and Espenshade, P. J. (2010). Sterol regulatory element binding proteins in fungi: hypoxic transcription factors linked to pathogenesis. *Eukaryot. Cell* 9, 352–359. doi: 10.1128/EC.00358

Bueid, A., Howard, S. J., Moore, C. B., Richardson, M. D., Harrison, E., Bowyer, P., et al. (2010). Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J. Antimicrob. Chemother.* 65, 2116–2118. doi: 10.1093/jac/dkq279

Camps, S. M., Dutilh, B. E., Arendrup, M. C., Rijs, A. J., Snelders, E., Huynen, M. A., et al. (2012a). Discovery of a HapE mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS ONE* 7:e50034. doi: 10.1371/journal.pone.0050034

Camps, S. M., Rijs, A. J., Klaassen, C. H., Meis, J. F., O'Gorman, C. M., Dyer, P. S., et al. (2012b). Molecular epidemiology of *Aspergillus fumigatus* isolates harboring the TR34/L98H azole resistance mechanism. *J. Clin. Microbiol.* 50, 2674–2680. doi: 10.1128/JCM.00335-12

Chowdhary, A., Kathuria, S., Randhawa, H. S., Gaur, S. N., Klaassen, C. H., and Meis, J. F. (2012). Isolation of multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR/L98H mutations in the cyp51A gene in India. *J. Antimicrob. Chemother.* 67, 362–366. doi: 10.1093/jac/dkr443

Chowdhary, A., Kathuria, S., Xu, J., and Meis, J. F. (2013). Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog.* 9:e1003633. doi: 10.1371/journal.ppat.1003633

Coleman, S. T., Tseng, E., and Moye-Rowley, W. S. (1997). *Saccharomyces cerevisiae* basic region-leucine zipper protein regulatory networks converge at the ATR1 structural gene. *J. Biol. Chem.* 272, 23224–23230. doi: 10.1074/jbc.272.37.23224

Costa, C., Dias, P. J., Sa-Correia, I., and Teixeira, M. C. (2014). MFS multidrug transporters in pathogenic fungi: do they have real clinical impact? *Front. Physiol.* 5:197. doi: 10.3389/fphys.2014.00197

Coste, A. T., Karababa, M., Ischer, F., Bille, J., and Sanglard, D. (2004). TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters CDR1 and CDR2. *Eukaryot. Cell* 3, 1639–1652. doi: 10.1128/EC.3.6.1639-1652.2004

da Silva Ferreira, M. E., Malavazi, I., Savoldi, M., Brakhage, A. A., Goldman, M. H., Kim, H. S., et al. (2006). Transcriptome analysis of *Aspergillus fumigatus* exposed to voriconazole. *Curr. Genet.* 50, 32–44. doi: 10.1007/s00294-006-0073-2

Denning, D. W., Venkateswarlu, K., Oakley, K. L., Anderson, M. J., Manning, N. J., Stevens, D. A., et al. (1997). Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 41, 1364–1368.

Dolfini, D., Gatta, R., and Mantovani, R. (2012). NF-Y and the transcriptional activation of CCAAT promoters. *Crit. Rev. Biochem. Mol. Biol.* 47, 29–49. doi: 10.3109/10409238.2011.628970

Fraczek, M. G., Bromley, M., Buied, A., Moore, C. B., Rajendran, R., Rautemaa, R., et al. (2013). The cdr1B efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* 68, 1486–1496. doi: 10.1093/jac/dkt075

Guttler, T., and Gorlich, D. (2011). Ran-dependent nuclear export mediators: a structural perspective. *EMBO J.* 30, 3457–3474. doi: 10.1038/emboj.2011.287

Howard, S. J., Cerar, D., Anderson, M. J., Albarrag, A., Fisher, M. C., Pasqualotto, A. C., et al. (2009). Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* 15, 1068–1076. doi: 10.3201/eid1507.090043

Kovalchuk, A., and Driessen, A. J. (2010). Phylogenetic analysis of fungal ABC transporters. *BMC Genomics* 11:177. doi: 10.1186/1471-2164-11-177

Kuge, S., Jones, N., and Nomoto, A. (1997). Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J.* 16, 1710–1720. doi: 10.1093/emboj/16.7.1710

Lamping, E., Ranchod, A., Nakamura, K., Tyndall, J. D., Niimi, K., Holmes, A. R., et al. (2009). Abc1p is a multidrug efflux transporter that tips the balance in favor of innate azole resistance in *Candida krusei*. *Antimicrob. Agents Chemother.* 53, 354–369. doi: 10.1128/AAC.01095

Lessing, F., Kniemeyer, O., Wozniok, I., Loeffler, J., Kurzai, O., Haertl, A., et al. (2007). The *Aspergillus fumigatus* transcriptional regulator AfYap1 represents the major regulator for defense against reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. *Eukaryot. Cell* 6, 2290–2302. doi: 10.1128/EC.00267

MacCallum, D. M., Coste, A., Ischer, F., Jacobsen, M. D., Odds, F. C., and Sanglard, D. (2010). Genetic dissection of azole resistance mechanisms in *Candida albicans* and their validation in a mouse model of disseminated infection. *Antimicrob. Agents Chemother.* 54, 1476–1483. doi: 10.1128/AAC.01645

Marger, M. D., and Saier, M. H. Jr. (1993). A major superfamily of transmembrane facilitators that catalyse uniport, symport, and antiport. *Trends Biochem. Sci.* 18, 13–20. doi: 10.1016/0968-0004(93)90081-W

Mellado, E., Diaz-Guerra, T. M., Cuenca-Estrella, M., and Rodriguez-Tudela, J. L. (2001). Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in *Aspergillus fumigatus* and other *Aspergillus species*. *J. Clin. Microbiol.* 39, 2431–2438. doi: 10.1128/JCM.39.7.2431-2438.2001

Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Melchers, W. J., Verweij, P. E., Cuenca-Estrella, M., et al. (2007). A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrob. Agents Chemother.* 51, 1897–1904. doi: 10.1128/AAC.01092-06

Mellado, E., Garcia-Effron, G., Buitrago, M. J., Alcazar-Fuoli, L., Cuenca-Estrella, M., and Rodriguez-Tudela, J. L. (2005). Targeted gene disruption of the 14-alpha sterol demethylase (cyp51A) in *Aspergillus fumigatus* and its role in azole drug susceptibility. *Antimicrob. Agents Chemother.* 49, 2536–2538. doi: 10.1128/AAC.49.6.2536-2538.2005

Monk, B. C., Tomasiak, T. M., Keniya, M. V., Huschmann, F. U., Tyndall, J. D., O'connell, J. D., et al. (2014). Architecture of a single membrane spanning cytochrome P450 suggests constraints that orient the catalytic domain relative to a bilayer. *Proc. Natl. Acad. Sci. U.S.A.* 111, 3865–3870. doi: 10.1073/pnas.1324245111

Morano, K. A., Grant, C. M., and Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics* 190, 1157–1195. doi: 10.1534/genetics.111.128033

Morio, F., Loge, C., Besse, B., Hennequin, C., and Le Pape, P. (2010). Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. *Diagn. Microbiol. Infect. Dis.* 66, 373–384. doi: 10.1016/j.diagmicrobio.2009.11.006

Morschhauser, J. (2002). The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochim. Biophys. Acta* 1587, 240–248. doi: 10.1016/S0925-4439(02)00087-X

Morschhauser, J. (2010). Regulation of multidrug resistance in pathogenic fungi. *Fungal Genet. Biol.* 47, 94–106. doi: 10.1016/j.fgb.2009.08.002

Mortensen, K. L., Jensen, R. H., Johansen, H. K., Skov, M., Pressler, T., Howard, S. J., et al. (2011). *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J. Clin. Microbiol.* 49, 2243–2251. doi: 10.1128/JCM.0021311

Nascimento, A. M., Goldman, G. H., Park, S., Marras, S. A., Delmas, G., Oza, U., et al. (2003). Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob. Agents Chemother.* 47, 1719–1726. doi: 10.1128/AAC.47.5.1719-1726.2003

Paul, S., Diekema, D., and Moye-Rowley, W. S. (2013). Contributions of *Aspergillus fumigatus* ATP-binding cassette transporter proteins to drug resistance and virulence. *Eukaryot. Cell* 12, 1619–1628. doi: 10.1128/EC.00171-13

Paul, S., and Moye-Rowley, W. S. (2014). Multidrug resistance in fungi: regulation of transporter-encoding gene expression. *Front. Physiol.* 5:143. doi: 10.3389/fphys.2014.00143

Perea, S., Lopez-Ribot, J. L., Kirkpatrick, W. R., Mcatee, R. K., Santillan, R. A., Martinez, M., et al. (2001). Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* 45, 2676–2684. doi: 10.1128/AAC.45.10.2676-2684.2001

Pham, C. D., Reiss, E., Hagen, F., Meis, J. F., and Lockhart, S. R. (2014). Passive surveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011–2013. *Emerg. Infect. Dis.* 20, 1498–1503. doi: 10.3201/eid2009.140142

Prasad, R., and Goffeau, A. (2012). Yeast ATP-binding cassette transporters conferring multidrug resistance. *Annu. Rev. Microbiol.* 66, 39–63. doi: 10.1146/annurev-micro-092611-150111

Qiao, J., Liu, W., and Li, R. (2010). Truncated Afyap1 attenuates antifungal susceptibility of *Aspergillus fumigatus* to voriconazole and confers adaptation of the fungus to oxidative stress. *Mycopathologia* 170, 155–160. doi: 10.1007/s11046-010-9309-2

Rognon, B., Kozovska, Z., Coste, A. T., Pardini, G., and Sanglard, D. (2006). Identification of promoter elements responsible for the regulation of MDR1 from *Candida albicans*, a major facilitator transporter involved in azole resistance. *Microbiology* 152, 3701–3722. doi: 10.1099/mic.0.29277-0

Ruping, M. J., Vehreschild, J. J., and Cornely, O. A. (2008). Patients at high risk of invasive fungal infections: when and how to treat. *Drugs* 68, 1941–1962. doi: 10.2165/00003495-200868140-00002

Samaranayake, Y. H., Cheung, B. P., Wang, Y., Yau, J. Y., Yeung, K. W., and Samaranayake, L. P. (2013). Fluconazole resistance in *Candida glabrata* is associated with increased bud formation and metallothionein production. *J. Med. Microbiol.* 62, 303–318. doi: 10.1099/jmm.0.044123-0

Sanglard, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M., and Bille, J. (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob. Agents Chemother.* 40, 2378–2386. doi: 10.1128/AAC.39.11.2378

Selmecki, A., Gerami-Nejad, M., Paulson, C., Forche, A., and Berman, J. (2008). An isochromosome confers drug resistance in vivo by amplification of two genes, ERG11 and TAC1. *Mol. Microbiol.* 68, 624–641. doi: 10.1111/j.1365-2958.2008.06176.x

Slaven, J. W., Anderson, M. J., Sanglard, D., Dixon, G. K., Bille, J., Roberts, I. S., et al. (2002). Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal Genet. Biol.* 36, 199–206. doi: 10.1016/S1087-1845(02)00016-6

Snelders, E., Van Der Lee, H. A., Kuijpers, J., Rijs, A. J., Varga, J., Samson, R. A., et al. (2008). Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med.* 5:e219. doi: 10.1371/journal.pmed.0050219

Tobin, M. B., Peery, R. B., and Skatrud, P. L. (1997). Genes encoding multiple drug resistance-like proteins in *Aspergillus fumigatus* and *Aspergillus flavus*. *Gene* 200, 11–23. doi: 10.1016/S0378-1119(97)00281-3

van der Linden, J. W., Snelders, E., Kampinga, G. A., Rijnders, B. J., Mattsson, E., Debets-Ossenkopp, Y. J., et al. (2011). Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007–2009. *Emerg. Infect. Dis.* 17, 1846–1854. doi: 10.3201/eid1710.110226

Vermeulen, E., Lagrou, K., and Verweij, P. E. (2013). Azole resistance in *Aspergillus fumigatus*: a growing public health concern. *Curr. Opin. Infect. Dis.* 26, 493–500. doi: 10.1097/QCO.0000000000000005

Verweij, P. E., Mellado, E., and Melchers, W. J. (2007). Multiple-triazole-resistant aspergillosis. *N. Engl. J. Med.* 356, 1481–1483. doi: 10.1056/NEJMc061720

Verweij, P. E., Snelders, E., Kema, G. H., Mellado, E., and Melchers, W. J. (2009). Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect. Dis.* 9, 789–795. doi: 10.1016/S1473-3099(09)70265-8

Willger, S. D., Puttikamonkul, S., Kim, K. H., Burritt, J. B., Grahl, N., Metzler, L. J., et al. (2008). A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathog.* 4:e1000200. doi: 10.1371/journal.ppat.1000200

Yan, C., Lee, L. H., and Davis, L. I. (1998). Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. *EMBO J.* 17, 7416–7429. doi: 10.1093/emboj/17.24.7416

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 November 2014; accepted: 20 January 2015; published online: 10 February 2015.

Citation: Moye-Rowley WS (2015) Multiple mechanisms contribute to the development of clinically significant azole resistance in Aspergillus fumigatus. Front. Microbiol. ***6****:70. doi: 10.3389/fmicb.2015.00070*

This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology.

Copyright © 2015 Moye-Rowley. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

REVIEW
published: 16 April 2015
doi: 10.3389/fmicb.2015.00325

The *Aspergillus fumigatus* cell wall integrity signaling pathway: drug target, compensatory pathways, and virulence

Vito Valiante[1], Juliane Macheleidt[1], Martin Föge[1,2] and Axel A. Brakhage[1,2]*

[1] Molecular Biotechnology of Natural Products, Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute, Jena, Germany, [2] Department of Microbiology and Molecular Biology, Institute of Microbiology, Friedrich Schiller University, Jena, Germany

OPEN ACCESS

Edited by:
Praveen Rao Juvvadi, Duke University Medical Center, USA

Reviewed by:
Vishukumar Aimanianda, Pasteur Institute, France
Johannes Wagener, Ludwig Maximilian University Munich, Germany

***Correspondence:**
Vito Valiante, Molecular Biotechnology of Natural Products, Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute, Beutenbergstrasse 11a, 07745 Jena, Germany
vito.valiante@hki-jena.de

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *10 February 2015*
Accepted: *31 March 2015*
Published: *16 April 2015*

Citation:
Valiante V, Macheleidt J, Föge M and Brakhage AA (2015) The Aspergillus fumigatus cell wall integrity signaling pathway: drug target, compensatory pathways, and virulence. Front. Microbiol. 6:325. doi: 10.3389/fmicb.2015.00325

Aspergillus fumigatus is the most important airborne fungal pathogen, causing severe infections with invasive growth in immunocompromised patients. The fungal cell wall (CW) prevents the cell from lysing and protects the fungus against environmental stress conditions. Because it is absent in humans and because of its essentiality, the fungal CW is a promising target for antifungal drugs. Nowadays, compounds acting on the CW, i.e., echinocandin derivatives, are used to treat *A. fumigatus* infections. However, studies demonstrating the clinical effectiveness of echinocandins in comparison with antifungals currently recommended for first-line treatment of invasive aspergillosis are still lacking. Therefore, it is important to elucidate CW biosynthesis pathways and their signal transduction cascades, which potentially compensate the inhibition caused by CW- perturbing compounds. Like in other fungi, the central core of the cell wall integrity (CWI) signaling pathway in *A. fumigatus* is composed of three mitogen activated protein kinases. Deletion of these genes resulted in severely enhanced sensitivity of the mutants against CW-disturbing compounds and in drastic alterations of the fungal morphology. Additionally, several cross-talk interactions between the CWI pathways and other signaling pathways are emerging, raising the question about their role in the CW compensatory mechanisms. In this review we focused on recent advances in understanding the CWI signaling pathway in *A. fumigatus* and its role during drug stress response and virulence.

Keywords: *Aspergillus fumigatus*, cell wall integrity, signaling pathways, virulence, mitogen activated protein kinases (MAPKs)

Overview About Cell Wall Organization in *Aspergillus fumigatus*

The fungal cell wall (CW) is the exoskeleton of fungal cells. Besides its starkness and firmness, it has a very flexible structure, which, therefore, can alter rapidly and efficiently in response to external and internal stimuli. In particular, filamentous fungi adapt their internal pressure and turgor in concert with CW-biosynthesis enzymes, in order to direct hyphal growth, following gradients of nutrients, and chemo attractants (e.g., hormones), or to avoid adverse habitats (Brand and Gow, 2009; Lew, 2011).

In recent years, the composition of the fungal CW was extensively studied. It varies among different species but is mostly composed of polymers of sugars, which show a high degree of branching. Although the CW composition varies among different fungal species, there are conserved parts such as a common core composed of branched β-1,3-glucan-chitin. An exception was found in zygomycetes, which contain chitosan instead of chitin (Singh et al., 2011). The polysaccharide-based three-dimensional network in *Aspergillus fumigatus* is completed by the addition of sugar-chains composed of α-1,3-glucan, galactofuran, and mannan, which make the structure rather variable compared to other fungi (Latge and Beauvais, 2014). Besides the polysaccharide structure, the CW is adorned with a variety of proteins. In *A. fumigatus* dormant conidia, the cell surface is covered by a rodlet layer, which is composed of regularly arranged RodA hydrophobin proteins. This hydrophobin envelope was found to be essential to immunologically mask the conidia, which are continuously inhaled by humans (Aimanianda et al., 2009). Other hydrophobins, which are produced at different developmental stages in *A. fumigatus*, were associated with drug response (Gautam et al., 2008) and biofilm-growth conditions (Bruns et al., 2010).

In the CW biosynthesis, the β-1,3-glucan synthase (Fks1) plays the major role (Beauvais et al., 2001). Fks1 is an integral plasma membrane protein having 16 putative *trans*-membrane helices. The genome of the majority of fungi normally contains only a single β-1,3-glucan synthase gene. Consequently, the deletion of this gene was supposed to be lethal (Firon et al., 2002; Henry et al., 2012). However, a recent publication reported the characterisation of an *A. fumigatus* Δ*fks1* mutant, which appeared to be viable besides showing a severe growth phenotype (Dichtl et al., 2015).

The *A. fumigatus* genome harbors many putative genes responsible for CW modifications. Different β-glucanases and branching enzymes were identified (Mouyna et al., 2013). These genes are more difficult to analyze by classical reverse genetics, because some of them are apparently functionally redundant. An example is given by the β-1,3-glucanosyltransferase (Gel) family, which, in *A. fumigatus*, is composed of seven different members. Among them, only the deletion of *gel4* was reported to be lethal (Gastebois et al., 2010), whereas the Δ*gel2*, Δ*gel1*, and Δ*gel7* deletion mutant strains were viable (Mouyna et al., 2005; Zhao et al., 2014).

For chitin, which is the second most abundant polysaccharide of the CW, a very similar situation was found. Among the eight putative chitin synthase genes identified in *A. fumigatus*, only the deletion of *csmA* and *csmB* led to a significantly altered phenotype, i.e., reduction of the colony radial growth rate and decrease in chitin content (Aufauvre-Brown et al., 1997; Jimenez-Ortigosa et al., 2012), while the mutation of the remaining genes did not result in a significant phenotype, and obvious phenotypical changes of mutants were only reported upon multiple simultaneous gene disruptions (Rogg et al., 2011; Muszkieta et al., 2014).

The knowledge about genes involved in the biosynthesis of the CW is steadily increasing. In total, more than 30% of the *A. fumigatus* genome still encodes genes with unknown function (Cerqueira et al., 2014). Thus, it is reasonable to assume that in the future further enzymes involved in CW biosynthesis will be discovered, likely having novel enzymatic functions. As an example, it was predicted that *A. fumigatus* potentially expresses more than 100 different glycosylphosphatidylinositol (GPI) anchored proteins (Cao et al., 2009), which are likely to form a bridge between the membrane lipid bilayer and the CW. The majority of these proteins are supposed to play a role in the CW formation, but only very few have been characterized so far (Li et al., 2007).

Activity of Antifungal Drugs Targeting the Fungal Cell Wall and Resistance Mechanisms

From all the different classes of potential antifungal drugs, only three of them have a wide clinical use against invasive and systemic infections caused by *A. fumigatus*. These compounds belong to the polyenes, azoles, and echinocandins (Ullmann and Cornely, 2006).

Polyenes and azoles are mainly targeting ergosterol and its biosynthesis, respectively, while echinocandins specifically inhibit β-1,3-glucan formation (**Figure 1**). However, it is increasingly acknowledged that defense mechanisms against these drugs have common elements. Consistently, a genome-wide screen conducted in *Schizosaccharomyces pombe* indicated that different ergosterol biosynthesis deficient mutants were also sensitive to β-1,3-glucan synthase inhibitor echinocandins and β-glucanase (Fang et al., 2012). A similar observation was made for *Candida albicans* biofilms, which normally exhibit higher resistance to polyenes depending on ergosterol and β-1,6-glucan synthesis (Khot et al., 2006).

Polyenes belong to the oldest known antifungal compounds. Amphothericin B (AmB), first isolated from *Streptomyces nodosus*, is the most commonly used polyene against systemic infections (Blum et al., 2013). AmB possesses a name-giving amphoteric character, which allows the binding of the lipophilic compound to the cytoplasmic membrane. The mode of action of polyenes is not fully understood. They theoretically bind to all kinds of sterols, with a significant higher affinity to ergosterol (Bolard, 1986; **Figure 1A**). The fungicidal activity of AmB is potentially based on the formation of channels in the cell membrane, which lead to increased membrane permeability and leakage of small molecules, inhibition of aerobic and anaerobic respiration, and accumulation of reactive oxygen species (ROS) (Mesa-Arango et al., 2014; **Figure 1A**). It was suggested that eight AmB molecules are assembled to a ring-like structure, showing both a hydrophobic interaction to ergosterol with its conjugated double-bond system and forming a hydrophilic pore with its inward directed hydroxyl groups (Baginski et al., 1997). Thus, the sensitivity toward polyenes is dependent on the composition of membranes, with regard to sterol content, and chemical structure of phospholipid fatty acyl chains.

Surprisingly, despite the long-term use of polyenes, resistance to AmB and others polyene drugs remains rare. Nevertheless, there are resistant species of *Candida* (*C. glabrata*, *C. tropicalis*, *C. lusitaniae*), which are characterized by an intrinsically low

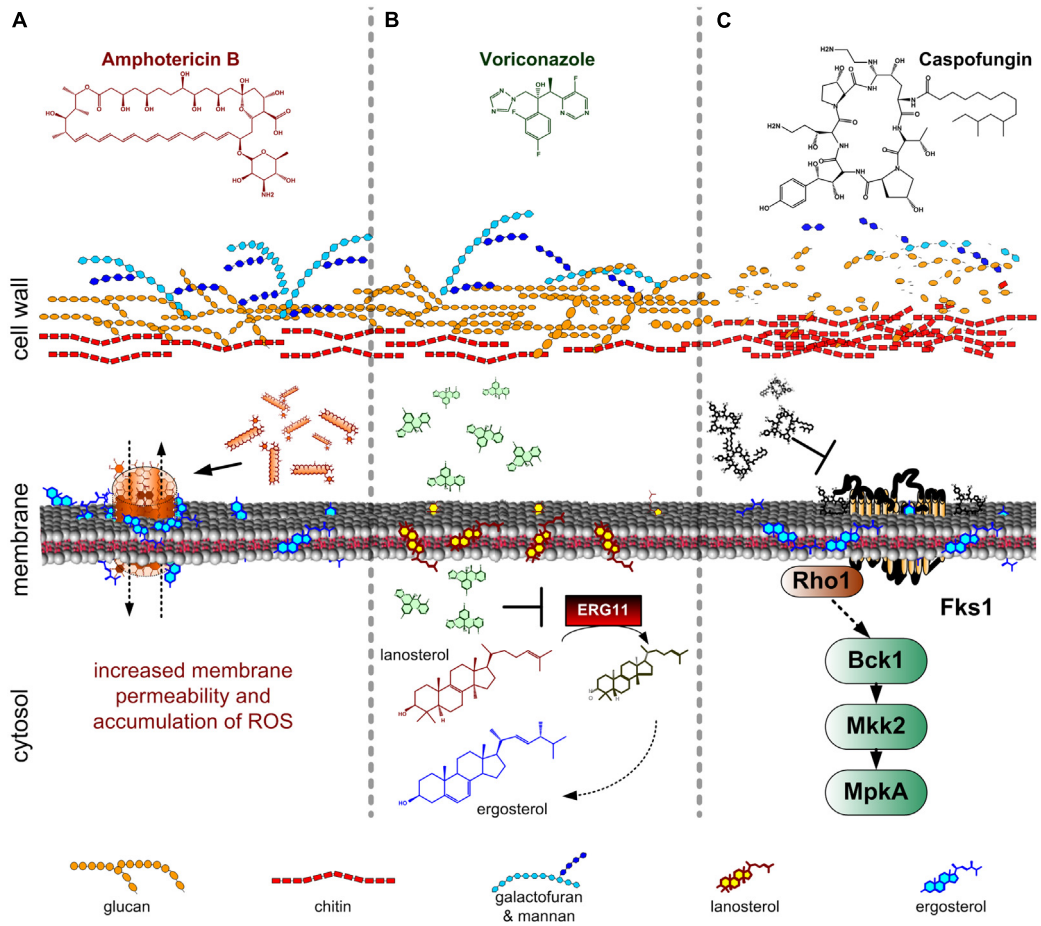


FIGURE 1 | Mode of actions of antifungal drugs commonly used against invasive and systemic *Aspergillus fumigatus* infection (Ullmann and Cornely, 2006). (A) Polyenes (in this case amphotericin **B**) bind to sterols, forming pores in the cell membrane. The formation of these pores leads to increased membrane permeability. Additionally, amphotericin B induces the accumulation of reactive oxygen species (ROS), which have multiple toxic effects on fungal cells; **(B)** Azoles (in this case voriconazole) inhibit the ERG11 enzyme thereby blocking the ergosterol biosynthesis. ERG11 catalyses the formation of 4,4-dimethylcholesta-8,14,24-trienol from lanosterol. The lack of ergosterol and the subsequent accumulation of lanosterol, results in high toxicity for the cell; **(C)** Echinocandins (in this case caspofungin) inhibit the β-1,3-glucan synthesis. As a response to the reduction of glucan content there is an increase in chitin biosynthesis.

ergosterol content (Young et al., 2003; Vandeputte et al., 2008; Gray et al., 2012; Eddouzi et al., 2013). The resistance to AmB in *Aspergillus terreus* was attributed to a higher level of catalase expression that counteracts the AmB-induced formation of ROS (Blum et al., 2013). Additionally, resistant environmental *Aspergillus flavus* strains were isolated, which were characterized by an alteration of CW composition and higher levels of α-1,3-glucan (Seo et al., 1999).

The group of azoles comprise a plethora of synthetic compounds, characterized by the presence of either a 2-N-containing imidazole ring or a 3-N-containing triazole ring with complex side-chains of at least one halogenated phenyl group (Bruggemann et al., 2009). In general, azoles show a broad spectrum of antifungal activity, a good toleration by humans and a relative low cytotoxicity, making them the major agents for treatment, and prevention of *Aspergillus* infections. There are many licensed azole antifungal drugs, but only four, namely fluconazole, itraconazole, posaconazole, and voriconazole, are mainly used for treatment of fungal infections (Bruggemann et al., 2009). They all act by inhibiting the fungal cytochrome P450 14α-sterol demethylase, which leads to a decrease in ergosterol in the fungal cytoplasmic membrane and thus to inhibition of growth (**Figure 1B**). In *A. fumigatus*, two genes have been assigned to this enzymatic function: *cyp51A* and *cyp51B* (*erg11*) (Mellado et al., 2001). The deduced proteins catalyze the oxidative removal of the 14α-methyl group of lanosterol or eburicol, respectively, (**Figure 1B**). However, single deletions of genes involved in either demethylation or desaturation of ergosterol intermediates such as *cyp51A*, *cyp51B*, and *erg3A*, *erg3B* and *erg3C*, respectively, showed differences in relative sterol composition, but further tests indicated that neither of these genes is individually essential for *A. fumigatus* survival and virulence (Mellado et al., 2005; Hu et al., 2007; Alcazar-Fuoli and Mellado, 2012).

The main mechanism of resistance of *A. fumigatus* against azole antifungal drugs is based on a mutation of the *cyp51A* gene locus with a conversion of leucine at position 98 into histidine (L98H) in combination with the appearance of a 34-bp tandem repeat in *cyp51A* promoter region (TR_{34}). In addition to TR34/L98H, also other hot spots for point mutations in the *cyp51A* gene have been reported, e.g., the exchange of glycine at pos. 54, with different impact on azole resistance in clinical isolates (Lelievre et al., 2013). Recently, a novel azole resistance mechanism was reported, which was caused by a mutation in the CCAAT-binding transcription factor (TF) complex subunit HapE (Camps et al., 2012). HapE interacts with the Hap-complex, which is, besides other functions, also important for adaptation to iron starvation and iron excess stress (Hortschansky et al., 2007).

The third and most recent class of clinically used antimycotic drugs are echinocandins. These molecules are composed of a cyclic hexapeptide core linked to a lipid side chain. Echinocandins act as non-competitive inhibitors of the β-1,3-glucan synthase (**Figure 1C**; Perez et al., 1983; Sawistowska-Schroder et al., 1984). Caspofungin was the first clinically applied echinocandin (CANCIDAS®, caspofungin acetate), which specifically targets the fungal CW assembly (Bowman et al., 2002). Inhibition of β-1,3-glucan synthesis results in inhibition of growth, increased osmotic sensitivity, and can even lead to lysis of cells. Besides their specificity, the use of echinocandins in clinical therapy is influenced by two drawbacks: the emergence of resistant strains, and the occurrence of the so-called paradoxical effect, which makes the drug less effective when used at high concentrations (Rocha et al., 2007; Wiederhold, 2009).

Until today, only little is known about naturally occurring resistance mechanisms against echinocandins. The most common mechanism is over-expression of the *fks1* gene in clinical isolates of *A. fumigatus* (Arendrup et al., 2009). Additionally, mutation of the Fks1 protein by substitution of a serine in position 678 by a proline (S678P) makes *A. fumigatus* resistant to caspofungin (Rocha et al., 2007). Apart from these changes, the adaptation of *A. fumigatus* to caspofungin is accompanied by a change in the CW sugar composition. As reported for other fungi, the exposition of *A. fumigatus* to caspofungin led as well to a decrease in β-glucan content and to an increase of chitin (Cowen and Steinbach, 2008; Verwer et al., 2012). In particular, these mechanisms seem to be related to the paradoxical effect exerted by this drug (Fortwendel et al., 2010). Potential mechanisms that have been suggested to induce chitin synthesis include manipulation of signaling pathways, such as the up-regulation of protein kinase C-encoding gene *pkcA*, a key component of the cell wall integrity (CWI) pathway, and elements acting in the calcineurin pathway (Wiederhold, 2007; Fortwendel et al., 2010).

The MAPK Cell Wall Integrity Signaling Pathway in *A. fumigatus*

Several of the major findings about signaling pathways were firstly reported in the model fungus *Saccharomyces cerevisiae* (Levin, 2011). The advances in genome sequencing allowed the identification of similar or identical signaling pathways in different fungal species, revealing the presence of highly conserved proteins that can be potentially used as antifungal drug targets (Horn et al., 2012). In particular, general signaling cascades such as the mitogen activated protein kinases (MAPKs), calcineurin, cAMP, and target of rapamycin (Tor) pathways are highly conserved in the fungal kingdom (Grosse et al., 2008; Rispail et al., 2009; Baldin et al., 2015).

Among the different signaling pathways, the MAPK CWI signaling plays a major role in CW maintenance. The CWI signal pathway in *A. fumigatus* is composed of a highly conserved module formed by three MAPKs, namely Bck1 (MAPK kinase kinase), Mkk2 (MAPK kinase), and MpkA (MAPK), which sequentially phosphorylate each other (**Figure 2**; Valiante et al., 2009; Dirr et al., 2010). Upon phosphorylation, MpkA moves into the nucleus, where it likely activates transcriptional regulators (Jain et al., 2011). The deletion of one of the three kinases of the MpkA module led to the lack of MpkA phosphorylation, which is the bottleneck in the activation of the entire pathway (Valiante et al., 2008). The three mutants appear phenotypically identical (**Figure 2**).

The lack of phosphorylation of MpkA yields mutants with a typically impaired CW, showing compact colonies and reduced filamentation (Valiante et al., 2009). Additionally, mutants of all three MAPKs are equally sensitive to all known CW-disturbing compounds, which highlight their incompetence to recover from CW stress (Valiante et al., 2008, 2009).

RNA-seq analysis performed on the Δ*mpkA* mutant of *A. fumigatus* showed that the deletion of this important kinase globally changed the expression of genes putatively involved in primary metabolism. Many genes involved in sugar and amino acid metabolism were differentially regulated, whereas many well-known CW biosynthesis genes were not affected (Müller et al., 2012). This unexpected finding suggested that the CWI pathway regulates CW biosynthesis by directly affecting the synthesis of sugars, which are the essential bricks for the CW formation. As a consequence, the role of the CWI pathway seems to be not only the mere structuring of the CW, but it is likely involved in fine-tuning the balance between stress responses and energy consumption in cellular processes required for growth and development. The latter statement is strengthened by the involvement of the CWI pathway in stress responses that are not directly connected to the CW biosynthesis. As an example, MpkA was activated during iron depletion (Jain et al., 2011). When *A. fumigatus* grows under iron starvation conditions, the fungus produces secondary metabolites named siderophores, which are derived from ornithine metabolism. Siderophores are secreted to bind external iron from the environment. The production of such metabolites is rather energy-consuming (Haas, 2014). The lack of *mpkA* in *A. fumigatus* increased the global siderophore production during iron depletion by positively regulating the metabolic flux toward the ornithine metabolic pathway. Living cells use ornithine and its precursor arginine for polyamine production. The functions of these small aliphatic molecules like putrescine and spermidine are still a matter of debate, but it was shown that their concentration

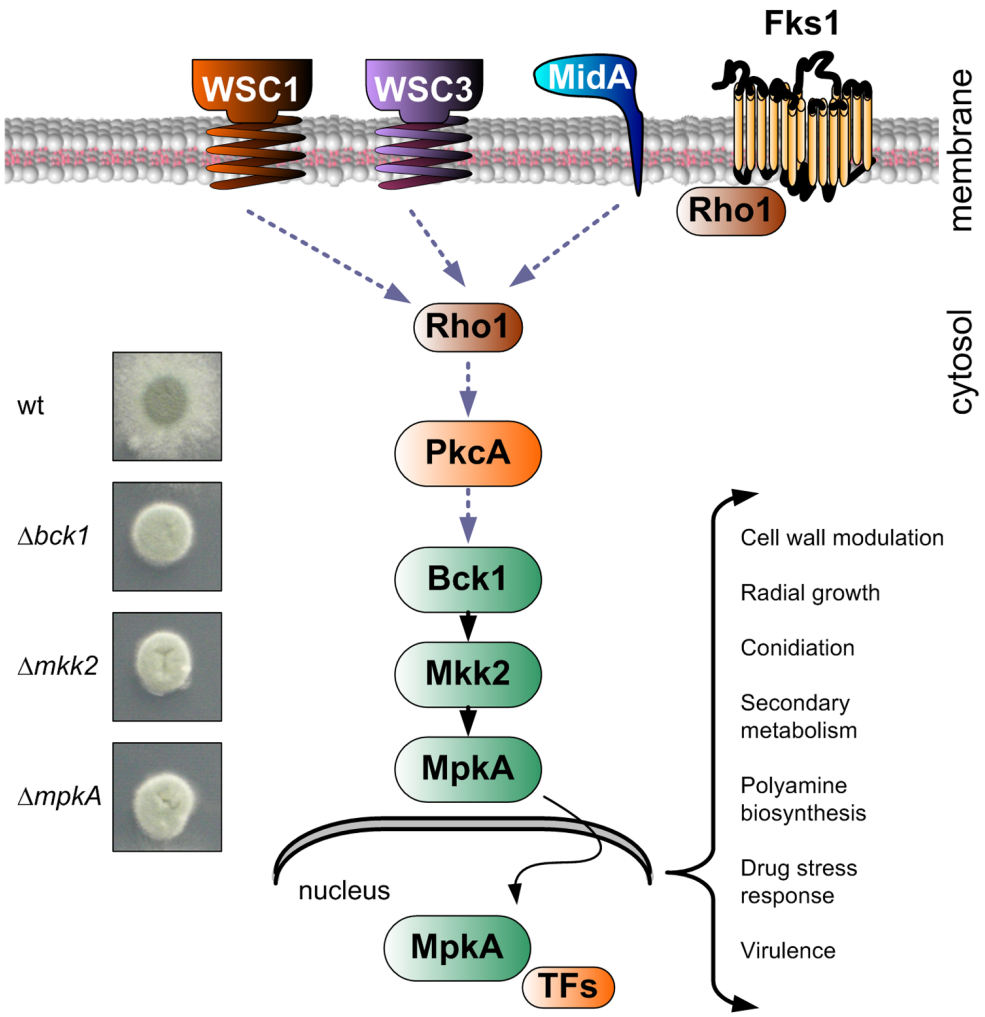


FIGURE 2 | Schematic representation of the cell wall integrity (CWI) signaling pathway in *A. fumigatus* and colony growth of indicated deletion mutants on agar plates in comparison to the wild type. The picture shows those elements that were experimentally proven to be part of the *A. fumigatus* CWI pathway, plus some important elements, whose action was predicted based on data reported in other organisms (Fks1, Rho1, and PkcA). Once phosphorylated, MpkA translocates in the nucleus, where specific transcription factors (TFs) are activated. The deletion of genes constituting the central MAPK module have identical phenotypes, resulting in a compact colony and sensitivity to all cell wall (CW)-acting compounds (Valiante et al., 2008, 2009; Jain et al., 2011; Dichtl et al., 2012).

accompanies certain developmental transitions or exposure to stress conditions (Minguet et al., 2008). In fact, the synthesis of putrescine and spermidine has been proven to be essential for life (Chattopadhyay et al., 2002). As a result, the increased production of siderophores occurring during iron starvation conditions negatively influenced the global polyamine production and storage (Jain et al., 2011). These results suggested that during iron starvation, a functional MpkA is required to act as a central regulator to balance essential polyamine formation and *de novo* siderophore production. Additionally, the latter mechanism seems to be independent from the well-known HapX-SreA iron regulation system in *A. fumigatus* (Hortschansky et al., 2007; Gsaller et al., 2014), suggesting that the role of this kinase is epistatic to HapX-SreA in the stress response process.

Besides siderophore production, the CWI pathway seems to be an important regulator of secondary metabolism in *A. fumigatus*. Transcriptome data revealed that more than 50% of the identified secondary metabolite gene clusters were affected by the lack of *mpkA* (Müller et al., 2012). This also included the production of important virulence determinants such as gliotoxin and melanins (Jain et al., 2011). Melanins are a class of brown-pigmented molecules, which are often associated with the CW. Their main role is to protect the organisms from exogenous stresses, contributing to the first line of defense against external hazards (Heinekamp et al., 2012). *A. fumigatus* produces two kinds of melanins: the dihydroxynaphthalene (DHN) melanin, which is responsible for the characteristic gray-greenish color of the conidia (Langfelder et al., 2003), and pyomelanin, which is produced during tyrosine catabolism (Schmaler-Ripcke et al.,

2009). The expression of the DHN-melanin biosynthesis gene cluster was found to be decreased in the Δ*mpkA* mutant strain. However, these mutants were still able to produce DHN-melanin, suggesting that the role of the CWI pathway in the regulation of the cluster is significant but not decisive. Concerning pyomelanin, a higher production of this compound was found in the Δ*mpkA* mutant strain (Valiante et al., 2009). These data confirmed that pyomelanin exerts a protective role, potentially compensating CW stress effects in a dysfunctional CWI background.

As summarized here, the *A. fumigatus* CWI pathway is required for many physiological states, and the CW maintenance is not its only function. It is still a matter of debate whether the CWI signaling is actively involved in the regulation of such a variety of processes or whether the observed phenotypes of corresponding deletion mutant strains are a consequence of the strong physiological impairment derived by the lack of a functional phosphorylation cascade. The meaning of the CWI signaling in *A. fumigatus* for virulence is unclear. The lack of *mpkA* did not affect virulence in a cyclophosphamide-cortisone-acetate murine infection model (Valiante et al., 2008). By contrast, the Δ*mkk2* mutant strain showing the same phenotypes as the Δ*mpkA* mutant *in vitro*, displayed reduced virulence in a murine systemic infection model (Dirr et al., 2010). These results suggest that in spite of the strongly reduced growth, the presence of neutrophil granulocytes is needed to clear the infection caused by these mutants.

Comparative analysis of signaling cascades between different fungi highlighted that the central core of signaling, i.e., kinases, are conserved, while upstream (receptors), and downstream (TFs) elements are more diverse (Rispail et al., 2009). In particular, receptor families are conserved, but vary in number. This suggests that activation of signaling is species-specific.

One of the most studied class of receptors acting upstream to the CWI signaling are the so-called WSC receptors (CWI and stress response components) (Nanduri and Tartakoff, 2001), which are characterized by the presence of highly repetitive domains containing serine and threonine residues. The WSC receptors also contain a putative carbohydrate-binding domain, which suggests that they could function as a bridge between the CW itself and the cytoplasmic membrane. Studies on the *S. cerevisiae* Wsc1 receptor suggested that these receptors work as a nanospring, able to detect/sense pulses derived from mechanical stress (Dupres et al., 2009). BLAST analysis revealed that the genome of *A. fumigatus* contains four putative genes coding for WSC receptors (*wsc1*, *wsc2*, *wsc3*, and *mid2*; Dichtl et al., 2012). All these proteins were found in the cytoplasmic membrane. However, their activity in *A. fumigatus* appears to be partially redundant, and a clearer CW phenotype was observed when multiple genes were simultaneously deleted. Additionally, the function of *wsc2* could not be related to the CWI signaling.

Other receptors putatively acting upstream of MAPK signaling pathways are the G protein coupled receptors (GPCRs). The number of these receptors also varies between different species (Grice et al., 2013). Deletion of the *gprC* and *gprD* genes in *A. fumigatus* produced mutant strains impaired in CW-related stress response (Gehrke et al., 2010).

In *S. cerevisiae*, receptors activating the MAPK signaling cascade are connected to the MAPK module *via* different signaling elements. BLAST analysis revealed that those elements are also conserved in the *A. fumigatus* genome (Rispail et al., 2009). Two of them, Rho1 and PkcA, are supposed to be essentials (Dichtl et al., 2012; De Souza et al., 2013); thus, their action should not only be related to the CWI signaling (**Figure 2**; Dichtl et al., 2010). Rho1 belongs to the Ras homolog family, which is a group of important signaling proteins (Levin, 2011). In *A. fumigatus*, Rho1 is localized in the hyphal tip, and it is misplaced by addition of farnesol (Dichtl et al., 2010), which suggests that this Ras protein interacts with putative membrane receptors to transduce external stimuli and to activate signaling cascades. A more evident phenotype was reported for the Rho guanyl-nucleotide exchange factor Rom2, which clearly localizes to the hyphal tips and septa, and its reduced expression strongly effects CW shape and stability (Samantaray et al., 2013).

Concerning PkcA (protein kinase C), there are no studies describing its function in *A. fumigatus*, but the repression of this gene in the closely related fungus *Aspergillus nidulans* affected the production of penicillin, which supports the involvement of the CWI pathway in the regulation of secondary metabolism (Herrmann et al., 2006). However, although these results suggested that the role of Rho1 and PkcA is conserved in fungi, the link between membrane receptors and the activation of the CWI pathway was shown in model yeasts, but not experimentally confirmed in *Aspergillus* species until now (Levin, 2011).

As mentioned before, the MAPK module is supposed to modulate the activity of transcriptional factors, associated with CWI signaling. Transcriptional regulators that act downstream of the CWI signaling pathway in *S. cerevisiae* were identified by BLAST analysis also in the *A. fumigatus* genome (Rispail et al., 2009; Levin, 2011), but were mostly not analyzed in detail yet. Deletion of two genes coding for putative zinc-finger TFs, named *dvrA* and *ace2*, resulted in phenotypes affecting the CW. In particular, mutants lacking *dvrA* were more resistant to nikkomycin Z, a well-known chitinase inhibitor, and more virulent compared to the wild-type strain (Ejzykowicz et al., 2010; Verwer et al., 2012). A similar phenotype was observed for the Δ*ace2* strain, which displayed abnormal pigmentation, as well as increased virulence (Ejzykowicz et al., 2009). Nonetheless, besides these phenotypes, it remains to be shown whether these TFs act downstream of the CWI signaling pathway in a MAPK-dependent manner.

Cross Talk Between Different Signaling Pathways

Aspergillus fumigatus is challenged by a multitude of external stimuli, each of them needing an appropriate response. Although several different signal transduction pathways exist to initiate the required transcriptional changes, they would not

be able to respond to environmental signals in a balanced way if they only acted separately in a linear manner. Therefore, an interaction between the pathways is likely. For *S. cerevisiae*, cross talk between the CWI pathway and other signal transduction pathways under different conditions have been described (Fuchs and Mylonakis, 2009), but in *A. fumigatus* this interesting and complex field of research is just emerging.

In *S. cerevisiae*, the connection between the high osmolarity glycerol (HOG) and the CWI signaling pathway has been elucidated in detail. Under CW stress, the pathways can display co-regulatory roles depending on the stress-inducing agent. It was shown that Hog1 (the yeast SakA ortholog) was activated in the absence of Slt2 (the yeast MpkA ortholog) under conditions causing CW stress (Bermejo et al., 2008; Garcia et al., 2009), indicating an inhibitory effect of the CWI signaling on the HOG pathway.

Candida albicans also shows the previously mentioned paradoxical effect in response to high dose exposure to caspofungin. When analyzing the response to this antifungal drug, the involvement of several signal transduction cascades was discovered, including CWI, HOG, and calcium/calmodulin-dependent calcineurin signaling, suggesting an interaction of these three pathways (Wiederhold et al., 2005; Munro et al., 2007; Walker et al., 2008). For *A. fumigatus* the participation of calcium-mediated signaling in the paradoxical growth in response to caspofungin has been described as well (Fortwendel et al., 2010). Calcium signaling involves the Ca^{2+}-binding protein calmodulin and the serine/threonine protein phosphatase calcineurin (Carafoli, 2005). Deletion of the calcineurin subunit A-encoding gene *cnaA* (also named *calA*) in *A. fumigatus* resulted in the loss of the paradoxical growth phenotype under caspofungin stress, which was attributed to the transcriptional regulation of chitin synthase-encoding genes by this signal transduction pathway (Fortwendel et al., 2010). This finding shows that the caspofungin response in *A. fumigatus*, apart from CWI and HOG signaling, also involves the calcium signal transduction cascade suggesting an interaction between all three pathways in this filamentous fungus, as it was described for *C. albicans*. This conclusion was supported by the finding that in *A. nidulans* the constitutive over-expression of protein kinase C (PkcA) can in part restore the wild-type phenotype of a Δ*cnaA* deletion mutant (Colabardini et al., 2014). In addition, CnaA affects CWI signaling by regulating MpkA phosphorylation. Moreover, PkcA has an influence on the transcription of calcium-related processes as well as on the maintenance of normal intracellular calcium levels (Colabardini et al., 2014). For the basidiomycete *Cryptococcus neoformans*, an interaction of the CWI pathway and calcium signaling has also been described (Kraus et al., 2003). This suggests that the cross talk between both pathways could be common to different fungal species.

Another survey shows that not only inhibitors of the calcineurin signal transduction cascade but also rapamycin, the inhibitor of the Tor signal transduction pathway, dramatically increased the effect of caspofungin on *A. fumigatus* (Kontoyiannis et al., 2003). As another component involved in the response to caspofungin, TOR might as well interact with CWI, HOG, or calcium signaling to coordinate the response to this drug. Altogether, this model raises the question whether in *A. fumigatus* the cross talk between signaling pathways is more pronounced than it is thought today.

In the last years, several transcriptomics studies of *A. fumigatus* signal transduction mutants were published (Malavazi et al., 2009; Jain et al., 2011; Müller et al., 2012; Macheleidt et al., 2015). The generated data suggest potential interactions of the CWI signaling cascade with other signal transduction pathways. For example, in a microarray analysis of the Δ*mpkA* strain, two calcium/calmodulin dependent kinases were found to be differentially regulated in the mutant compared to the wild type under stress conditions induced by glucanex, which lyses the CW (Jain et al., 2011). Furthermore, a microarray hybridisation approach comparing the transcriptional profile of the calcineurin mutant Δ*calA* with the wild type, found the two MAP kinase kinase-encoding genes *mkk2*, involved in CWI signaling, and *pbs2*, involved in the HOG pathway, to be significantly down-regulated in the mutant strain (Malavazi et al., 2009). Data indicate once more a potential co-regulation of central signaling pathways in *A. fumigatus*. The increasing number of transcriptome analyses will lead to the identification of further interactions between signaling cascades, and most likely reveal the complexity of signal transduction in *A. fumigatus*.

Cell Wall Impaired Mutants and Virulence

It is common praxis in infection biology to test the virulence of mutant strains in mouse infection models, in order to define whether genes have a potential function as virulence determinant. Among the different *A. fumigatus* mutant strains having a defect in the CW, more than 20 were affected in virulence (**Table 1**). Surprisingly, many mutant strains with defects in CW biosynthesis enzymes were still virulent in the applied infection models. As an example, both the α-1,3-glucan synthase mutant strains, Δ*ags1,* and Δ*ags2*, were still pathogenic, although their α-1,3-glucan content was reduced by 50% compared to the wild-type strain (Beauvais et al., 2005). Moreover, the Δ*ags3* mutant was reported to be even hypervirulent (Maubon et al., 2006). To our knowledge, only the deletion of the genes encoding for a β-1,3-glucanosyltransferase (*gel2*) and a α-1,2-mannosyltransferase (*mnt1*) resulted in a decrease of virulence (Mouyna et al., 2005; Wagener et al., 2008).

As reported above, structuring of the CW depends on different signaling pathways. The observation that these pathways are connected to each other is increasingly acknowledged. Deletion of a single gene often affected more than one specific signaling pathway. In fact, many of the reported mutants with impaired CW, which displayed decreased virulence, were

TABLE 1 | The table lists all *A. fumigatus* mutant strains that show an altered CW structure and were reported to be involved in virulence.

Accession numbers	Associate function	Name	Function related to the cell wall (CW)	Virulence of loss-of-function mutant	Reference
AFUA_1G05800	MAP kinase kinase	*mkk2*	Essential for cell CWI signaling	Decreased	Dirr et al. (2010)
AFUA_1G09280	Protein phosphatase 2C	*ptcB*	Deletion strain is more sensitive to CW-acting compounds	Decreased	Winkelstroter et al. (2015)
AFUA_1G10880	P-type calcium ATPase	*pmcA*	Gene deletion affects normal growth, CW shape conidiation, and virulence	Decreased	Dinamarco et al. (2012)
AFUA_1G14660	Methyltransferase	*laeA*	Gene deletion affects external hydrophobin layer	Decreased	Bok et al. (2005); Dagenais et al. (2010)
AFUA_1G15440	α-1,3-glucan synthase	*ags3*	Mutants showed an increased rate of germination and melanin production	Increased	Maubon et al. (2006)
AFUA_1G16950	Protein required for glycosylphosphatidylinositol (GPI)-anchor biosynthesis	*pig-a*	Required for the CWI	Decreased	Li et al. (2007)
AFUA_2G07770	Small monomeric GTPase Ras	*rasB*	Gene deletion led to irregular hyphal morphology	Decreased	Fortwendel et al. (2005)
AFUA_2G12200	cAMP-dependent protein kinase catalytic subunit 1	*pkaC1*	Important for regulation of germination, CW homeostasis, and growth	Decreased	Fuller et al. (2011)
AFUA_2G12640	G-protein coupled receptor	*gprD*	Lack of function produces CW impaired mutants	Decreased	Gehrke et al. (2010)
AFUA_2G17600	Polyketide synthase	*pksP*	Deletion blocks DHN-melanin production, production of white conidia	Decreased	Jahn et al. (1997)
AFUA_3G05650	αα-trehalose-phosphate synthase subunit TPS2	*orlA*	Gene deletion affects CWI and tolerance to high temperature	Decreased	Puttikamonkul et al. (2010)
AFUA_3G09820	C2H2 zinc finger domain protein	*dvrA*	Deletion strain is more resistant to nikkomycin Z	Increased	Ejzykowicz et al. (2010)
AFUA_3G11250	C2H2 transcription factor (TF)	*ace2*	Deletion strain displayed an abnormal conidial CW architecture	Increased	Ejzykowicz et al. (2009)
AFUA_3G12690	Putative UDP-galactopyranose mutase	*glfA*	Mutant results in a thinner CW	Decreased	Schmalhorst et al. (2008)
AFUA_4G06820	Related to sporulation-specific gene SPS2	*ecm33*	Putative glycophosphatidylinositol (GPI)-anchored CW protein	Increased	Romano et al. (2006)
AFUA_5G04170	Heat shock protein	*hsp90*	Over-expression leads to hypersensitivity to caspofungin	Decreased	Lamoth et al. (2012)
AFUA_5G08570	protein kinase A catalytic subunit 2	*pkaC2*	Important for regulation of germination, CW homeostasis, and growth	Decreased	Fuller et al. (2011)
AFUA_5G09360	Calcineurin A	*calA*	Gene deletion affects normal growth, CWI, conidiation, and virulence	Decreased	Steinbach et al. (2006)
AFUA_5G09580	Conidial hydrophobin	*rodA*	Deletion increases surface exposure of β1,3-glucan and α-mannose	Decreased	Carrion Sde et al. (2013)
AFUA_5G10760	α-1,2-mannosyltransferase	*mnt1*	Deletion leads to a higher sensitivity to calcofluor white and congo red	Decreased	Wagener et al. (2008)
AFUA_5G11230	Small monomeric GTPase Ras	*rasA*	Lack of function produces CW impaired mutants	Decreased	Fortwendel et al. (2012)
AFUA_6G10240	Sensor histidine kinase/response regulator	*fos-1 (tcsA)*	Putative histidine kinase, two-component signal transduction protein	Decreased	Clemons et al. (2002)
AFUA_6G11390	β-1,3-glucanosyltransferase	*gel2*	This gene exerts a role in conidiogenesis and CW composition	Decreased	Mouyna et al. (2005)
AFUA_7G04800	G-protein coupled receptor	*gprC*	Lack of function results in CW impaired mutants	Decreased	Gehrke et al. (2010)

obtained by deleting genes putatively involved in signaling, such as Δ*calA*, Δ*rasA*, and *B*, and Δ*pkaC* mutants (Fortwendel et al., 2005, 2012; Steinbach et al., 2006; Fuller et al., 2011). Recently, it was reported that the deletion of *ptcB*, a putative protein phosphatase 2C, positively affected the phosphorylation status of both MpkA and SakA, resulting in decreased virulence (Winkelstroter et al., 2015). Similarly, blocking of CWI signaling led to reduced virulence (Dirr et al., 2010). However, blocking of CWI signaling also resulted in various physiological alterations, which are apparently not directly connected to the CW (e.g., alteration of secondary metabolism; Jain et al., 2011).

Perspective

A major question concerns the selection of suitable and effective targets for future antifungal drugs. The three clinically important classes of antifungal drugs used against *A. fumigatus* target either enzymatic steps involved in cell membrane/CW biosynthesis or ergosterol as part of the cytoplasmic membrane. These drugs show some limitations either because they cannot really clear *A. fumigatus* infection, or because resistant strains were isolated and are therefore of major clinical concern. The increase of life-threatening mycoses accompanied with the lack of effective drugs, has fostered the search for new, broad-spectrum fungicidal agents. Drug efficacy could also be increased by the reformulation of existing antifungals as well as the search for synergistically acting compounds that can be used for treatment and prophylaxis. As an example, the effectiveness of caspofungin in prophylaxis is still a matter of debate, and there are studies indicating that caspofungin did not decrease the mortality rate of patients with diagnosed invasive aspergillosis (Karthaus, 2011). In future, because of their low toxicity, echinocandins might well be used in a combinatorial antifungal therapy with other synergistically acting drugs (Deresinski and Stevens, 2003). In line, it was already reported that the combination of caspofungin with azoles or AmB increased caspofungin activity *in vitro* (Dannaoui et al., 2004; Liu et al., 2012). Additionally, the combination of these drugs improved the efficacy of treatments of patients with severe fungal infections, in which the first line therapy failed (Nivoix et al., 2006).

The fungal CW still remains a very powerful target for antifungal drugs. However, recent studies suggest that the signaling pathways responsible for CW formation have not been completely elucidated. It remains to be shown which signaling pathways act as compensatory pathways that decrease the effectiveness of drug treatments. The identification of such pathways could lead to the discovery of new targets and new modes of action that can be exploited to potentiate the efficiency of known drugs, and to improve prophylaxis against invasive mycoses.

Acknowledgments

This work was supported by the excellence graduate school *Jena School for Microbial Communication* (JSMC) and the ERA-NET PathoGenoMics program ANTIFUN (The CW as a target to improve antifungal therapy against Aspergillosis), BFU2008-04709-E/BMC (German Federal Ministry of Education and Research: BMBF FKZ 0315439).

References

Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S. R., et al. (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 460, 1117–1121. doi: 10.1038/nature08264

Alcazar-Fuoli, L., and Mellado, E. (2012). Ergosterol biosynthesis in *Aspergillus fumigatus*: its relevance as an antifungal target and role in antifungal drug resistance. *Front. Microbiol.* 3:439. doi: 10.3389/fmicb.2012.00439

Arendrup, M. C., Garcia-Effron, G., Buzina, W., Mortensen, K. L., Reiter, N., Lundin, C., et al. (2009). Breakthrough *Aspergillus fumigatus* and *Candida albicans* double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. *Antimicrob. Agents Chemother.* 53, 1185–1193. doi: 10.1128/AAC.01292-08

Aufauvre-Brown, A., Mellado, E., Gow, N. A., and Holden, D. W. (1997). *Aspergillus fumigatus* chsE: a gene related to CHS3 of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. *Fungal. Genet. Biol.* 21, 141–152. doi: 10.1006/fgbi.1997.0959

Baginski, M., Resat, H., and Mccammon, J. A. (1997). Molecular properties of amphotericin B membrane channel: a molecular dynamics simulation. *Mol. Pharmacol.* 52, 560–570.

Baldin, C., Valiante, V., Krüger, T., Schafferer, L., Haas, H., Kniemeyer, O., et al. (2015). Comparative proteomics of a tor inducible *Aspergillus fumigatus* mutant reveals involvement of the Tor kinase in iron regulation. *Proteomics* doi: 10.1002/pmic.201400584 [Epub ahead of print].

Beauvais, A., Bruneau, J. M., Mol, P. C., Buitrago, M. J., Legrand, R., and Latge, J. P. (2001). Glucan synthase complex of *Aspergillus fumigatus*. *J. Bacteriol.* 183, 2273–2279. doi: 10.1128/JB.183.7.2273-2279.2001

Beauvais, A., Maubon, D., Park, S., Morelle, W., Tanguy, M., Huerre, M., et al. (2005). Two alpha(1-3) glucan synthases with different functions in *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 71, 1531–1538. doi: 10.1128/AEM.71.3.1531-1538.2005

Bermejo, C., Rodriguez, E., Garcia, R., Rodriguez-Pena, J. M., Rodriguez De La Concepcion, M. L., Rivas, C., et al. (2008). The sequential activation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. *Mol. Biol. Cell* 19, 1113–1124. doi: 10.1091/mbc.E07-08-0742

Blum, G., Hortnagl, C., Jukic, E., Erbeznik, T., Pumpel, T., Dietrich, H., et al. (2013). New insight into amphotericin B resistance in *Aspergillus terreus*. *Antimicrob. Agents Chemother.* 57, 1583–1588. doi: 10.1128/AAC.01283-12

Bok, J. W., Balajee, S. A., Marr, K. A., Andes, D., Nielsen, K. F., Frisvad, J. C., et al. (2005). LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryot. Cell* 4, 1574–1582. doi: 10.1128/EC.4.9.1574-1582.2005

Bolard, J. (1986). How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim. Biophys. Acta* 864, 257–304. doi: 10.1016/0304-4157(86)90002-X

Bowman, J. C., Hicks, P. S., Kurtz, M. B., Rosen, H., Schmatz, D. M., Liberator, P. A., et al. (2002). The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. *Antimicrob. Agents Chemother.* 46, 3001–3012. doi: 10.1128/AAC.46.9.3001-3012.2002

Brand, A., and Gow, N. A. (2009). Mechanisms of hypha orientation of fungi. *Curr. Opin. Microbiol.* 12, 350–357. doi: 10.1016/j.mib.2009.05.007

Bruggemann, R. J., Alffenaar, J. W., Blijlevens, N. M., Billaud, E. M., Kosterink, J. G., Verweij, P. E., et al. (2009). Clinical relevance of the pharmacokinetic interactions of azole antifungal drugs with other coadministered agents. *Clin. Infect. Dis.* 48, 1441–1458. doi: 10.1086/598327

Bruns, S., Seidler, M., Albrecht, D., Salvenmoser, S., Remme, N., Hertweck, C., et al. (2010). Functional genomic profiling of *Aspergillus fumigatus* biofilm reveals enhanced production of the mycotoxin gliotoxin. *Proteomics* 10, 3097–3107. doi: 10.1002/pmic.201000129

Camps, S. M., Dutilh, B. E., Arendrup, M. C., Rijs, A. J., Snelders, E., Huynen, M. A., et al. (2012). Discovery of a HapE mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. *PLoS ONE* 7:e50034. doi: 10.1371/journal.pone.0050034

Cao, W., Maruyama, J., Kitamoto, K., Sumikoshi, K., Terada, T., Nakamura, S., et al. (2009). Using a new GPI-anchored-protein identification system to mine the protein databases of *Aspergillus fumigatus*, *Aspergillus nidulans*, and *Aspergillus oryzae*. *J. Gen. Appl. Microbiol.* 55, 381–393. doi: 10.2323/jgam.55.381

Carafoli, E. (2005). Calcium–a universal carrier of biological signals. Delivered on 3 July 2003 at the special FEBS meeting in Brussels. *FEBS J.* 272, 1073–1089. doi: 10.1111/j.1742-4658.2005.04546.x

Carrion Sde, J., Leal, S. M. Jr., Ghannoum, M. A., Aimanianda, V., Latge, J. P., and Pearlman, E. (2013). The RodA hydrophobin on *Aspergillus fumigatus* spores masks dectin-1- and dectin-2-dependent responses and enhances fungal survival in vivo. *J. Immunol.* 191, 2581–2588. doi: 10.4049/jimmunol.1300748

Cerqueira, G. C., Arnaud, M. B., Inglis, D. O., Skrzypek, M. S., Binkley, G., Simison, M., et al. (2014). The *Aspergillus* genome database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations. *Nucleic Acids Res.* 42, D705–D710. doi: 10.1093/nar/gkt1029

Chattopadhyay, M. K., Tabor, C. W., and Tabor, H. (2002). Absolute requirement of spermidine for growth and cell cycle progression of fission yeast (*Schizosaccharomyces pombe*). *Proc. Natl. Acad. Sci. U.S.A.* 99, 10330–10334. doi: 10.1073/pnas.162362899

Clemons, K. V., Miller, T. K., Selitrennikoff, C. P., and Stevens, D. A. (2002). fos-1, a putative histidine kinase as a virulence factor for systemic aspergillosis. *Med. Mycol.* 40, 259–262. doi: 10.1080/714031112

Colabardini, A. C., Ries, L. N., Brown, N. A., Savoldi, M., Dinamarco, T. M., Von Zeska, M. R., et al. (2014). Protein kinase C overexpression suppresses calcineurin-associated defects in *Aspergillus nidulans* and is involved in mitochondrial function. *PLoS ONE* 9:e104792. doi: 10.1371/journal.pone.0104792

Cowen, L. E., and Steinbach, W. J. (2008). Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance. *Eukaryot. Cell* 7, 747–764. doi: 10.1128/EC.00041-08

Dagenais, T. R., Giles, S. S., Aimanianda, V., Latge, J. P., Hull, C. M., and Keller, N. P. (2010). *Aspergillus fumigatus* LaeA-mediated phagocytosis is associated with a decreased hydrophobin layer. *Infect. Immun.* 78, 823–829. doi: 10.1128/IAI.00980-09

Dannaoui, E., Lortholary, O., and Dromer, F. (2004). In vitro evaluation of double and triple combinations of antifungal drugs against *Aspergillus fumigatus* and *Aspergillus terreus*. *Antimicrob. Agents Chemother.* 48, 970–978. doi: 10.1128/AAC.48.3.970-978.2004

Deresinski, S. C., and Stevens, D. A. (2003). Caspofungin. *Clin. Infect. Dis.* 36, 1445–1457. doi: 10.1086/375080

De Souza, C. P., Hashmi, S. B., Osmani, A. H., Andrews, P., Ringelberg, C. S., Dunlap, J. C., et al. (2013). Functional analysis of the *Aspergillus* nidulans kinome. *PLoS ONE* 8:e58008. doi: 10.1371/journal.pone.0058008

Dichtl, K., Ebel, F., Dirr, F., Routier, F. H., Heesemann, J., and Wagener, J. (2010). Farnesol misplaces tip-localized Rho proteins and inhibits cell wall integrity signalling in *Aspergillus fumigatus*. *Mol. Microbiol.* 76, 1191–1204. doi: 10.1111/j.1365-2958.2010.07170.x

Dichtl, K., Helmschrott, C., Dirr, F., and Wagener, J. (2012). Deciphering cell wall integrity signalling in *Aspergillus fumigatus*: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. *Mol. Microbiol.* 83, 506–519. doi: 10.1111/j.1365-2958.2011.07946.x

Dichtl, K., Samantaray, S., Aimanianda, V., Zhu, Z., Prevost, M. C., Latge, J. P., et al. (2015). *Aspergillus fumigatus* devoid of cell wall beta-1,3-glucan is viable, massively sheds galactomannan and is killed by septum formation inhibitors. *Mol. Microbiol.* 95, 458–471. doi: 10.1111/mmi.12877

Dinamarco, T. M., Freitas, F. Z., Almeida, R. S., Brown, N. A., Dos Reis, T. F., Ramalho, L. N., et al. (2012). Functional characterization of an *Aspergillus fumigatus* calcium transporter (PmcA) that is essential for fungal infection. *PLoS ONE* 7:e37591. doi: 10.1371/journal.pone.0037591

Dirr, F., Echtenacher, B., Heesemann, J., Hoffmann, P., Ebel, F., and Wagener, J. (2010). AfMkk2 is required for cell wall integrity signaling, adhesion, and full virulence of the human pathogen *Aspergillus fumigatus*. *Int. J. Med. Microbiol.* 300, 496–502. doi: 10.1016/j.ijmm.2010.03.001

Dupres, V., Alsteens, D., Wilk, S., Hansen, B., Heinisch, J. J., and Dufrene, Y. F. (2009). The yeast Wsc1 cell surface sensor behaves like a nanospring in vivo. *Nat. Chem. Biol.* 5, 857–862. doi: 10.1038/nchembio.220

Eddouzi, J., Parker, J. E., Vale-Silva, L. A., Coste, A., Ischer, F., Kelly, S., et al. (2013). Molecular mechanisms of drug resistance in clinical Candida species isolated from Tunisian hospitals. *Antimicrob. Agents Chemother.* 57, 3182–3193. doi: 10.1128/AAC.00555-13

Ejzykowicz, D. E., Cunha, M. M., Rozental, S., Solis, N. V., Gravelat, F. N., Sheppard, D. C., et al. (2009). The *Aspergillus fumigatus* transcription factor Ace2 governs pigment production, conidiation and virulence. *Mol. Microbiol.* 72, 155–169. doi: 10.1111/j.1365-2958.2009.06631.x

Ejzykowicz, D. E., Solis, N. V., Gravelat, F. N., Chabot, J., Li, X., Sheppard, D. C., et al. (2010). Role of *Aspergillus fumigatus* DvrA in host cell interactions and virulence. *Eukaryot. Cell* 9, 1432–1440. doi: 10.1128/EC.00055-10

Fang, Y., Hu, L., Zhou, X., Jaiseng, W., Zhang, B., Takami, T., et al. (2012). A genomewide screen in *Schizosaccharomyces* pombe for genes affecting the sensitivity of antifungal drugs that target ergosterol biosynthesis. *Antimicrob. Agents Chemother.* 56, 1949–1959. doi: 10.1128/AAC.05126-11

Firon, A., Beauvais, A., Latge, J. P., Couve, E., Grosjean-Cournoyer, M. C., and D'enfert, C. (2002). Characterization of essential genes by parasexual genetics in the human fungal pathogen *Aspergillus fumigatus*: impact of genomic rearrangements associated with electroporation of DNA. *Genetics* 161, 1077–1087.

Fortwendel, J. R., Juvvadi, P. R., Perfect, B. Z., Rogg, L. E., Perfect, J. R., and Steinbach, W. J. (2010). Transcriptional regulation of chitin synthases by calcineurin controls paradoxical growth of *Aspergillus fumigatus* in response to caspofungin. *Antimicrob. Agents Chemother.* 54, 1555–1563. doi: 10.1128/AAC.00854-09

Fortwendel, J. R., Juvvadi, P. R., Rogg, L. E., Asfaw, Y. G., Burns, K. A., Randell, S. H., et al. (2012). Plasma membrane localization is required for RasA-mediated polarized morphogenesis and virulence of *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 966–977. doi: 10.1128/EC.00091-12

Fortwendel, J. R., Zhao, W., Bhabhra, R., Park, S., Perlin, D. S., Askew, D. S., et al. (2005). A fungus-specific ras homolog contributes to the hyphal growth and virulence of *Aspergillus fumigatus*. *Eukaryot. Cell* 4, 1982–1989. doi: 10.1128/EC.4.12.1982-1989.2005

Fuchs, B. B., and Mylonakis, E. (2009). Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. *Eukaryot. Cell* 8, 1616–1625. doi: 10.1128/EC.00193-09

Fuller, K. K., Richie, D. L., Feng, X., Krishnan, K., Stephens, T. J., Wikenheiser-Brokamp, K. A., et al. (2011). Divergent Protein Kinase A isoforms co-ordinately regulate conidial germination, carbohydrate metabolism and virulence in *Aspergillus fumigatus*. *Mol. Microbiol.* 79, 1045–1062. doi: 10.1111/j.1365-2958.2010.07509.x

Garcia, R., Rodriguez-Pena, J. M., Bermejo, C., Nombela, C., and Arroyo, J. (2009). The high osmotic response and cell wall integrity pathways cooperate to regulate transcriptional responses to zymolyase-induced cell wall stress in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 284, 10901–10911. doi: 10.1074/jbc.M808693200

Gastebois, A., Mouyna, I., Simenel, C., Clavaud, C., Coddeville, B., Delepierre, M., et al. (2010). Characterization of a new beta(1-3)-glucan branching activity of *Aspergillus fumigatus*. *J. Biol. Chem.* 285, 2386–2396. doi: 10.1074/jbc.M109.077545

Gautam, P., Shankar, J., Madan, T., Sirdeshmukh, R., Sundaram, C. S., Gade, W. N., et al. (2008). Proteomic and transcriptomic analysis of *Aspergillus fumigatus* on exposure to amphotericin B. *Antimicrob. Agents Chemother.* 52, 4220–4227. doi: 10.1128/AAC.01431-07

Gehrke, A., Heinekamp, T., Jacobsen, I. D., and Brakhage, A. A. (2010). Heptahelical receptors GprC and GprD of *Aspergillus fumigatus* Are essential regulators of colony growth, hyphal morphogenesis, and virulence. *Appl. Environ. Microbiol.* 76, 3989–3998. doi: 10.1128/AEM.00052-10

Gray, K. C., Palacios, D. S., Dailey, I., Endo, M. M., Uno, B. E., Wilcock, B. C., et al. (2012). Amphotericin primarily kills yeast by simply binding ergosterol. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2234–2239. doi: 10.1073/pnas.1117280109

Grice, C. M., Bertuzzi, M., and Bignell, E. M. (2013). Receptor-mediated signaling in *Aspergillus fumigatus*. *Front. Microbiol.* 4:26. doi: 10.3389/fmicb.2013.00026

Grosse, C., Heinekamp, T., Kniemeyer, O., Gehrke, A., and Brakhage, A. A. (2008). Protein kinase A regulates growth, sporulation, and pigment formation in *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 74, 4923–4933. doi: 10.1128/AEM.00470-08

Gsaller, F., Hortschansky, P., Beattie, S. R., Klammer, V., Tuppatsch, K., Lechner, B. E., et al. (2014). The Janus transcription factor HapX controls fungal adaptation to both iron starvation and iron excess. *EMBO J.* 33, 2261–2276. doi: 10.15252/embj.201489468

Haas, H. (2014). Fungal siderophore metabolism with a focus on *Aspergillus fumigatus*. *Nat. Prod. Rep.* 31, 1266–1276. doi: 10.1039/C4NP00071D

Heinekamp, T., Thywissen, A., Macheleidt, J., Keller, S., Valiante, V., and Brakhage, A. A. (2012). *Aspergillus fumigatus* melanins: interference with the

host endocytosis pathway and impact on virulence. *Front. Microbiol.* 3:440. doi: 10.3389/fmicb.2012.00440

Henry, C., Latge, J. P., and Beauvais, A. (2012). alpha1,3 glucans are dispensable in *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 26–29. doi: 10.1128/EC.05270-11

Herrmann, M., Sprote, P., and Brakhage, A. A. (2006). Protein kinase C (PkcA) of *Aspergillus* nidulans is involved in penicillin production. *Appl. Environ. Microbiol.* 72, 2957–2970. doi: 10.1128/AEM.72.4.2957-2970.2006

Horn, F., Heinekamp, T., Kniemeyer, O., Pollmacher, J., Valiante, V., and Brakhage, A. A. (2012). Systems biology of fungal infection. *Front. Microbiol.* 3:108. doi: 10.3389/fmicb.2012.00108

Hortschansky, P., Eisendle, M., Al-Abdallah, Q., Schmidt, A. D., Bergmann, S., Thon, M., et al. (2007). Interaction of HapX with the CCAAT-binding complex–a novel mechanism of gene regulation by iron. *EMBO J.* 26, 3157–3168. doi: 10.1038/sj.emboj.7601752

Hu, W., Sillaots, S., Lemieux, S., Davison, J., Kauffman, S., Breton, A., et al. (2007). Essential gene identification and drug target prioritization in *Aspergillus fumigatus*. *PLoS Pathog* 3:e24. doi: 10.1371/journal.ppat.0030024

Jahn, B., Koch, A., Schmidt, A., Wanner, G., Gehringer, H., Bhakdi, S., et al. (1997). Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence. *Infect. Immun.* 65, 5110–5117.

Jain, R., Valiante, V., Remme, N., Docimo, T., Heinekamp, T., Hertweck, C., et al. (2011). The MAP kinase MpkA controls cell wall integrity, oxidative stress response, gliotoxin production and iron adaptation in *Aspergillus fumigatus*. *Mol. Microbiol.* 82, 39–53. doi: 10.1111/j.1365-2958.2011.07778.x

Jimenez-Ortigosa, C., Aimanianda, V., Muszkieta, L., Mouyna, I., Alsteens, D., Pire, S., et al. (2012). Chitin synthases with a myosin motor-like domain control the resistance of *Aspergillus fumigatus* to echinocandins. *Antimicrob. Agents Chemother.* 56, 6121–6131. doi: 10.1128/AAC.00752-12

Karthaus, M. (2011). Prophylaxis and treatment of invasive aspergillosis with voriconazole, posaconazole and caspofungin: review of the literature. *Eur. J. Med. Res.* 16, 145–152. doi: 10.1186/2047-783X-16-4-145

Khot, P. D., Suci, P. A., Miller, R. L., Nelson, R. D., and Tyler, B. J. (2006). A small subpopulation of blastospores in candida albicans biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and beta-1,6-glucan pathway genes. *Antimicrob. Agents Chemother.* 50, 3708–3716. doi: 10.1128/AAC.00997-06

Kontoyiannis, D. P., Lewis, R. E., Osherov, N., Albert, N. D., and May, G. S. (2003). Combination of caspofungin with inhibitors of the calcineurin pathway attenuates growth in vitro in *Aspergillus* species. *J. Antimicrob. Chemother.* 51, 313–316. doi: 10.1093/jac/dkg090

Kraus, P. R., Fox, D. S., Cox, G. M., and Heitman, J. (2003). The Cryptococcus neoformans MAP kinase Mpk1 regulates cell integrity in response to antifungal drugs and loss of calcineurin function. *Mol. Microbiol.* 48, 1377–1387. doi: 10.1046/j.1365-2958.2003.03508.x

Lamoth, F., Juvvadi, P. R., Fortwendel, J. R., and Steinbach, W. J. (2012). Heat shock protein 90 is required for conidiation and cell wall integrity in *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 1324–1332. doi: 10.1128/EC.00032-12

Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhage, A. A. (2003). Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal. Genet. Biol.* 38, 143–158. doi: 10.1016/S1087-1845(02)00526-1

Latge, J. P., and Beauvais, A. (2014). Functional duality of the cell wall. *Curr. Opin. Microbiol.* 20, 111–117. doi: 10.1016/j.mib.2014.05.009

Lelievre, L., Groh, M., Angebault, C., Maherault, A. C., Didier, E., and Bougnoux, M. E. (2013). Azole resistant *Aspergillus fumigatus*: an emerging problem. *Med. Mal. Infect.* 43, 139–145. doi: 10.1016/j.medmal.2013.02.010

Levin, D. E. (2011). Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. *Genetics* 189, 1145–1175. doi: 10.1534/genetics.111.128264

Lew, R. R. (2011). How does a hypha grow? The biophysics of pressurized growth in fungi. *Nat. Rev. Microbiol.* 9, 509–518. doi: 10.1038/nrmicro2591

Li, H., Zhou, H., Luo, Y., Ouyang, H., Hu, H., and Jin, C. (2007). Glycosylphosphatidylinositol (GPI) anchor is required in *Aspergillus fumigatus* for morphogenesis and virulence. *Mol. Microbiol.* 64, 1014–1027. doi: 10.1111/j.1365-2958.2007.05709.x

Liu, W., Li, L., Sun, Y., Chen, W., Wan, Z., Li, R., et al. (2012). Interaction of the echinocandin caspofungin with amphotericin B or voriconazole against *Aspergillus biofilms* in vitro. *Antimicrob. Agents Chemother.* 56, 6414–6416. doi: 10.1128/AAC.00687-12

Macheleidt, J., Scherlach, K., Neuwirth, T., Schmidt-Heck, W., Straßburger, M., Spraker, J., et al. (2015). Transcriptome analysis of cAMP-dependent protein kinase A regulated genes reveals the production of the novel natural compound fumipyrrole by *Aspergillus fumigatus*. *Mol. Microbiol.* 96, 148–162. doi: 10.1111/mmi.12926

Malavazi, I., Da Silva Ferreira, M. E., Soriani, F. M., Dinamarco, T. M., Savoldi, M., Uyemura, S. A., et al. (2009). Phenotypic analysis of genes whose mRNA accumulation is dependent on calcineurin in *Aspergillus fumigatus*. *Fungal Genet. Biol.* 46, 791–802. doi: 10.1016/j.fgb.2009.06.009

Maubon, D., Park, S., Tanguy, M., Huerre, M., Schmitt, C., Prevost, M. C., et al. (2006). AGS3, an alpha(1-3)glucan synthase gene family member of *Aspergillus fumigatus*, modulates mycelium growth in the lung of experimentally infected mice. *Fungal Genet. Biol.* 43, 366–375. doi: 10.1016/j.fgb.2006.01.006

Mellado, E., Diaz-Guerra, T. M., Cuenca-Estrella, M., and Rodriguez-Tudela, J. L. (2001). Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in *Aspergillus fumigatus* and other *Aspergillus* species. *J. Clin. Microbiol.* 39, 2431–2438. doi: 10.1128/JCM.39.7.2431-2438.2001

Mellado, E., Garcia-Effron, G., Buitrago, M. J., Alcazar-Fuoli, L., Cuenca-Estrella, M., and Rodriguez-Tudela, J. L. (2005). Targeted gene disruption of the 14-alpha sterol demethylase (cyp51A) in *Aspergillus fumigatus* and its role in azole drug susceptibility. *Antimicrob. Agents Chemother.* 49, 2536–2538. doi: 10.1128/AAC.49.6.2536-2538.2005

Mesa-Arango, A. C., Trevijano-Contador, N., Roman, E., Sanchez-Fresneda, R., Casas, C., Herrero, E., et al. (2014). The production of reactive oxygen species is a universal action mechanism of Amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug. *Antimicrob. Agents Chemother.* 58, 6627–6638. doi: 10.1128/AAC.03570-14

Minguet, E. G., Vera-Sirera, F., Marina, A., Carbonell, J., and Blazquez, M. A. (2008). Evolutionary diversification in polyamine biosynthesis. *Mol. Biol. Evol.* 25, 2119–2128. doi: 10.1093/molbev/msn161

Mouyna, I., Hartl, L., and Latge, J. P. (2013). beta-1,3-glucan modifying enzymes in *Aspergillus fumigatus*. *Front. Microbiol.* 4:81. doi: 10.3389/fmicb.2013.00081

Mouyna, I., Morelle, W., Vai, M., Monod, M., Lechenne, B., Fontaine, T., et al. (2005). Deletion of GEL2 encoding for a beta(1-3)glucanosyltransferase affects morphogenesis and virulence in *Aspergillus fumigatus*. *Mol. Microbiol.* 56, 1675–1688. doi: 10.1111/j.1365-2958.2005.04654.x

Müller, S., Baldin, C., Groth, M., Guthke, R., Kniemeyer, O., Brakhage, A. A., et al. (2012). Comparison of transcriptome technologies in the pathogenic fungus *Aspergillus fumigatus* reveals novel insights into the genome and MpkA dependent gene expression. *BMC Genomics* 13:519. doi: 10.1186/1471-2164-13-519

Munro, C. A., Selvaggini, S., De Bruijn, I., Walker, L., Lenardon, M. D., Gerssen, B., et al. (2007). The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. *Mol. Microbiol.* 63, 1399–1413. doi: 10.1111/j.1365-2958.2007.05588.x

Muszkieta, L., Aimanianda, V., Mellado, E., Gribaldo, S., Alcazar-Fuoli, L., Szewczyk, E., et al. (2014). Deciphering the role of the chitin synthase families 1 and 2 in the in vivo and in vitro growth of *Aspergillus fumigatus* by multiple gene targeting deletion. *Cell Microbiol.* 16, 1784–1805. doi: 10.1111/cmi.12326

Nanduri, J., and Tartakoff, A. M. (2001). The arrest of secretion response in yeast: signaling from the secretory path to the nucleus via Wsc proteins and Pkc1p. *Mol. Cell.* 8, 281–289. doi: 10.1016/S1097-2765(01)00312-4

Nivoix, Y., Zamfir, A., Lutun, P., Kara, F., Remy, V., Lioure, B., et al. (2006). Combination of caspofungin and an azole or an amphotericin B formulation in invasive fungal infections. *J. Infect.* 52, 67–74. doi: 10.1016/j.jinf.2005.01.006

Perez, P., Garcia-Acha, I., and Duran, A. (1983). Effect of papulacandin B on the cell wall and growth of *Geotrichum* lactis. *J. Gen. Microbiol.* 129, 245–250. doi: 10.1099/00221287-129-1-245

Puttikamonkul, S., Willger, S. D., Grahl, N., Perfect, J. R., Movahed, N., Bothner, B., et al. (2010). Trehalose 6-phosphate phosphatase is required for cell wall integrity and fungal virulence but not trehalose biosynthesis in the human fungal pathogen *Aspergillus fumigatus*. *Mol. Microbiol.* 77, 891–911. doi: 10.1111/j.1365-2958.2010.07254.x

Rispail, N., Soanes, D. M., Ant, C., Czajkowski, R., Grunler, A., Huguet, R., et al. (2009). Comparative genomics of MAP kinase and calcium-calcineurin signalling components in plant and human pathogenic fungi. *Fungal Genet. Biol.* 46, 287–298. doi: 10.1016/j.fgb.2009.01.002

Rocha, E. M., Garcia-Effron, G., Park, S., and Perlin, D. S. (2007). A Ser678Pro substitution in Fks1p confers resistance to echinocandin drugs in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 51, 4174–4176. doi: 10.1128/AAC.00917-07

Rogg, L. E., Fortwendel, J. R., Juvvadi, P. R., Lilley, A., and Steinbach, W. J. (2011). The chitin synthase genes chsA and chsC are not required for cell wall stress responses in the human pathogen *Aspergillus fumigatus. Biochem. Biophys. Res. Commun.* 411, 549–554. doi: 10.1016/j.bbrc.2011.06.180

Romano, J., Nimrod, G., Ben-Tal, N., Shadkchan, Y., Baruch, K., Sharon, H., et al. (2006). Disruption of the *Aspergillus fumigatus* ECM33 homologue results in rapid conidial germination, antifungal resistance and hypervirulence. *Microbiology* 152, 1919–1928. doi: 10.1099/mic.0.28936-0

Samantaray, S., Neubauer, M., Helmschrott, C., and Wagener, J. (2013). Role of the guanine nucleotide exchange factor Rom2 in cell wall integrity maintenance of *Aspergillus fumigatus*. *Eukaryot. Cell* 12, 288–298. doi: 10.1128/EC.00246-12

Sawistowska-Schroder, E. T., Kerridge, D., and Perry, H. (1984). Echinocandin inhibition of 1,3-beta-D-glucan synthase from *Candida albicans*. *FEBS Lett.* 173, 134–138. doi: 10.1016/0014-5793(84)81032-7

Schmaler-Ripcke, J., Sugareva, V., Gebhardt, P., Winkler, R., Kniemeyer, O., Heinekamp, T., et al. (2009). Production of pyomelanin, a second type of melanin, via the tyrosine degradation pathway in *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 75, 493–503. doi: 10.1128/AEM.02077-08

Schmalhorst, P. S., Krappmann, S., Vervecken, W., Rohde, M., Müller, M., Braus, G. H., et al. (2008). Contribution of galactofuranose to the virulence of the opportunistic pathogen *Aspergillus fumigatus. Eukaryot. Cell* 7, 1268–1277. doi: 10.1128/EC.00109-08

Seo, K., Akiyoshi, H., and Ohnishi, Y. (1999). Alteration of cell wall composition leads to amphotericin B resistance in *Aspergillus flavus*. *Microbiol. Immunol.* 43, 1017–1025. doi: 10.1111/j.1348-0421.1999.tb01231.x

Singh, R., Shivaprakash, M. R., and Chakrabarti, A. (2011). Biofilm formation by zygomycetes: quantification, structure and matrix composition. *Microbiology* 157, 2611–2618. doi: 10.1099/mic.0.048504-0

Steinbach, W. J., Cramer, R. A. Jr., Perfect, B. Z., Asfaw, Y. G., Sauer, T. C., Najvar, L. K., et al. (2006). Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. *Eukaryot. Cell* 5, 1091–1103. doi: 10.1128/EC.00139-06

Ullmann, A. J., and Cornely, O. A. (2006). Antifungal prophylaxis for invasive mycoses in high risk patients. *Curr. Opin. Infect. Dis.* 19, 571–576. doi: 10.1097/QCO.0b013e3280108e45

Valiante, V., Heinekamp, T., Jain, R., Hartl, A., and Brakhage, A. A. (2008). The mitogen-activated protein kinase MpkA of *Aspergillus fumigatus* regulates cell wall signaling and oxidative stress response. *Fungal Genet. Biol.* 45, 618–627. doi: 10.1016/j.fgb.2007.09.006

Valiante, V., Jain, R., Heinekamp, T., and Brakhage, A. A. (2009). The MpkA MAP kinase module regulates cell wall integrity signaling and pyomelanin formation in *Aspergillus fumigatus*. *Fungal Genet. Biol.* 46, 909–918. doi: 10.1016/j.fgb.2009.08.005

Vandeputte, P., Tronchin, G., Larcher, G., Ernoult, E., Berges, T., Chabasse, D., et al. (2008). A nonsense mutation in the ERG6 gene leads to reduced susceptibility to polyenes in a clinical isolate of *Candida glabrata*. *Antimicrob. Agents Chemother.* 52, 3701–3709. doi: 10.1128/AAC.00423-08

Verwer, P. E., Van Duijn, M. L., Tavakol, M., Bakker-Woudenberg, I. A., and Van De Sande, W. W. (2012). Reshuffling of *Aspergillus fumigatus* cell wall components chitin and beta-glucan under the influence of caspofungin or nikkomycin Z alone or in combination. *Antimicrob. Agents Chemother.* 56, 1595–1598. doi: 10.1128/AAC.05323-11

Wagener, J., Echtenacher, B., Rohde, M., Kotz, A., Krappmann, S., Heesemann, J., et al. (2008). The putative alpha-1,2-mannosyltransferase AfMnt1 of the opportunistic fungal pathogen *Aspergillus fumigatus* is required for cell wall stability and full virulence. *Eukaryot. Cell* 7, 1661–1673. doi: 10.1128/EC.00221-08

Walker, L. A., Munro, C. A., De Bruijn, I., Lenardon, M. D., Mckinnon, A., and Gow, N. A. (2008). Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. *PLoS Pathog* 4:e1000040. doi: 10.1371/journal.ppat.1000040

Wiederhold, N. P. (2007). Attenuation of echinocandin activity at elevated concentrations: a review of the paradoxical effect. *Curr. Opin. Infect. Dis.* 20, 574–578. doi: 10.1097/QCO.0b013e3282f1be7f

Wiederhold, N. P. (2009). Paradoxical echinocandin activity: a limited in vitro phenomenon? *Med. Mycol.* 47(Suppl. 1), S369–S375. doi: 10.1080/13693780802428542

Wiederhold, N. P., Kontoyiannis, D. P., Prince, R. A., and Lewis, R. E. (2005). Attenuation of the activity of caspofungin at high concentrations against *Candida albicans*: possible role of cell wall integrity and calcineurin pathways. *Antimicrob. Agents Chemother.* 49, 5146–5148. doi: 10.1128/AAC.49.12.5146-5148.2005

Winkelstroter, L. K., Bom, V. L., De Castro, P. A., Ramalho, L. N., Goldman, M. H., Brown, N. A., et al. (2015). High Osmolarity Glycerol response (HOG) PtcB phosphatase is important for *Aspergillus fumigatus* virulence. *Mol. Microbiol.* 96, 42–54. doi: 10.1111/mmi.12919

Young, L. Y., Hull, C. M., and Heitman, J. (2003). Disruption of ergosterol biosynthesis confers resistance to amphotericin B in *Candida lusitaniae*. *Antimicrob. Agents Chemother.* 47, 2717–2724. doi: 10.1128/AAC.47.9.2717-2724.2003

Zhao, W., Li, C., Liang, J., and Sun, S. (2014). The *Aspergillus fumigatus* beta-1,3-glucanosyltransferase Gel7 plays a compensatory role in maintaining cell wall integrity under stress conditions. *Glycobiology* 24, 418–427. doi: 10.1093/glycob/cwu003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Valiante, Macheleidt, Föge and Brakhage. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 26 February 2015
doi: 10.3389/fmicb.2015.00128

Exploration of *Aspergillus fumigatus* Ras pathways for novel antifungal drug targets

Qusai Al Abdallah and Jarrod R. Fortwendel*

Department of Microbiology and Immunology, University of South Alabama, Mobile, AL, USA

Edited by:
Praveen R. Juvvadi, Duke University Medical Center, USA

Reviewed by:
Deborah Hogan, Geisel School of Medicine at Dartmout, USA
Connie B. Nichols, Duke University Medical Center, USA

***Correspondence:**
Jarrod R. Fortwendel, Department of Microbiology and Immunology, University of South Alabama, 5851 USA Drive North, Medical Sciences Building 2102, Mobile, AL 36688, USA
e-mail: jfortwendel@jaguar1.usouthal.edu

Ras pathway signaling is a critical virulence determinant for pathogenic fungi. Localization of Ras to the plasma membrane (PM) is required for Ras network interactions supporting fungal growth and virulence. For example, loss of *Aspergillus fumigatus* RasA signaling at the PM via inhibition of palmitoylation leads to decreased growth, altered hyphal morphogenesis, decreased cell wall integrity and loss of virulence. In order to be properly localized and activated, Ras proteins must transit a series of post-translational modification (PTM) steps. These steps include farnesylation, proteolytic cleavage of terminal amino acids, carboxymethylation, and palmitoylation. Because Ras activation drives tumor development, Ras pathways have been extensively studied in mammalian cells as a potential target for anti-cancer therapy. Inhibitors of mammalian Ras interactions and PTM components have been, or are actively being, developed. This review will focus on the potential for building upon existing scaffolds to exploit fungal Ras proteins for therapy, synthesizing data from studies employing both mammalian and fungal systems.

Keywords: Ras protein, G domain, hypervariable region, post-translational modifications, spatio-temporal regulation, fungal pathogenesis, antifungal therapeutics

INTRODUCTION

Aspergillus fumigatus is the most common fungal pathogen of invasive aspergillosis in immunocompromised patients. Despite the introduction of several antifungal drugs, infections related to invasive aspergillosis are usually severe and fatal (Latge, 1999). Therefore, there is an increasing demand for new drugs against *A. fumigatus* infections, and thereby identification of target proteins for therapeutic drug design.

Ras-mediated signaling pathways play key roles in regulating cell responses to different stresses via a wide range of effector proteins (for more information, refer to Rajalingam et al., 2007). In pathogenic fungi, Ras signaling pathways control virulence in host cells (reviewed in detail in Fortwendel, 2012). Therefore Ras proteins, and their effectors, represent potential targets of intervention for novel antifungal therapies. Due to their role in tumor formation, mammalian Ras post-translational modification (PTM) pathway proteins have been studied in detail for their potential as targets for anticancer therapeutics (refer to Downward, 2003; Adjei and Hidalgo, 2005; Spiegel et al., 2014). In this minireview, we discuss these studies in the context of development of antifungal therapy. Since RasA is the major Ras protein in *A. fumigatus*, this brief review focuses on the RasA signaling pathway.

A. Fumigatus Ras PROTEINS

Ras proteins are low molecular weight monomeric G-proteins, which localize to the plasma membrane (PM) and exhibit GTPase activities (Wennerberg et al., 2005). They are induced by extracellular stimuli and function primarily as signal mediators for several downstream cascades. Such cascades activate transcription factors, which control a wide range of cellular processes such as cell growth, division, differentiation and survival (Weeks and Spiegelman, 2003). In contrast to human cells, which typically express three Ras isoforms (HRas, KRas, and NRas), only two Ras homologs (RasA and RasB) are produced in *A. fumigatus* (Fortwendel et al., 2004). Based on sequence similarity, RasA is more closely related to the human H-Ras with homologs found in most eukaryotes. In contrast, RasB is only produced by filamentous fungi (Fortwendel et al., 2004). Both, RasA, and RasB, exhibit distinct but overlapping roles in conidial germination, mycelial growth, conidiogenesis, and cell mitosis (Fortwendel et al., 2004, 2005, 2008, 2012). Additionally, both proteins modulate virulence in *A. fumigatus* and other pathogenic fungi (Fortwendel et al., 2005, 2012; Fortwendel, 2012).

DOMAIN STRUCTURE OF Ras PROTEINS

The domain structure of human Ras proteins has been reviewed in detail previously (Sprang, 1997; Vetter and Wittinghofer, 2001; Wittinghofer and Vetter, 2011). Briefly, the approximately 190-amino acid protein is divided into 165 highly conserved amino acids (90–100% identical) at the N-terminus (known as the G domain or GTPase domain) and a C-terminal hypervariable region (HVR) that encompasses the remaining amino acids (Hancock, 2003). In the next sections, we will discuss the domain structure of both regions and their role in mediating Ras activation, transmembrane localization and cell signaling.

THE G DOMAIN FACILITATES PROTEIN CONFIRMATION AND DOWNSTREAM SIGNALING

Numerous biochemical, molecular and structural studies, involving both yeast and mammalian cells, have shown that the

G domain of Ras-like proteins houses the amino acid sequences required for binding guanine nucleotides [i.e., guanosine diphosphate (GDP) and guanosine triphosphate (GTP)], GTPase-activating protein (GAP), guanine nucleotide exchange factor (GEF), and downstream effectors (Ahearn et al., 2012). The G domain is organized into six β sheets and five α helices. Additionally, two loop regions, designated switch I and switch II, mediate Ras transformation between its two interchangeable activity states via conformational change during binding of guanine nucleotides (Vetter and Wittinghofer, 2001).

The Ras activation mechanism involves GEF proteins, which promote the release of GDP. GTP, which exists in the cytoplasm at concentrations 10 times higher than that of GDP, binds to the GDP-free form of Ras. GTP association with Ras releases energy, which changes protein conformation at the switch I and II regions. This transforms Ras to the active state and allows binding of the effector proteins to its G domain. Active Ras proteins are negatively regulated by GAP proteins. Binding of GAP to Ras protein increases its intrinsic GTPase activity 10^5 fold and hydrolyzes GTP to GDP. The hydrolysis of GTP depletes the released energy, causing conformational changes at the switch domains, and subsequently releasing the effector (Vetter and Wittinghofer, 2001; Wennerberg et al., 2005; Kyriakis, 2009; Ahearn et al., 2012; Prior and Hancock, 2012).

THE HVR GOVERNS Ras MEMBRANE LOCALIZATION AND ANCHORING

The HVR of RasA homologs is divided into two regions: an anchor and a linker. The anchor region is highly conserved among Ras isoforms and is composed of a CAAX box—where C is cysteine, AA are two aliphatic amino acids, and X is any amino acid—and a palmitoylation motif (Gao et al., 2009).

The anchor plays an essential role in Ras subcellular trafficking and membrane localization. Protein trafficking and subsequent membrane association of many proteins is typically mediated by hydrophobic transmembrane domains. However, Ras proteins lack such domains, and therefore the protein undergoes several PTMs at the CAAX box and the palmitoylated cysteine motif which convert the HVR to a hydrophobic, membrane-associated domain (**Figure 1A**) (Takai et al., 2001; Hancock, 2003; Larsen et al., 2006; Iwasaki and Ōmura, 2007).

Ras PTM mechanisms have been studied in detail in human and yeast cells. In spite of lack of similar studies in *A. fumigatus*, homologous proteins have been identified in the *A. fumigatus* genome (**Figure 1B**), implying conservation of RasA PTM processes in *Aspergillus* species. The first step of the Ras PTM series is prenylation, which is the process of covalent addition of a farnesyl (farnesylation) or geranylgeranyl group (geranylgeranylation) at the cysteine residue of the CAAX box via farnesyl transferase (FT) or geranylgeranyl transferases (GGT I and II), respectively (Berndt and Sebti, 2011). Prenylation of the CAAX box facilitates the association of Ras protein to the endoplasmic reticulum (ER) membrane (Omerovic et al., 2007). At the ER membrane, the farnesylated (or geranylgeranylated) Ras protein is further processed by AAX cleavage via type I (Ste24) and type II CAAX prenyl endopeptidase (Rce1) (Manolaridis et al., 2013).

The remaining prenylated cysteine residue of the CAAX box is then methylated by isoprenylcysteine carboxyl methyltransferase (ICMT) in the ER (Chiu et al., 2004). Prenylation, proteolysis, and methylation suffice weak binding of the CAAX motif cysteine residue to the ER membrane. Such unstable association causes Ras to encounter a constant exchange between the ER membrane and the cytoplasm (Greaves and Chamberlain, 2007). Therefore, a second moiety is required to stabilize Ras association to the ER membrane. Such a signal varies and can be a lysine polybasic domain in K-Ras(B), a single palmitoylation site in N-Ras and K-Ras(A), or a double palmitoylation site in H-Ras (Hancock et al., 1989, 1990). Palmitoylation, specifically S-palmitoylation, is the addition of palmitate to the cysteine residue(s) via thioester bond (Smotrys and Linder, 2004; Wan et al., 2007). In Ras proteins, palmitoylation cysteines are located in the anchor region and are adjacent to the CAAX box cysteine residue (Linder and Deschenes, 2007). Palmitoylated Ras is transported from the Golgi to the PM via the exocytic vesicular pathway (Goodwin et al., 2005). Unlike prenylation, palmitoylation is a reversible step, as Ras can be depalmitoylated via thioesterase on the PM. Depalmitoylated Ras recycles back to the Golgi via a non-vesicular pathway. This cycle of palmitoylation and depalmitoylation is used by the cell to avoid unnecessary accumulation of Ras proteins on the PM (Goodwin et al., 2005; Salaun et al., 2010).

In fungi, the amino acid composition of the anchor region varies among species in different ways. First, the number of cysteine residues in the palmitoylation region varies from one cysteine in *S. cerevisiae*, *S. pombe*, and *Candida albicans*; to two cysteine residues in *Cryptococcus neoformans* and other filamentous fungi such as *A. fumigatus*. Second, variations in the anchor region might exist between different Ras homologs within one fungal species. For example, unlike RasA, *A. fumigatus* RasB does not contain the palmitoylation cysteine residues. Although no experimental evidence for RasB localization exists, the differences in anchor region amino acid sequence might indicate different subcellular localizations of RasA and RasB (Fortwendel, 2012).

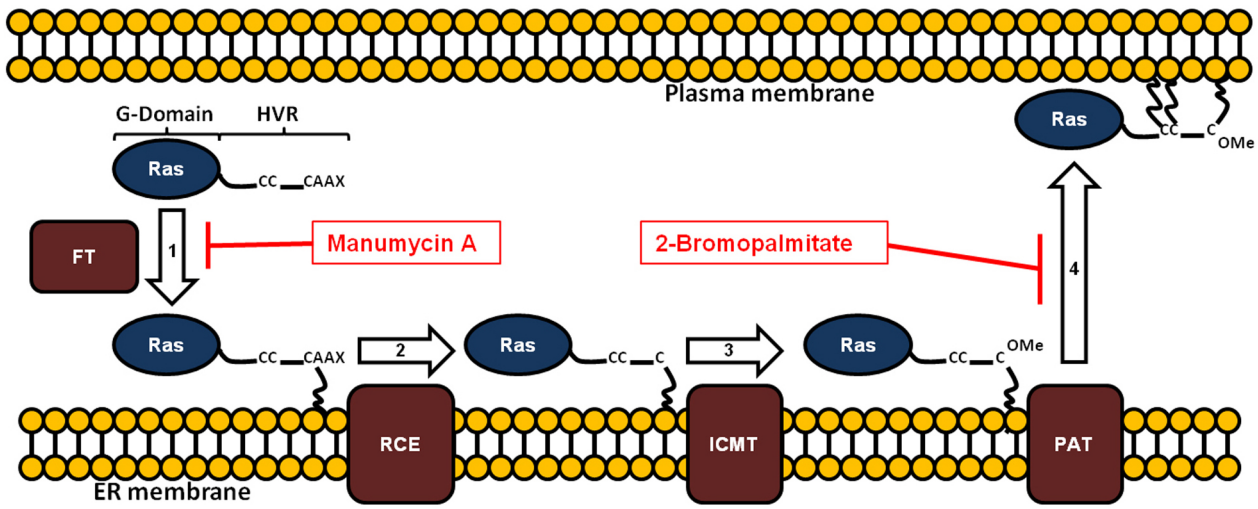
Ras-MEDIATED FUNGAL MORPHOGENESIS AND VIRULENCE DEPEND ON THE SPATIO-TEMPORAL REGULATION OF Ras ACTIVITY

Like their human counterparts, the cellular functions of fungal Ras GTPases depend on the spatio-temporal regulation of the protein on the PM. Understanding the spatio-temporal organization of RasA has been a necessary step in selecting potential targets that are predicted to interfere with its ability to properly transmit signals for growth, stress response and virulence. The following sections will briefly summarize our current knowledge of RasA spatial and temporal regulation mechanisms.

THE TEMPORAL REGULATION OF RasA ACTIVITY

The essential role of temporal activation of RasA has been investigated by comparing the phenotype of *A. fumigatus* mutants that express dominant negative RasA (DNrasA) or dominant activate RasA (DArasA) to that of a *rasA* (Δ*rasA*) deletion mutant. The DNrasA and DArasA mutants provide the ability to study the phenotypic effects of improper temporal inactivation or activation of RasA, respectively, during development. DNrasA, and Δ*rasA* strains show similar phenotypes (i.e., delayed in germination), whereas the DArasA mutant initiates germination in the absence

A



B

Process	GenBank Accession (*Protein Length*)		Identity (%)	*A. fumigatus* Af293 Systematic Name
	Human	*A. fumigatus*		
1. Farnesylation	NP_002019 (*437 aa*) NP_002018 (*379 aa*)	XP_751793 (*519 aa*) XP_752045 (*353 aa*)	43.6 35.6	Afu4g10330 (Ram1) Afu4g07800 (Ram 2)
2. CAAX Proteolysis	NP_005124 (*329 aa*) NP_005848 (*475 aa*)	XP_747589 (*337 aa*) XP_752066 (*456 aa*)	33.1 39.9	Afu6g04890 (Rce1) Afu4g07590 (Ste24)
3. Methylation	NP_036537 (*284 aa*)	XP_755176 (*287 aa*)	40.5	Afu2g08420 (Ste14)
4. Palmitoylation	NP_057116 (*364 aa*)	XP_754959 (*607 aa*)	32.4	Afu3g06470 (Erf2)

FIGURE 1 | Conservation of the Ras post-translational modification pathway in *Aspergillus fumigatus*. (A) Ras proteins transit a series of post-translational modifications to reach the plasma membrane. These include: (1) farnesylation of cytoplasmic Ras on a conserved cysteine residue by a dual subunit, protein farnesyltransferase enzyme complex; (2) cleavage of the C-terminal CAAX motif; (3) methylation of the processed C-terminus; and (4) palmitoylation of conserved cysteine residues upstream of the CAAX motif. Farnesylation is prerequisite for association with the endoplasmic reticulum, whereas palmitoylation is required for stable association with the plasma membrane. Inhibitors with activity against these processes in *A. fumigatus* include manumycin A and 2-bromopalmitate, targeting farnesylation and palmitoylation, respectively. FT = farnesyltransferase; RCE = Ras converting enzyme; ICMT = isoprenylcysteine carboxymethyltransferase; PAT = palmitoyltransferase. **(B)** Homologs of the protein components of the Ras post-translational modification pathway are shown. Protein lengths in amino acids (aa) are included with the GenBank accession numbers. Identity (%) was determined using protein alignments in Lasergene software (DNAstar). For reference, homologs of the yeast pathway are given in parentheses next to the *A. fumigatus* Af293 systematic name (right column).

of a germinant. Additionally, mycelia of all three strains grow slower than wild type and exhibit defects in polarity maintenance. Although constitutive Ras activation delays germ tube formation and reduces colony outgrowth, the DArasA mutant also displays hyphal swelling and spontaneous lysis during fully polarized growth (Fortwendel, 2012). These data show the importance of temporal regulation of RasA activity for proper hyphal morphogenesis, since the inability to modulate RasA activity during developmental progression causes abnormalities in fungal growth.

THE SPATIAL REGULATION OF RasA SIGNALING

In addition to the previously described temporal regulation, spatial regulation of Ras signaling plays an essential role in RasA function. Evidence for the cellular mechanisms that control *A. fumigatus* RasA PTM and localization was obtained from subcellular localization analyses of RasA using GFP tagging. Similar to human Ras, RasA localizes to the PM of *A. fumigatus*. However, when RasA farnesylation is blocked by exchange mutagenesis of the CAAX box cysteine residue (C210) to serine, RasA aborts the PM localization and accumulates in the cytoplasm. Additionally, expression of RasA in an *A. fumigatus* deletion strain of the putative palmitoyltransferase subunit gene (Δ*erfD*) shows a punctate localization of RasA, implying palmitoylation of RasA is required for PM localization. Furthermore, mutation of the palmitoylation double cysteine motif (C206 and C207) to serine mislocalizes RasA to endomembranes. Phenotype analysis of these mutants showed that *A. fumigatus* strains expressing either a non-farnesylated or non-palmitoylated

RasA exhibit a full or partial Δ*rasA* phenotype, respectively (Fortwendel et al., 2012; Norton and Fortwendel, 2014). Consistent with these data, similar results have been obtained in other pathogenic fungi. For example, in *C. neoformans*, farnesylation and palmitoylation are both required for normal Ras1 localization and morphogenesis (Nichols et al., 2009). In *C. albicans*, both PTMs are required for localization, but farnesylation plays the greater role in Ras-mediated growth and morphogenesis (Piispanen et al., 2011). Taken together, these data support the importance of RasA PTM processes for fungal growth and virulence.

APPROACHES FOR DEVELOPING Ras-TARGETED ANTIFUNGAL THERAPEUTICS

Mammalian Ras signaling pathways have been the target of extensive research for developing anticancer therapeutics. This accumulated knowledge could be translated into novel strategies to treat *Aspergillus* infections since the mechanisms of Ras activation and PTMs are shared by both human and fungi. The development of Ras-targeted anticancer therapy has focused on: (1) targeting Ras proteins directly, (2) blockade of Ras upstream and downstream signaling pathways, and (3) inhibition of Ras PTMs. There are many Ras inhibitors that have tested in mammalian cell as anticancer therapeutics. Details on specific successes and failures have been reviewed extensively (Adjei and Hidalgo, 2005; Berndt et al., 2011; Spiegel et al., 2014). Although all three of these have been pursued as potentially viable channels for inhibition of Ras function, most progress has been achieved in the development of anti-Ras compounds targeting the PTM pathway. In the following section, we will briefly describe these approaches, focusing mainly on inhibition of Ras PTMs and the published data that support this pathway as a potential anti-*Aspergillus* therapeutic target.

TARGETING Ras PROTEINS AND Ras PROTEIN INTERACTIONS

In *A. fumigatus*, RasA modulates fungal pathogenesis and has been considered an attractive target for antifungal agents. Directly targeting Ras proteins for anticancer therapies has proven a difficult task. For example, inhibition of Ras-GTP binding is difficult to achieve, since the Ras-GTP interaction is very high affinity, occurring in the picomolar range (Gysin et al., 2011). However, recent approaches have generated small molecules that inhibit K-Ras activation (Maurer et al., 2012). This method relied on in-depth structural analyses of the K-Ras protein, the level of which have not been accomplished for any fungal Ras protein. As such, the applicability of such inhibitors remains unknown for fungal Ras proteins.

In general, fungal Ras proteins appear to share similar signaling mechanisms with their human homologs, and therefore inhibitors of these signaling events might also have great potential for thwarting invasive fungal infections. Examples of these are protein kinases that modulate downstream Ras signaling pathways. Several kinase inhibitors have been applied successfully to treat cancer (reviewed in detail by Downward, 2003), however, these molecules have not been tested in models of fungal infection. Although the signaling mechanisms are similar between humans and fungi, a deeper understanding of the biochemistry underpinning Ras-mediated signaling in pathogenic fungi is required for the identification of fungal-specific, selective targets in this area.

INHIBITION OF Ras PTM

Inhibition of farnesylation

In contrast to the limited number of therapeutics directly targeting Ras proteins, multiple compounds are being developed and employed to inhibit steps in the PTM pathway of human Ras proteins. Importantly, the individual elements of the PTM pathway are conserved between humans and fungal pathogens, the first step of which is the lipidation of Ras with a farnesyl moiety (**Figure 1B**). There are several farnesyl transferase inhibitors (FTIs) that are used or being clinically tested for use as anticancer agents (described in detail by Appels et al., 2005). The antifungal activities of some of these FTIs have been evaluated against several pathogenic fungi. For example, when the wild type strain of *C. neoformans* was treated with six different FTIs, one inhibitor, i.e., Manumycin A, showed inhibitory activities comparable to Amphotericin B—an antifungal agent. Additionally, when a *C. neoformans* mutant that lacks the cell wall capsule, i.e., cap59 mutant, was used, two additional FTIs, namely ethylenediamine inhibitor #2 and tipifarnib, showed elevated inhibitory activities. Interestingly, treatment of *C. neoformans* with high concentrations of Manumycin A caused a shift in Ras1 localization from the PM to the cytosol (Hast et al., 2011). Similar experiments have been carried out to study antifungal activities of Manumycin A against several species from *Aspergillus* and *Candida*. Compared to *C. neoformans*, the FTI minimal inhibitory concentrations (MIC) were 80–160-fold and 5–10-fold higher for *Aspergillus* and for *Candida*, respectively. (Hast et al., 2011; Qiao et al., 2013). However, it is unclear whether such differences in MIC are caused by fungal resistance to Manumycin A; or variations in experimental procedure, media composition, or pH of the media. In a similar experiment, the inhibition of protein farnesylation in *C. albinans* by farnesyl transferase inhibitor III (FPT inhibitor III) blocked the development of yeast to hyphae (McGeady et al., 2002), which is a Ras-mediated virulence step (Cutler, 1991; Feng et al., 1999). Additionally, FPT Inhibitor III blocks hyphal differentiation in a dose-dependent manner in *C. neoformans* (Vallim et al., 2004).

To better understand the protein–protein interactions between FT and their inhibitors (FTIs), structural studies of inhibitor-bound *A. fumigatus* and *C. neoformans* FT were compared to their human homolog. These studies revealed that the substrate-binding groove residues are highly conserved between human and fungal FT, while the product exit groove displays a high sequence divergence. Importantly, these studies reveal fungal-specific attributes of the highly conserved Ras PTM pathway. For example, both grooves are wider in fungal FT in comparison to their human homolog, causing weaker binding activities of inhibitors toward *Aspergillus* FT in comparison to human FT (Hast et al., 2011; Mabanglo et al., 2014). Therefore, modification of anticancer FTIs is required for optimal antifungal efficiency. Re-purposing FTIs developed for anticancer treatments may represent a novel area for antifungal drug development.

In addition to targeting Ras farnesylation, mapping the fungal farnesylome, i.e., proteins that are farnesylated by

farnesyltransferase, will open the door for numerous potential drug targets. One example is the Ras-related protein, Rheb, which is, like Ras proteins, farnesylated before transmembrane localization (Clark et al., 1997). The cellular functions of mammalian Rheb are inhibited by cell treatment with FTI (Castro et al., 2003). In *A. fumigatus*, the Rheb homolog, i.e., RhbA, plays an important role in fungal pathogenesis and vegetative growth. An *A. fumigatus rhbA* deletion mutant, Δ*rhbA*, exhibits impaired virulence in mouse model and reduced growth on minimal media supplemented with poor nitrogen sources. Additionally, this strain displays higher sensitivity to the rapamycin antibiotic, which inhibits TOR kinases (Panepinto et al., 2003). Taken together, this suggests that fungal Rheb, i.e., Rhb, could serve as potential target for antifungal therapy.

Inhibition of palmitoylation

Palmitoylation is another putative target for antifungal therapeutics since RasA palmitoylation is important for mycelial polarized growth and virulence. Targeting Ras palmitoylation is still at the beginning stages as an anticancer therapeutic. Palmitoylation inhibitors have only recently been developed and have not yet been employed in disease models (reviewed in Chavda et al., 2014). To our knowledge, only one study assessed the potential of inhibition of RasA palmitoylation as antifungal target. In this study, blocking RasA palmitoylation in *A. fumigatus* by 2-bromopalmitate disrupts RasA transmembrane localization and reduces fungal growth in liquid culture (Fortwendel et al., 2012). Therefore, additional studies that aim at designing, developing and assessing novel compounds that target fungal RasA palmitoylation are warranted.

CONCLUSION

The potential for developing antifungal therapeutics by targeting the Ras signaling pathway is a promising avenue of research. Ras signaling has been studied intensively in humans, and the accumulated knowledge can be utilized as a scaffold for the development of antifungal agents with selective toxicity. In support of this, the individual components of the Ras PTM pathway share only partial sequence similarity with their human homologs (**Figure 1B**). Additionally, further characterization of Ras regulatory pathways in pathogenic fungi is necessary to deepen our understanding of fungal growth and virulence.

ACKNOWLEDGMENT

This work is supported by NIH grant 5R01AI106925 to JRF.

REFERENCES

Adjei, A. A., and Hidalgo, M. (2005). Intracellular signal transduction pathway proteins as targets for cancer therapy. *J. Clin. Oncol.* 23, 5386–5403. doi: 10.1200/JCO.2005.23.648

Ahearn, I. M., Haigis, K., Bar-Sagi, D., and Philips, M. R. (2012). Regulating the regulator: post-translational modification of RAS. *Nat. Rev. Mol. Cell Biol.* 13, 39–51. doi: 10.1038/nrm3255

Appels, N. M., Beijnen, J. H., and Schellens, J. H. M. (2005). Development of farnesyl transferase inhibitors: a review. *Oncologist* 10, 565–578. doi: 10.1634/theoncologist.10-8-565

Berndt, N., Hamilton, A. D., and Sebti, S. M. (2011). Targeting protein prenylation for cancer therapy. *Nat. Rev. Cancer* 11, 775–791. doi: 10.1038/nrc3151

Berndt, N., and Sebti, S. M. (2011). Measurement of protein farnesylation and geranylgeranylation in vitro, in cultured cells and in biopsies, and the effects of prenyl transferase inhibitors. *Nat. Protoc.* 6, 1775–1791. doi: 10.1038/nprot.2011.387

Castro, A. F., Rebhun, J. F., Clark, G. J., and Quilliam, L. A. (2003). Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J. Biol. Chem.* 278, 32493–32496. doi: 10.1074/jbc.C300226200

Chavda, B., Arnott, J. A., and Planey, S. L. (2014). Targeting protein palmitoylation: selective inhibitors and implications in disease. *Expert Opin. Drug Discov.* 9, 1005–1019. doi: 10.1517/17460441.2014.933802

Chiu, V. K., Silletti, J., Dinsell, V., Wiener, H., Loukeris, K., Ou, G., et al. (2004). Carboxyl methylation of Ras regulates membrane targeting and effector engagement. *J. Biol. Chem.* 279, 7346–7352. doi: 10.1074/jbc.M311602200

Clark, G. J., Kinch, M. S., Rogers-Graham, K., Sebti, S. M., Hamilton, A. D., and Der, C. J. (1997). The Ras-related protein rheb is farnesylated and antagonizes Ras signaling and transformation. *J. Biol. Chem.* 272, 10608–10615. doi: 10.1074/jbc.272.16.10608

Cutler, J. E. (1991). Putative virulence factors of *Candida albicans*. *Annu. Rev. Microbiol.* 45, 187–218. doi: 10.1146/annurev.mi.45.100191.001155

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* 3, 11–22. doi: 10.1038/nrc969

Feng, Q., Summers, E., Guo, B., and Fink, G. (1999). Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*. *J. Bacteriol.* 181, 6339–6346.

Fortwendel, J. R. (2012). Ras-mediated signal transduction and virulence in human pathogenic fungi. *Fungal Genomics Biol.* 2, 105. doi: 10.4172/2165-8056.1000105

Fortwendel, J. R., Fuller, K. K., Stephens, T. J., Bacon, W. C., Askew, D. S., and Rhodes, J. C. (2008). *Aspergillus fumigatus* RasA regulates asexual development and cell wall integrity. *Eukaryot. Cell* 7, 1530–1539. doi: 10.1128/EC.00080-08

Fortwendel, J. R., Juvvadi, P. R., Rogg, L. E., Asfaw, Y. G., Burns, K. A., Randell, S. H., et al. (2012). Plasma membrane localization is required for RasA-mediated polarized morphogenesis and virulence of *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 966–977. doi: 10.1128/EC.00091-12

Fortwendel, J. R., Panepinto, J. C., Seitz, A. E., Askew, D. S., and Rhodes, J. C. (2004). *Aspergillus fumigatus* rasA and rasB regulate the timing and morphology of asexual development. *Fungal Genet. Biol.* 41, 129–139. doi: 10.1016/j.fgb.2003.10.004

Fortwendel, J. R., Zhao, W., Bhabhra, R., Park, S., Perlin, D. S., Askew, D. S., et al. (2005). A fungus-specific Ras homolog contributes to the hyphal growth and virulence of *Aspergillus fumigatus*. *Eukaryot. Cell* 4, 1982–1989. doi: 10.1128/EC.4.12.1982-1989.2005

Gao, J., Liao, J., and Yang, G.-Y. (2009). CAAX-box protein, prenylation process and carcinogenesis. *Am. J. Transl. Res.* 1, 312–325.

Goodwin, J. S., Drake, K. R., Rogers, C., Wright, L., Lippincott-Schwartz, J., Philips, M. R., et al. (2005). Depalmitoylated Ras traffics to and from the Golgi complex via a non-vesicular pathway. *J. Cell Biol.* 170, 261–272. doi: 10.1083/jcb.200502063

Greaves, J., and Chamberlain, L. H. (2007). Palmitoylation-dependent protein sorting. *J. Cell Biol.* 176, 249–254. doi: 10.1083/jcb.200610151

Gysin, S., Salt, M., Young, A., and McCormick, F. (2011). Therapeutic strategies for targeting Ras proteins. *Genes Cancer* 2, 359–372. doi: 10.1177/1947601911412376

Hancock, J. F. (2003). Ras proteins: different signals from different locations. *Nat. Rev. Mol. Cell Biol.* 4, 373–385. doi: 10.1038/nrm1105

Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989). All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* 57, 1167–1177. doi: 10.1016/0092-8674(89)90054-8

Hancock, J. F., Paterson, H., and Marshall, C. J. (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* 63, 133–139. doi: 10.1016/0092-8674(90)90294-O

Hast, M. A., Nichols, C. B., Armstrong, S. M., Kelly, S. M., Hellinga, H. W., Alspaugh, J. A., et al. (2011). Structures of *Cryptococcus neoformans* protein farnesyltransferase reveal strategies for developing inhibitors that target fungal pathogens. *J. Biol. Chem.* 286, 35149–35162. doi: 10.1074/jbc.M111.250506

Iwasaki, S., and Ōmura, S. (2007). Search for protein farnesyltransferase inhibitors of microbial origin: our strategy and results as well as the results obtained by other groups. *J. Antibiot. (Tokyo)* 60, 1–12. doi: 10.1038/ja.2007.1

Kyriakis, J. M. (2009). Thinking outside the box about Ras. *J. Biol. Chem.* 284, 10993–10994. doi: 10.1074/jbc.R800085200

Larsen, M. R., Trelle, M. B., Thingholm, T. E., and Jensen, O. N. (2006). Analysis of post-translational modifications of proteins by tandem mass spectrometry. *Biotechniques* 40, 790–798. doi: 10.2144/000112201

Latge, J.-P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12, 310–350.

Linder, M. E., and Deschenes, R. J. (2007). Palmitoylation: policing protein stability and traffic. *Nat. Rev. Mol. Cell Biol.* 8, 74–84. doi: 10.1038/nrm2084

Mabanglo, M. F., Hast, M. A., Lubock, N. B., Hellinga, H. W., and Beese, L. S. (2014). Crystal structures of the fungal pathogen *Aspergillus fumigatus* protein farnesyltransferase complexed with substrates and inhibitors reveal features for antifungal drug design. *Protein Sci.* 23, 289–301. doi: 10.1002/pro.2411

Manolaridis, I., Kulkarni, K., Dodd, R. B., Ogasawara, S., Zhang, Z., Bineva, G., et al. (2013). Mechanism of farnesylated CAAX protein processing by the integral membrane protease Rce1. *Nature* 504, 301–305. doi: 10.1038/nature12754

Maurer, T., Garrenton, L. S., Oh, A., Pitts, K., Anderson, D. J., Skelton, N. J., et al. (2012). Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. *Proc. Natl. Acad. Sci. U.S.A.* 109, 5299–5304. doi: 10.1073/pnas.1116510109

McGeady, P., Logan, D. A., and Wansley, D. L. (2002). A protein-farnesyl transferase inhibitor interferes with the serum-induced conversion of *Candida albicans* from a cellular yeast form to a filamentous form. *FEMS Microbiol. Lett.* 213, 41–44. doi: 10.1111/j.1574-6968.2002.tb11283.x

Nichols, C. B., Ferreyra, J., Ballou, E. R., and Alspaugh, J. A. (2009). Subcellular localization directs signaling specificity of the *Cryptococcus neoformans* Ras1 protein. *Eukaryot. Cell* 8, 181–189. doi: 10.1128/EC.00351-08

Norton, T. S., and Fortwendel, J. R. (2014). Control of Ras-mediated signaling in *Aspergillus fumigatus*. *Mycopathologia* 178, 325–330. doi: 10.1007/s11046-014-9765-1

Omerovic, J., Laude, A. J., and Prior, I. A. (2007). Ras proteins: paradigms for compartmentalised and isoform specific signalling. *Cell. Mol. Life Sci.* 64, 2575–2589. doi: 10.1007/s00018-007-7133-8

Panepinto, J. C., Oliver, B. G., Fortwendel, J. R., Smith, D. L. H., Askew, D. S., and Rhodes, J. C. (2003). Deletion of the *Aspergillus fumigatus* gene encoding the Ras-related protein RhbA reduces virulence in a model of invasive pulmonary aspergillosis. *Infect. Immun.* 71, 2819–2826. doi: 10.1128/IAI.71.5.2819-2826.2003

Piispanen, A. E., Bonnefoi, O., Carden, S., Deveau, A., Bassilana, M., and Hogan, D. A. (2011). Roles of Ras1 membrane localization during *Candida albicans* hyphal growth and farnesol response. *Eukaryot. Cell* 10, 1473–1484. doi: 10.1128/EC.05153-11

Prior, I. A., and Hancock, J. F. (2012). Ras trafficking, localization and compartmentalized signalling. *Semin. Cell Dev. Biol.* 23, 145–153. doi: 10.1016/j.semcdb.2011.09.002

Qiao, J., Gao, P., Jiang, X., and Fang, H. (2013). In vitro antifungal activity of farnesyltransferase inhibitors against clinical isolates of Aspergillus and Candida. *Ann. Clin. Microbiol. Antimicrob.* 12, 37. doi: 10.1186/1476-0711-12-37

Rajalingam, K., Schreck, R., Rapp, U. R., and Albert, Š. (2007). Ras oncogenes and their downstream targets. *Biochim. Biophys. Acta* 1773, 1177–1195. doi: 10.1016/j.bbamcr.2007.01.012

Salaun, C., Greaves, J., and Chamberlain, L. H. (2010). The intracellular dynamic of protein palmitoylation. *J. Cell Biol.* 191, 1229–1238. doi: 10.1083/jcb.201008160

Smotrys, J. E., and Linder, M. E. (2004). PALMITOYLATION OF INTRACELLULAR SIGNALING PROTEINS: regulation and function. *Annu. Rev. Biochem.* 73, 559–587. doi: 10.1146/annurev.biochem.73.011303.073954

Spiegel, J., Cromm, P. M., Zimmermann, G., Grossmann, T. N., and Waldmann, H. (2014). Small-molecule modulation of Ras signaling. *Nat. Chem. Biol.* 10, 613–622. doi: 10.1038/nchembio.1560

Sprang, S. R. (1997). G PROTEIN MECHANISMS: insights from structural analysis. *Annu. Rev. Biochem.* 66, 639–678. doi: 10.1146/annurev.biochem.66.1.639

Takai, Y., Sasaki, T., and Matozaki, T. (2001). Small GTP-binding proteins. *Physiol. Rev.* 81, 153–208.

Vallim, M. A., Fernandes, L., and Alspaugh, J. A. (2004). The RAM1 gene encoding a protein-farnesyltransferase β-subunit homologue is essential in *Cryptococcus neoformans*. *Microbiology* 150, 1925–1935. doi: 10.1099/mic.0.27030-0

Vetter, I. R., and Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. *Science* 294, 1299–1304. doi: 10.1126/science.1062023

Wan, J., Roth, A. F., Bailey, A. O., and Davis, N. G. (2007). Palmitoylated proteins: purification and identification. *Nat. Protoc.* 2, 1573–1584. doi: 10.1038/nprot.2007.225

Weeks, G., and Spiegelman, G. B. (2003). Roles played by Ras subfamily proteins in the cell and developmental biology of microorganisms. *Cell. Signal.* 15, 901–909. doi: 10.1016/S0898-6568(03)00073-1

Wennerberg, K., Rossman, K. L., and Der, C. J. (2005). The Ras superfamily at a glance. *J. Cell Sci.* 118, 843–846. doi: 10.1242/jcs.01660

Wittinghofer, A., and Vetter, I. R. (2011). Structure-function relationships of the G domain, a canonical switch motif. *Annu. Rev. Biochem.* 80, 943–971. doi: 10.1146/annurev-biochem-062708-134043

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 January 2015; accepted: 03 February 2015; published online: 26 February 2015.

*Citation: Al Abdallah Q and Fortwendel JR (2015) Exploration of Aspergillus fumigatus Ras pathways for novel antifungal drug targets. Front. Microbiol. **6**:128. doi: 10.3389/fmicb.2015.00128*

This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology.

Copyright © 2015 Al Abdallah and Fortwendel. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 March 2015
doi: 10.3389/fmicb.2015.00175

Identification and mutational analyses of phosphorylation sites of the calcineurin-binding protein CbpA and the identification of domains required for calcineurin binding in *Aspergillus fumigatus*

Praveen R. Juvvadi[1†], Yan Ma[2†], Amber D. Richards[1], Erik J. Soderblom[3], M. Arthur Moseley[3], Frédéric Lamoth[1,4,5] and William J. Steinbach[1,6*]*

[1] Division of Pediatric Infectious Diseases, Department of Pediatrics, Duke University Medical Center, Durham, NC, USA, [2] Department of Dermatology and Venereology, The Second Hospital of Shanxi Medical University, Taiyuan, Shanxi, China, [3] Duke Proteomics and Metabolomics Core Facility, Center for Genomic and Computational Biology, Duke University, Durham, NC, USA, [4] Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Lausanne, Switzerland, [5] Institute of Microbiology, Lausanne University Hospital, Lausanne, Switzerland, [6] Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA

Calcineurin is a key protein phosphatase required for hyphal growth and virulence in *Aspergillus fumigatus*, making it an attractive antifungal target. However, currently available calcineurin inhibitors, FK506 and cyclosporine A, are immunosuppressive, limiting usage in the treatment of patients with invasive aspergillosis. Therefore, the identification of endogenous inhibitors of calcineurin belonging to the calcipressin family is an important parallel strategy. We previously identified the gene *cbpA* as the *A. fumigatus* calcipressin member and showed its involvement in hyphal growth and calcium homeostasis. However, the mechanism of its activation/inhibition through phosphorylation and its interaction with calcineurin remains unknown. Here we show that *A. fumigatus* CbpA is phosphorylated at three distinct domains, including the conserved SP repeat motif (phosphorylated domain-I; PD-I), a filamentous fungal-specific domain (PD-II), and the C-terminal CIC motif (Calcipressin Inhibitor of Calcineurin; PD-III). While mutation of three phosphorylated residues (Ser208, Ser217, Ser223) in the PD-II did not affect CbpA function *in vivo,* mutation of the two phosphorylated serines (Ser156, Ser160) in the SP repeat motif caused reduced hyphal growth and sensitivity to oxidative stress. Mutational analysis in the key domains in calcineurin A (CnaA) and proteomic interaction studies confirmed the requirement of PxIxIT motif-binding residues (352-NIR-354) and the calcineurin B (CnaB)-binding helix residue (V371) for the binding of CbpA to CnaA. Additionally, while the calmodulin-binding residues (442-RVF-444) did not affect CbpA binding to CnaA, three mutations (T359P, H361L, and L365S) clustered between the CnaA catalytic and the CnaB-binding helix were also required for CbpA binding. This is the first study to analyze the phosphorylation status of calcipressin in filamentous fungi and identify the domains required for binding to calcineurin.

Keywords: ***Aspergillus fumigatus*, calcineurin, calcineurin-binding protein (CbpA), phosphorylation, mutation**

OPEN ACCESS

Edited by:
Ana Traven, Monash University, Australia

Reviewed by:
Alexander Idnurm, University of Missouri–Kansas City, USA
Hubertus Haas, Innsbruck Medical University, Austria

***Correspondence:**
Praveen R. Juvvadi and William J. Steinbach, Division of Pediatric Infectious Diseases, Department of Pediatrics, Duke University Medical Center, 427 Jones Building, Research Drive, Durham, NC 27710, USA
praveen.juvvadi@duke.edu; bill.steinbach@duke.edu

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *22 December 2014*
Accepted: *16 February 2015*
Published: *13 March 2015*

Citation:
Juvvadi PR, Ma Y, Richards AD, Soderblom EJ, Moseley MA, Lamoth F and Steinbach WJ (2015) Identification and mutational analyses of phosphorylation sites of the calcineurin-binding protein CbpA and the identification of domains required for calcineurin binding in Aspergillus fumigatus. Front. Microbiol. 6:175. doi: 10.3389/fmicb.2015.00175

Introduction

Calcineurin (also known as protein phosphatase 2B) is an important protein phosphatase essential for hyphal growth, development, and virulence in *Aspergillus fumigatus*, making it an attractive antifungal target (Steinbach et al., 2007). However, due to direct immunosuppressive effects, administration of currently available calcineurin inhibitors, FK506 and cyclosporine A, for the treatment of patients with invasive aspergillosis is not practical (Ho et al., 1996). Therefore, identification of endogenous inhibitors of calcineurin is important to more effectively inhibit calcineurin as part of future drug targeting strategies. One such group of endogenous inhibitors are the regulators of calcineurin (RCANs; RCAN1-3) or modulatory calcineurin-interacting proteins (MCIPs; MCIP1-3) belonging to the calcipressin family of proteins (Rothermel et al., 2003). This family is composed of key regulators of calcineurin-NFAT signaling in diverse organisms, ranging from the yeasts to humans (Kingsbury and Cunningham, 2000; Rothermel et al., 2003). Another group of scaffolding proteins, Cain/Cabin-1 and A-kinase anchoring protein 79 (AKAP79), have also been identified to interact and inhibit calcineurin function in a phosphorylation-dependent manner in mammalian cells (Coghlan et al., 1995; Kashishian et al., 1998; Lai et al., 1998). Despite the conservation of calcineurin from the yeasts to human, the fungal homologs of the endogenous mammalian calcineurin inhibitor Cabin 1/Cain have not yet been identified.

How RCAN proteins precisely modulate calcineurin function is debatable. Earlier reports in mammalian cells revealed that RCAN1, one of the target genes of NFAT, binds to calcineurin and inhibits its activity (Fuentes et al., 2000; Rothermel et al., 2000), but more recent reports suggest that RCAN1 actually facilitates the activation of the calcineurin-NFAT signaling pathway (Sanna et al., 2006). A study using a combination of *in silico* stimulations and single cell experiments indicated that RCAN1 inhibits calcineurin at lower concentrations, but functions as a facilitator when its levels increase within the cell (Shin et al., 2011).

Attempts to understand the molecular mechanism responsible for facilitation of calcineurin-NFAT signaling revealed that phosphorylation of RCAN1 switches it from an inhibitor to a facilitator (Shin et al., 2011). Earlier structural analysis of domains in RCAN1 revealed the requirement of PxIxIT and LxVP motifs in order to cause an inhibitory effect, and TxxP and ExxP motifs and a GSK3β phosphorylation site to act as a facilitator (Mehta et al., 2009). The hallmark of the members of the calcipressin family is the presence of a conserved serine-proline repeat (SP repeat; FxISPPxSPP motif) which is phosphorylated at the two serine residues by mitogen-activated kinase (MAPK), followed by glycogen synthase kinase 3 (GSK3β; Vega et al., 2002). Studies in *Saccharomyces cerevisiae* have shown that phosphorylation by GSK3β within the SP repeat is required for calcineurin activity (Hilioti et al., 2004). Previously, this GSK3β phosphorylation was shown to be reversed by the phosphatase action of calcineurin (Vega et al., 2002).

In the yeast *S. cerevisiae,* deletion of RCAN1 caused sensitivity to cation stress, as opposed to no cation sensitivity with deletion of *CBP1* (the RCAN1 ortholog) in *Cryptococcus neoformans* (Görlach et al., 2000). In the presence of the FK506-FKBP12 complex, binding of CBP1 to calcineurin was also inhibited in *C. neoformans* (Görlach et al., 2000). In addition to the coordinating role of Cbp1 with calcineurin in filamentation of *C. neoformans* during mating, the importance of phosphorylation and dephosphorylation of two serine residues in the SP repeat motif for directing activity of calcineurin during hyphal elongation was demonstrated through site-directed mutagenesis (Fox and Heitman, 2005). In *S. cerevisiae,* phosphorylation of RCAN1 promoted its degradation and decreased its inhibitory effect on calcineurin (Kishi et al., 2007).

We previously demonstrated the importance of the RCAN1 ortholog, CbpA, for hyphal growth and calcium homeostasis in *A. fumigatus* (Pinchai et al., 2009). While deletion of *cbpA* resulted in increased expression of the *vcxA* (vacuolar Ca^{2+}/H^{+} exchanger), *chsA* (chitin synthase A), and *cnaA* genes, it only slightly attenuated virulence. Although we showed that *cbpA* deletion or overexpression altered *cnaA* transcriptional response, the biochemical mechanisms underlying *A. fumigatus* CbpA phosphorylation and the mechanism of how it binds to calcineurin remain unknown. In this study, using phosphopeptide enrichment and tandem mass spectrometry we identified that CbpA is phosphorylated at three distinct domains, including a conserved SP repeat motif, a non-conserved filamentous fungal-specific domain, and a C-terminal region with low homology to yeast and human orthologs. Assessment of CbpA phosphorylation status in the absence of CnaA *in vivo* revealed the continual phosphorylation of the two serine residues (Ser156, Ser160) in the SP repeat motif and the two serine residues (Ser217, Ser223) in the filamentous fungal-specific domain, indicating the probable role for calcineurin in the dephosphorylation of these residues. While mutation of the phosphorylated residues in the non-conserved filamentous fungal-specific domain did not result in any growth defects, mutations in the SP repeat motif caused decreased hyphal growth and caused sensitivity to oxidative stress. Furthermore, although we could not identify a consensus PxIxIT motif in *A. fumigatus* CbpA, we found the absolute requirement of PxIxIT motif-binding residues (352-NIR-354) and the calcineurin B (CnaB)-binding helix residue (V371) for the binding of CbpA to CnaA. The binding of CbpA to CnaA was not inhibited by mutation of the calmodulin-binding residues (442-RVF-444) in CnaA, indicating that CbpA binds to calcineurin independent of calmodulin. We also identified three residues (T359, H361, and L365) that are clustered between the catalytic and the CnaB-binding helix that are required for CbpA binding.

Materials and Methods

Strains and Culture Conditions

Aspergillus fumigatus strain *akuB*^{KU80} and the isogenic *pyrG* auxotrophic strain *akuB*^{KU80} *pyrG*$^{-}$ were used in all experiments. *A. fumigatus* cultures were grown on glucose minimal medium (GMM) at 37°C. *Escherichia coli* DH5α competent cells were used

for subcloning. *A. fumigatus* was transformed as described earlier (Steinbach et al., 2006). For radial growth analyses, 10^4 conidia of the *akuB*^{KU80} and the other strains were cultured on GMM agar at 37°C, with colony diameters determined every 24 h over a period of 5 days. All growth experiments were performed in triplicate.

Generation of *cbpA* Deletion and CbpA-EGFP Expression Strains

While a Δ*cbpA* strain was previously reported by our group (Pinchai et al., 2009), we generated a second deletion strain in the *akuB*^{KU80} background for this study in order to maintain the same genetic background for all mutated strains. Deletion of *cbpA* was performed using a previously designed construct in the plasmid pJW24 with the *Aspergillus parasiticus pyrG* gene as the auxotrophic marker (Steinbach et al., 2006). The *cbpA* deletion construct contained 1 kb promoter and 780 bp terminator flanking regions to facilitate homologous recombination. The ∼5 kb *cbpA* deletion construct was PCR-amplified using the primers cbpA-promo-KpnI-F and cbpA-term-HindIII-R (Supplementary Table S1) and transformed into the *A. fumigatus akuB*^{KU80} *pyrG*$^{-}$ strain. Transformants selected in the absence of uracil/uridine (Steinbach et al., 2006) were screened by PCR (Supplementary Table S1) and Southern analyses to confirm deletion of the *cbpA* gene (Supplementary Figure S1).

In order to purify the CbpA protein for phosphorylation analyses, the *cbpA*-tagged *egfp* expression construct at the *cbpA* native locus was generated by a fusion PCR strategy. First, the 864 bp *cbpA* genomic DNA without the stop-codon and the 722 bp *egfp* gene were PCR-amplified separately. Next, the two PCR fragments were combined and used as templates to amplify the final fusion PCR product containing the *cbpA-egfp* fusion product, which was then digested with KpnI and NotI to clone at the KpnI-NotI sites in pUCGH (gift from Axel Brakhage) with the hygromycin B resistant gene (*hph*) as the selection marker. The cloned *cbpA* genomic DNA was sequenced to ensure accuracy (Primers listed in Supplementary Table S1). To facilitate homologous recombination, the 780 bp *cbpA* terminator region was cloned into the SbfI-HindIII sites in pUCGH (Langfelder et al., 2001). The final plasmid obtained was designated as pUCGH-CbpA, and the entire construct was linearized by KpnI-HindIII digestion to obtain a fragment of ∼6.7 kb. The linearized construct was then transformed into the *A. fumigatus akuB*^{KU80} strain as previously described and transformants selected by resistance to hygromycin B. The transformants obtained were verified for homologous integration by PCR and also by fluorescent microscopy to visualize GFP fluorescence.

Construction of *cbpA* Mutations

Site-directed mutation of phosphorylated residues in CbpA was performed using primers listed in Supplementary Table S1 and pUCGH-*cbpA* as a template. Briefly, the first PCR used complementary primers overlapping the regions to be mutated and the respective primers at the opposite ends to amplify two PCR fragments. Next, a fusion PCR was performed by using an equiproportional mixture of the two PCR fragments as a template and amplifying the mutated PCR fragment using primers at the opposite ends (Supplementary Table S1). Each fragment cloned into pUCGH was sequenced to confirm the respective mutation and then linearized for homologous integration. The linearized constructs were transformed into the *A. fumigatus akuB*^{KU80} strain as previously described and transformants selected by resistance to hygromycin B. The transformants obtained were verified for homologous integration by PCR and also by fluorescent microscopy to visualize GFP fluorescence.

Protein Extraction and GFP-Trap® Affinity Purification

The *A. fumigatus* strain expressing the *cbpA-egfp* fusion construct was grown in GMM liquid medium as shaking cultures for 24 h at 37°C. Cell extracts were prepared by homogenizing the mycelia using liquid nitrogen in buffer A (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 50 mM KCl, 0.01% Triton X-100, 1 mM PMSF, and 1:100 protease inhibitor cocktail). Total cell lysate was initially centrifuged at 5000 rpm to eliminate cell debris, and clarified by further centrifugation at 7000 rpm for 10 min at 4°C. The final supernatant fraction was collected and protein content determined by Bradford assay. Total protein was normalized to contain ∼10 mg protein in the sample before GFP-Trap affinity purification (Chromotek). GFP-Trap® resin (35 μl) was equilibrated by washing three times in 500 μl ice-cold dilution buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 1:100 Protease Inhibitory Cocktail) according to the manufacturer instructions and resuspended in 100 μl ice cold dilution buffer. The GFP-Trap® resin suspension was then mixed with total crude cell lysate containing ∼10 mg total protein and incubated at 4°C by gentle agitation for 2 h. The suspension was centrifuged at 2000 rpm for 10 min at 4°C and the pelleted GFP-Trap® resin was washed once in 500 μl of ice-cold dilution buffer and then twice with 500 μl of wash buffer (10 mM Tris-HCl pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 1:100 Protease Inhibitory Cocktail).

Sample Preparation for Mass Spectrometry

Protein-bound GFP-Trap® resins were washed three times with 200 μl of 50 mM ammonium bicarbonate, pH 8.0, and suspended in 30 μl 50 mM ammonium bicarbonate, pH 8.0, supplemented with 0.1% Rapigest SF surfactant (Waters Corp). Samples were reduced with 5 mM dithiothreitol for 30 min at 70°C and free sulfhydryls were alkylated with 10 mM iodoacetamide for 45 min at room temperature. Proteolytic digestion was accomplished by the addition of 500 ng sequencing grade trypsin (Promega) directly to the resin, with incubation at 37°C for 18 h. Supernatants were collected following a 2 min centrifugation at 1000 rpm, acidified to pH 2.5 with TFA, and incubated at 60°C for 1 h to hydrolyze the remaining Rapigest surfactant. Insoluble hydrolyzed surfactant was cleared by centrifugation at 15,000 rpm for 5 min. Ninety percent (by volume) of the sample was then removed for subsequent phosphopeptide analysis, and the remaining 10% (by volume) was subjected to an unbiased protein interaction analysis.

Phosphopeptide Enrichment and LC-MS/MS Analysis

For the phosphopeptide analysis, samples were lyophilized to dryness using vacuum centrifugation and resuspended in 65 μl 80% acetonitrile, 1% TFA. Peptides were subjected to phosphopeptide enrichment using a 10 μl GL Sciences TiO2 Spin Tip and subsequently washed with 80% acetonitrile, 1% TFA. Peptides were eluted in 50 μl 20% acetonitrile, 5% aqueous ammonia, pH 10.5, and then acidified to pH 2.5 with formic acid prior to lyophilization to dryness.

Samples were resuspended in 10 μl 2% acetonitrile, 10 mM citric acid 0.1% formic acid and subjected to chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 μm HSS T3 C18 75 μm I.D. x250 mm reversed-phase column. Phosphopeptide enriched samples were additionally supplemented with 10 mM citric acid. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 5 μl injection, peptides were trapped for 5 min on a 5 μm Symmetry C18 180 lm I.D. x20 mm column at 20 μl/min in 99.9% A.

The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA, USA) with a 10 μm tip orifice. Non-phosphopeptide enriched samples were analyzed on a Synapt G2 QToF mass spectrometer operating in a data-dependent mode of acquisition with a precursor MS scan from m/z 400–2000 followed by three MS/MS scans at a CID energy of 30% and a dynamic exclusion of 30 s. Phosphopeptide enriched samples were analyzed on a QExactive Plus mass spectrometer with a precursor MS scan from m/z 300–1600 with $r = 70{,}000$ at m/z 200 and a target AGC setting of 1e6 ions. In a data-dependent mode of acquisition, MS/MS spectra of the 10 most abundant precursor ions were with a CID energy setting of 27 and a dynamic exclusion of 20 s was employed for previously fragmented precursor ions.

Raw LC-MS/MS data files were processed in Mascot distiller (Matrix Science) and then submitted to independent Mascot searches (Matrix Science) against a custom NCBI_*Aspergillus* database containing both forward and reverse entries of each protein. Search tolerances were 5 or 10 ppm for precursor ions and 0.02 or 0.04 Da for product ions using trypsin specificity with up to two missed cleavages for phosphopeptide enriched or non-phosphopeptide enriched data, respectively. Carbamidomethylation (+57.0214 Da on C) was set as a fixed modification, whereas oxidation (+15.9949 Da on M), deamidation (+0.98 Da on NQ), and phosphorylation (+79.98 Da on STY) were allowed. All searched spectra were imported into Scaffold (v4.0, Proteome Software) and scoring thresholds were set to achieve a protein false discovery rate of 0% using the PeptideProphet algorithm. Normalized spectral counts were used to estimate relative protein abundances for interaction studies. This was accomplished by adjusting the sum of the selected quantitative value for all proteins in the list within each MS sample to the average of the sums of all MS samples present in the experiment.

Results and Discussion

Aspergillus fumigatus CbpA is phosphorylated at Three Domains, Including the FxSPPxSPP Motif

As a first step toward identifying the phosphorylation status of CbpA *in vivo* we initially utilized the *A. fumigatus* strain expressing *cnaA-egfp* fusion construct considering the fact that CbpA would co-purify with CnaA. As shown in Supplementary Table S2, purification of CnaA by GFP-Trap® affinity purification and phosphopeptide-enrichment (see Supplemental Materials and Methods) led to the identification of six phosphorylated residues in CbpA (Ser156, Ser160, Thr205, Ser208, Ser217, Ser223). Next, in order to validate this finding and more clearly verify the phosphorylation status of *A. fumigatus* CbpA *in vivo,* a strain expressing the *cbpA–egfp* fusion construct under the control of its native promoter was generated in the *akuB*^{KU80} strain. The CbpA–EGFP fusion protein was purified by GFP-Trap® affinity purification and subjected to protease digestion, followed by phosphopeptide-enrichment and tandem mass spectrometry. As shown in **Table 1**, a total of nine residues (Ser8, Thr133, Ser156, Ser160, Ser208, Ser217, Ser223, Ser257, and Ser266) were identified to be phosphorylated in *A. fumigatus* CbpA.

Based on the location of the majority of the phosphorylated residues, they were classified into three different domains: phosphorylated domain I (PD-I), phosphorylated domain II (PD-II), and phosphorylated domain III (PD-III; **Figure 1**). While the phosphorylation of the FxISPPxSPP motif in the PD-I (Ser156, Ser160; Supplementary Figure S2) is well-known from mammalian and the yeast RCANs (Vega et al., 2002; Hilioti et al., 2004; Abbasi et al., 2006), the identification of three residues phosphorylated in PD-II (Ser208, Ser217, Ser223) seems to be specific to *A. fumigatus* as this region was not conserved in the *C. neoformans* Cbp1 or in the Human MCIP1 (**Figure 1**). In addition, phosphorylation of two serine residues (Ser257 and Ser266) in PD-III, previously designated as the CIC motif (Calcipressin Inhibitor of Calcineurin), was also noted. The calcineurin-binding PxIxIT sequence (PSVVVH) together with the ELHA sequence comprises the CIC motif and is conserved in all vertebrate RCANs/MCIPs (underlined in **Figure 1**; Aubareda et al., 2006; Mulero et al., 2007). Both the ELHA and PxIxIT sequences are required for binding to calcineurin with high affinity (Martínez-Martínez et al., 2009; Mulero et al., 2009). As shown in Supplementary Figure S3, the two serine residues (Ser156 and Ser160) in the FxISPPxSPP motif (PD-I) are conserved in all the filamentous fungi. The fungal orthologs, however, show less conservation to mammalian RCAN in the ELHA and PxIxIT domains (PD-III; Supplementary Figure S3). However, it is interesting to note that phosphorylation of a conserved serine residue was observed within the CIC (PD-III) motif of MCIP1 (**Figure 1**; serine residue shown in white color) and the protein kinase CK2 was identified as the enzyme responsible for the phosphorylation to potentiate inhibition of NFATc signaling by disrupting the calcineurin-NFATc interaction (Martínez-Høyer et al., 2013). Although we do not have conservation of the ELHA or the PSVVVH residues in the *A. fumigatus* CbpA CIC motif (PD-III),

TABLE 1 | List of phosphorylated peptides identified in CbpA by LC-MS/MS analysis.

Phosphorylated peptides identified in CbpA (1)	Position	Ascore probability (2) %	Max mascot ion score in wild-type (spectral counts; 3)	Max mascot ion score in Δ*cnaA* strain (spectral counts; 3)
ADITTTS*PPHSLPQSPSFR	8	<50	51.1 (1)	
IYFGEPT*PLLDEGRPK	133	>99	49.1 (2)	60.7 (2)
LFFIS*PPPS*PPHGWVMR	156, 160	100	79.0 (5)	90.5 (4)
TEQSAPVSGPVDPGTPMS*MSDEKR	208	>99	72.3 (3)	
TGS*WPIAMS*GQR	217, 223	<50, >99	46.5 (3)	43.4 (3)
SSTLIYNPEDHGGSPGLPAVMVEDTTVDS*DDEDIEMMS*PIDMSVR	257, 266	<50, >99	44.7 (3)	

(1) Phosphorylated residues in the respective peptides are indicated by an asterisk. Phosphorylated residues in the phosphorylated domain I (PD-I) and phosphorylated domain II (PD-II) are colored red and green, respectively.
(2) Probability of residue specific phosphorylation localization determined from http://ascore.med.harvard.edu/
(3) Maximum ion scores were determined for each phosphorylated peptide within an individual sample. Spectral counts indicate total number of matching spectral assignments to that particular phosphorylated species within the individual LC-MS/MS analysis.

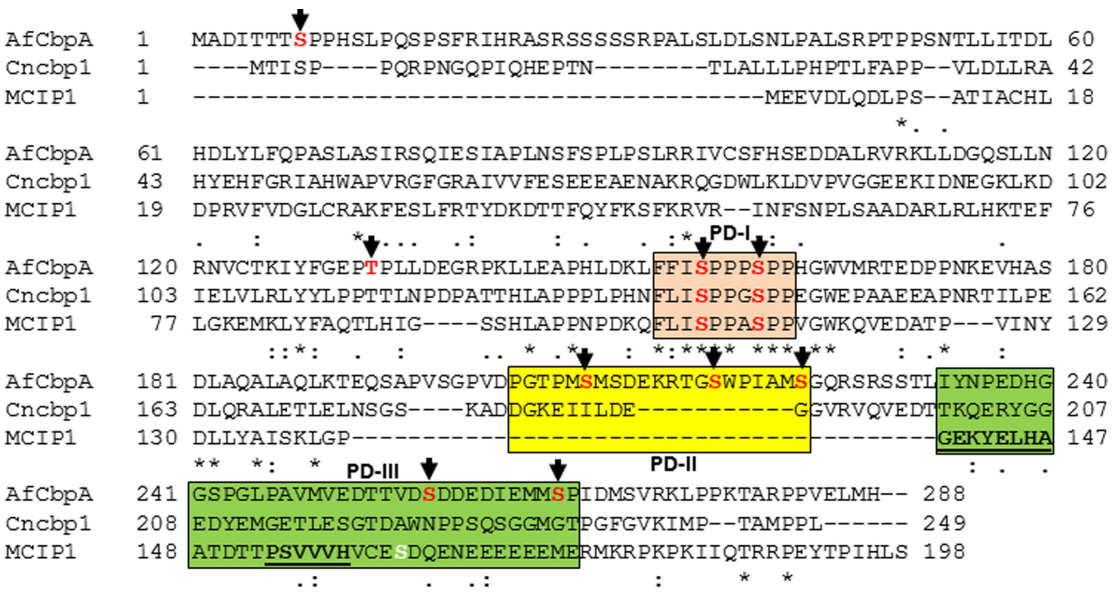


FIGURE 1 | Clustal alignment of *Aspergillus fumigatus* CbpA (AfCbpA) with *Cryptococcus neoformans* Cbp1 (Cncbp1) and Human MCIP1. Conserved residues are indicated in asterisks. Arrows and residues shown in red indicate the identified phosphorylation sites in CbpA. The respective phosphorylated domains PD-I (in pink), PD-II (in yellow) and PD-III (in green) are boxed. The ELHA and the PxIxIT motifs (PSVVVH) in MCIP1 are underlined. The phosphorylated residue within the CIC-motif of Human MCIP1 (PD-III) is shown in white color.

we do have the predicted CK2 target sequence (S/TxxE/D) within the PD-III.

While phosphorylation at the CIC motif (PD-III) was also suggested to be independent of the phosphorylation occurring at the FxISPPxSPP motif (PD-I) of the RCANs, the non-phosphorylated CIC (PD-III) caused increased NFATc (Martínez-Høyer et al., 2013). The absence of PD-II domain in the mammalian MCIP1, the non-conservation of the PD-III domain in the fungal orthologs (Supplementary Figure S3), and our recovery of PD-III phosphorylated CbpA from the wild-type strain all suggest possible differential regulation of the fungal Cbps through phosphorylation-dephosphorylation. In the future, it will be interesting to examine the effect of mutation of these residues on CbpA toward the CnaA–CrzA (ortholog of NFAT) interaction and activation.

Deletion of CnaA Caused Reduction in the Phosphorylated Residues of CbpA

Previous studies have suggested the dephosphorylation of RCANs by calcineurin (Fox and Heitman, 2005). In the mammalian RCANs, phosphorylation of the two serine residues within the FxISPPxSPP motif (PD-I; Ser108 and Ser112) occurs first at Ser112 through MAPK, which then triggers subsequent phosphorylation at Ser108 by GSK3β (Vega et al., 2002). The phosphorylated Ser108 can then be dephosphorylated by calcineurin. It was also previously shown that alterations in the FxISPPxSPP motif (PD-I) phosphorylation influence protein stability, with mutation of the FxISPPxSPP leading to a more stable protein (Genesca et al., 2003). Genetic evidence from *S. cerevisiae* suggests that phosphorylation of Ser108 by GSK3 can release the inhibitory activity of RCAN and convert the protein to

an activator of calcineurin, although the exact mechanism is unknown (Hilioti et al., 2004). Also it is unknown whether the phosphorylation at the CIC motif (PD-III) is subject to regulation by calcineurin protein phosphatase activity.

Considering these data, and the rationale that absence of calcineurin may inhibit dephosphorylation of the residues that undergo calcineurin-mediated dephosphorylation, we next determined if any of the phosphorylated residues are targets for calcineurin by deleting *cnaA* and monitoring the phosphorylation status of CbpA. The FxISPPxSPP residues (PD-I; Ser156, Ser160; **Figure 2**) and Ser217 and Ser223 in the PD-II domain remained phosphorylated, in addition to the single Thr133 residue (**Table 1**). In addition to the N-terminal Ser8 residue, the Ser208 in the PD-II domain, and the residues in the CIC motif (PD-III; Ser257, Ser266) were not phosphorylated in the *cnaA* deletion background, indicating the possibility of calcineurin involvement in the phosphorylation-dephosphorylation mechanism of CbpA. Specifically, the residues in the FxISPPxSPP motif (PD-I) and the PD-II domain may be amenable for dephosphorylation by calcineurin.

Phosphorylation and Dephosphorylation of CbpA at the FxSPPxSPP Motif (PD-I) is Important for Proper Hyphal Growth and Tolerance to Oxidative Stress

Phosphorylation of the FxISPPxSPP motif (PD-I) at both serine residues in *C. neoformans* Cbp1 was shown to be necessary for its stability, activity, and regulation of hyphal elongation during mating (Fox and Heitman, 2005). Interestingly, the phosphorylation of the FxISPPxSPP motif (PD-I) did not mediate the binding of calcineurin to Cbp1 (Fox and Heitman, 2005). In order to investigate the importance of phosphorylation of *A. fumigatus* CbpA at the FxISPPxSPP motif (PD-I), we next mutated the two phosphorylated residues within the FxISPPxSPP motif to alanine and aspartic acid residues, respectively, to mimic a non-phosphorylated state (*cbpA*^{mt}-PD-I-2SA) and a constitutively phosphorylated state (*cbpA*^{mt}-PD-I-2SD). Radial growth analyses of the strains under normal growth conditions over a period of 5 days indicated a slower growth rate in the *cbpA*^{mt}-PD-I-2SA strain compared to the *cbpA*^{mt}-PD-I-2SD strain (**Figure 3**). Unexpectedly, the Δ*cbpA* strain displayed a slightly better growth rate than the *cbpA*^{mt}-PD-I-2SA strain. Based on our previous report (Pinchai et al., 2009), it is possible that the complete deletion of *cbpA* induces higher expression of calcineurin or inappropriate activation of calcineurin that can negatively affect growth. Although the *cbpA*^{mt}-PD-I-2SD strain grew better than the *cbpA*^{mt}-PD-I-2SA strain, its growth was comparable to the Δ*cbpA* strain, indicating that both dephosphorylation and continued phosphorylation of CbpA at the two serine residues in the FxISPPxSPP motif (PD-I) affected its activity. A similar observation was made with *C. neoformans* Cbp1-phospho mimetic expression, wherein mutation of both the serines to glutamic acid abolished filamentation during mating, a phenotype also observed with complete deletion of Cbp1 (Görlach et al., 2000). Importantly, in contrast to the FxISPPxSPP motif mutations (PD-I), the mutation of the three phosphorylated residues in the PD-II domain did not have any effect on the growth under normal conditions (**Figure 3**).

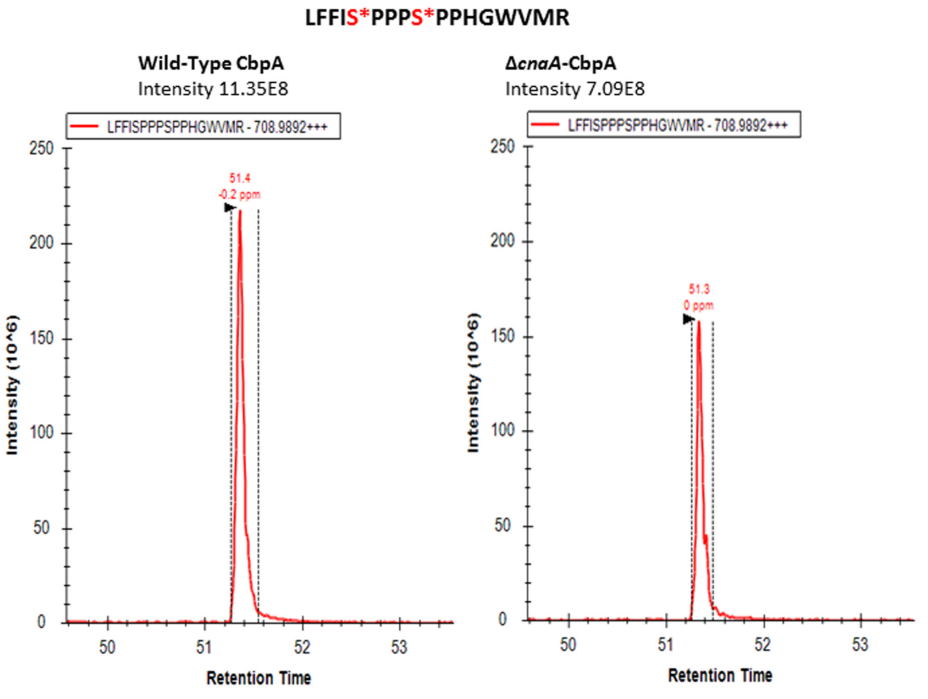


FIGURE 2 | Selected ion chromatogram of LFFI[pS]PPP[pS]PPHGWVMR (m/z [708.9892]$^{2+}$) from *A. fumigatus* CbpA in the *akuB*^{KU80} (reference strain) and the Δ*cnaA* background strains.

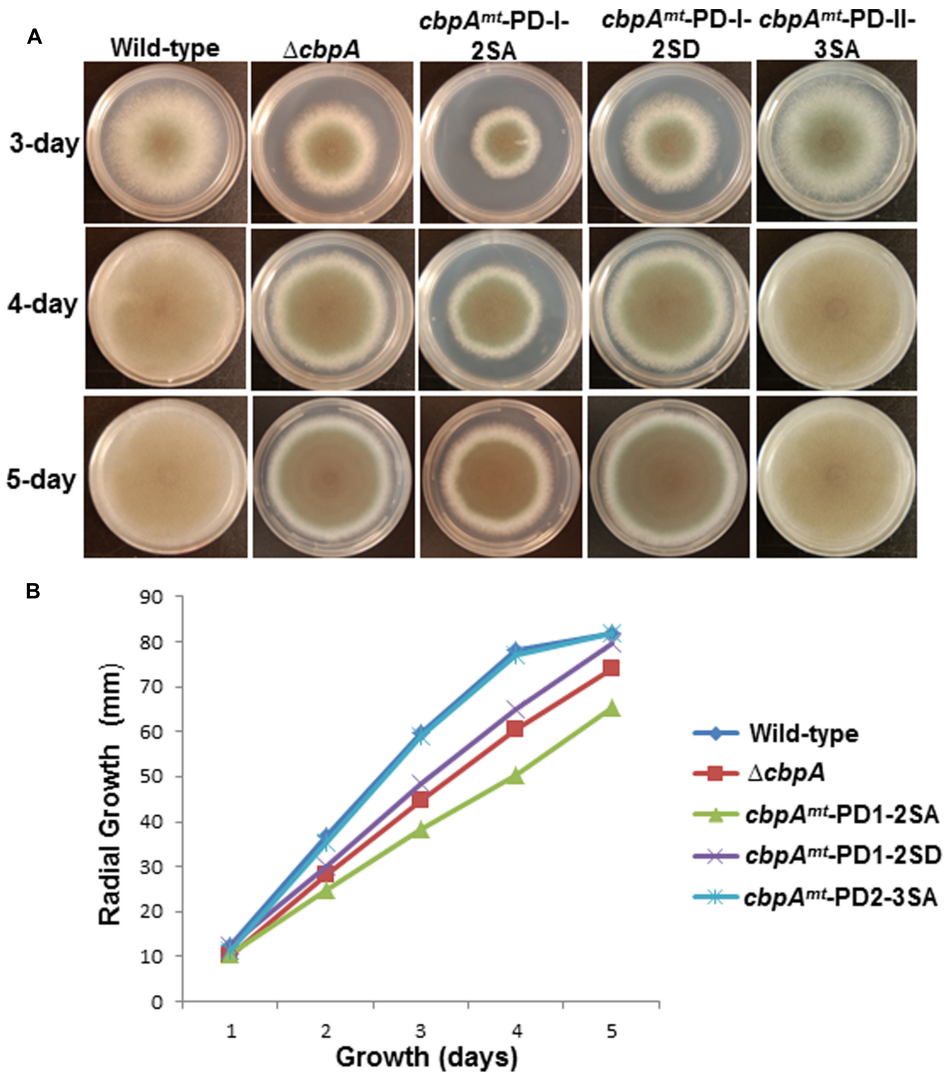


FIGURE 3 | (A) Radial growth of the reference strain (wild-type; *akuB*^{KU80}), the Δ*cbpA* strain, and the respective CbpA phosphorylation mutation strains (*cbpA*^{mt}-PD-I-2SA; *cbpA*^{mt}-PD-I-2SD and *cbpA*^{mt}-PD-II-3SA) was assessed over a period of 5-days at 37°C on GMM agar medium. Growth of the strains at 3, 4, and 5 days are shown. A total of 1 × 10^4 conidia were spotted for each strain. **(B)** Measurement of colony diameter of the strains over a period of 5 days. For radial growth quantification the strains were grown on GMM agar medium in triplicate and values are depicted as average colony diameter.

In mammalian cells, increased levels of RCAN1 were found to be protective against oxidative stress and suppress cell growth. It is not known whether these features of RCANs are a direct consequence of calcineurin inhibition or result from a yet unidentified action of RCAN (Leahy and Crawford, 2000). Furthermore, phosphorylation of the mammalian RCANs at the FxISPPxSPP motif (PD-I) was correlated with an increase in calcineurin-mediated transcriptional inhibition (Genesca et al., 2003). Hence, in order to determine the role of phosphorylated CbpA in counteracting oxidative stress, the respective *cbpA* mutant strains were assayed for growth in the presence of the oxidative stress inducer paraquat (**Figure 4**). Based on phenotypes observed under normal growth conditions, phosphorylation of CbpA at the FxISPPxSPP motif (PD-I) is important for proper hyphal growth, but the phosphorylation at the PD-II domain seemed unimportant for growth. Furthermore, both the *cbpA*^{mt}-PD-I-2SA and the *cbpA*^{mt}-PD-I-2SD strains were sensitive to oxidative stress induced by paraquat (**Figure 4**) and H_2O_2 (data not shown), indicating the importance of phosphorylation-dephosphorylation of CbpA for counteracting oxidative stress conditions. The possibility of other phosphatases regulating the dephosphorylation of CbpA cannot be precluded.

CnaA Requires the PxIxIT-Binding Motif and CnaB-Binding Helix But not the CaM-Binding Helix for Binding to CbpA

It was previously demonstrated that binding of mammalian RCANs to the calcineurin catalytic subunit (CnA) does not interfere with binding of either calmodulin (CaM) or the calcineurin regulatory subunit (CnB) to CnA (Vega et al., 2002).

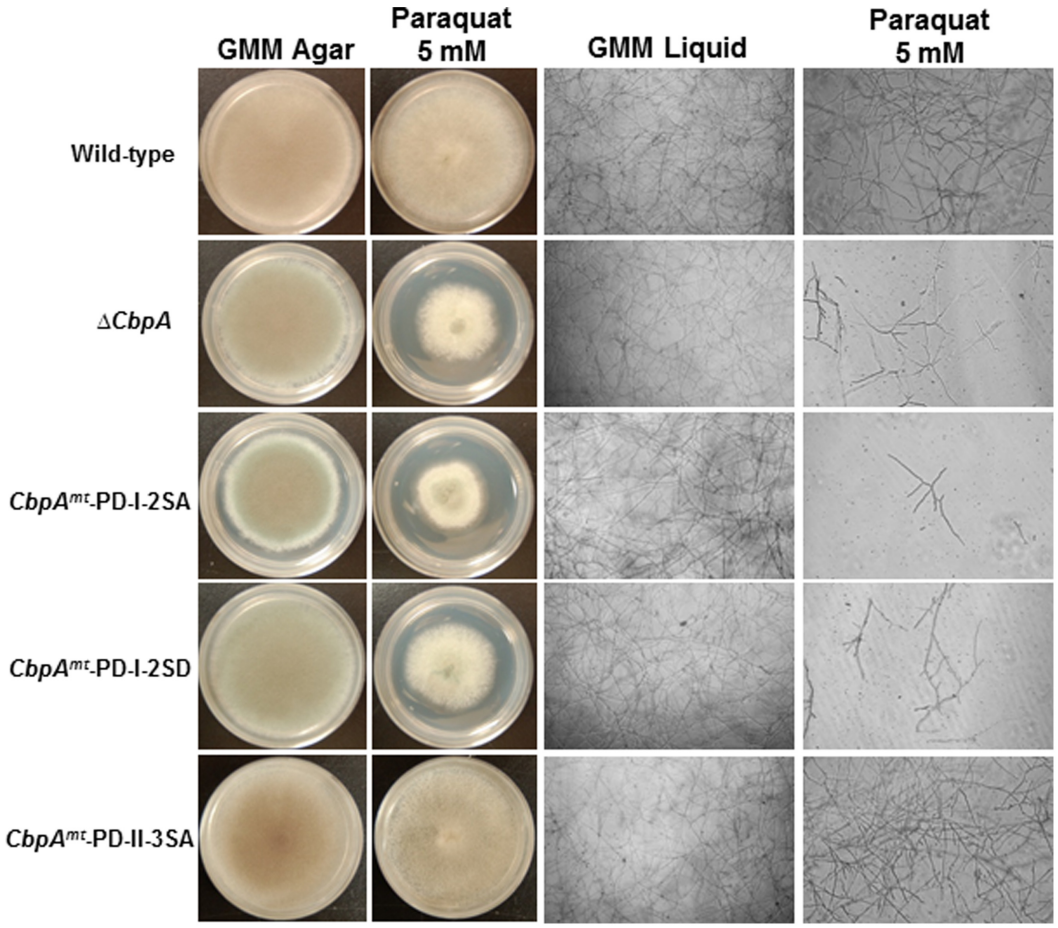


FIGURE 4 | The reference strain (wild-type; *akuB*^{KU80}), the Δ*cbpA* strain, and the respective CbpA phosphorylation mutation strains (*cbpA*^{mt}-PD-I-2SA; *cbpA*^{mt}-PD-I-2SD and *cbpA*^{mt}-PD-II-3SA) were cultured in the absence or presence of the oxidative stress inducer paraquat (5 mM). Strains were grown for a period of 5-days on GMM agar supplemented with the respective oxidative stress inducers at 37°C. To more clearly distinguish the susceptibility of the strains to oxidative stress agents the respective strains were cultured in GMM liquid medium supplemented with paraquat (5 mM) and light-microscopy photographs (×10 magnification) were taken after 48 h of growth at 37°C. For liquid growth assays 1 × 10^4 conidia of each strain were inoculated into 200 μl of GMM in multi-well plate.

In fact, most data indicate that RCANs bind preferentially to active calcineurin, suggesting that CaM binding facilitates RCAN binding (Martínez-Martínez et al., 2009). However, in *C. neoformans* it was shown that the regulatory subunit of calcineurin is required for the binding of CBP1 to drugs that inhibit calcineurin activity (cyclosporin A or FK506) and also disrupt the CBP1/CNA interaction (Görlach et al., 2000), suggesting that the site of CBP1 interaction with CNA overlaps with that of immunophilins.

We previously generated *A. fumigatus* strains harboring mutations in key domains of CnaA (the PxIxIT-binding motif; CnaB binding helix; CaM-binding domain) to understand their significance for calcineurin localization and function (Juvvadi et al., 2013). All the respective *cnaA* mutated constructs were tagged to *egfp* and verified for stable expression (Juvvadi et al., 2013). Because there is no predictable PxIxIT motif in the *A. fumigatus* CbpA, we utilized the *A. fumigatus* strain expressing CnaA-NIR^{mt}-AAA in which the PxIxIT motif-binding residues (352-NIR-354; Asn352 Ile353 Arg354) are mutated to alanines in CnaA to determine if the binding of CbpA to CnaA requires the PxIxIT motif. As shown in **Table 2**, we found that mutation of the PxIxIT motif-binding NIR residues in CnaA completely abolished the binding of CbpA to CnaA. The binding of CbpA to CnaA was also found to be dependent on CnaB binding to CnaA. This was demonstrated by utilizing the *A. fumigatus* mutant strains expressing CnaA^{mt}-V371D and CnaA-THL^{mt}-PLS constructs wherein the key CnaB-binding residue Val371 on CnaA is mutated to aspartic acid (V371D) and three other residues (THL^{mt}-PLS; Thr359 His361 Leu365 mutated to Pro359 Leu361 Ser361, respectively) located close to the CnaB binding helix completely inhibited the binding of CbpA to CnaA. Moreover, treatment with FK506 also inhibited the binding of CnaA to CbpA. Contrary to these findings, mutation of the CaM binding domain residues RVF (442-RVF-444; Arg442 Val443 Phe444 to alanines) on CnaA did not affect the binding of CbpA to CnaA. The strain expressing wild-type CnaA-EGFP was used as a

TABLE 2 | Binding analysis of CbpA to various mutated calcineurin A (CnaAs) *in vivo*.

Strains/treatment	Interacted with CnaA	Uniquely identified peptides (1)	Normalized total spectral counts (2)
Wild type	+	SQIESIAPLNSFSPLPSLR LLDGQSLLNR IYFGEPTPLLDEGRPK EVHASDLAQALAQL TGSWPIAMSGQR	7
Wild type + FK506	–	–	–
CnaA-NIR^{mt}-AAA	–	–	–
CnaA-RVF^{mt}-AAA	+	SQIESIAPLNSFSPLPSLR LLDGQSLLNR IYFGEPTPLLDEGRPK EVHASDLAQALAQL TGSWPIAMSGQR LLEAPHLDK	6
CnaA-V371D	–	–	–
CnaA-THL^{mt}-PLS	–	–	–

(1) Total number of unique peptide sequences identified to CbpA within the corresponding LC-MS/MS analysis.
(2) Normalized spectral counts reported from Scaffold following adjusting the sum of the selected quantitative value for all proteins in the list within each MS sample to a common value: the average of the sums of all MS samples present in the experiment.

comparative control to determine the CbpA peptides bound to CnaA. The binding of CbpA peptides to CnaA were validated in three independent experiments by mass spectrometry (**Table 2** and Supplementary Table S3). Taken together, these results indicated that CbpA binds to CnaA through a PxIxIT motif and requires the binding of CnaB to CnaA. Although we could not identify the PxIxIT motif in CbpA, it is possible that a PxIxIT-like sequence (PVDPGT) close to the newly identified phosphorylated domain II (PD II) is responsible for binding to CnaA. Future crystallization studies on the calcineurin-CbpA complex would shed light on the role of each of these domains and the functional motifs in the binding of CbpA to calcineurin. Furthermore, detailed knowledge of the molecular mechanisms governing the CbpA-calcineurin interaction may also prove useful in the rational design of future immunosuppressant drugs.

A recent study using human cell lines identified the phosphorylation of a serine residue at the CIC motif in all the RCANs (RCAN1, RCAN2, and RCAN3; Martínez-Høyer et al., 2013). Through *in vitro* and *in vivo* phosphorylation assays, CK2 was identified as the kinase phosphorylating this serine residue and this phosphorylation enhanced the disruptive potential toward the calcineurin-NFATc interaction. Interestingly, in the present study, the serine (Ser257) along with another serine residue at position 266 was identified to be phosphorylated the region spanning the CIC motif (PD-III) in *A. fumigatus* CbpA. In the future, it will be interesting to examine the effect of mutation of these residues on CbpA toward the CnaA-CrzA interaction and activation. Taken together, our results indicate that phosphorylation of CbpA at the FxISPPxSPP motif (SP repeat motif) is conserved in filamentous fungi and this phosphorylation is important for CbpA function *in vivo*. In addition, the binding of CbpA to CnaA occurs via a PxIxIT motif and the CnaB binding helix is essential for the interaction.

Author Contributions

PJ and WS conceived and designed research; PJ, YM, AR, and FL performed research; PJ, ES, and MM acquired and analyzed the proteomics data; PJ and WS wrote the paper; PJ, YM, AR, ES, MM, FL, and WS approved the final submission.

Acknowledgments

This work was funded by grant NIH/NIAID 1 R01 AI112595-01 to WS. YM was supported by funds from the National Natural Science Foundation of China (Grant No. 81101233), the Natural Science Foundation for youth in Shanxi province (Grant No. 2010021036-2). FL was supported by the Swiss National Science Foundation and the Swiss Foundation for Medical-Biological Grants (P3SMP3-151742).

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00175/abstract

References

Abbasi, S., Lee, J.-D., Su, B., Chen, X., Alcon, J. L., Yang, J., et al. (2006). Protein kinase-mediated regulation of calcineurin through the phosphorylation of modulatory calcineurin-interacting protein 1. *J. Biol. Chem.* 281, 7717–7726. doi: 10.1074/jbc.M510775200

Aubareda, A., Mulero, M. C., and Pérez-Riba, M. (2006). Functional characterization of the calcipressin 1 motif that suppresses calcineurin-mediated NFAT-dependent cytokine gene expression in human T cells. *Cell. Signal.* 18, 1430–1438. doi: 10.1016/j.cellsig.2005.11.006

Coghlan, V., Perrino, B., Howard, M., Langeberg, L., Hicks, J., Gallatin, W., et al. (1995). Association of protein kinase A and protein phosphatase 2B

with a common anchoring protein. *Science* 267, 108–111. doi: 10.1126/science.7528941

Fox, D. S., and Heitman, J. (2005). Calcineurin-binding protein cbp1 directs the specificity of calcineurin-dependent hyphal elongation during mating in cryptococcus neoformans. *Eukaryot. Cell* 4, 1526–1538. doi: 10.1128/ec.4.9.1526-1538.2005

Fuentes, J. J., Genescà, L., Kingsbury, T. J., Cunningham, K. W., Pérez-Riba, M., Estivill, X., et al. (2000). DSCR1, overexpressed in down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. *Hum. Mol. Genet.* 9, 1681–1690. doi: 10.1093/hmg/9.11.1681

Genesca, L., Aubareda, A., Fuentes, J. J., Estivill, X., and Perez-Riba, M. (2003). Phosphorylation of calcipressin 1 increases its ability to inhibit calcineurin and decreases calcipressin half-life. *Biochem. J.* 374, 567–575. doi: 10.1042/BJ20030267

Görlach, J., Fox, D. S., Cutler, N. S., Cox, G. M., Perfect, J. R., and Heitman, J. (2000). Identification and characterization of a highly conserved calcineurin binding protein, CBP1/calcipressin, in *Cryptococcus* neoformans. *EMBO J.* 19, 3618–3619. doi: 10.1093/emboj/19.14.3618

Hilioti, Z., Gallagher, D. A., Low-Nam, S. T., Ramaswamy, P., Gajer, P., Kingsbury, T. J., et al. (2004). GSK-3 kinases enhance calcineurin signaling by phosphorylation of RCNs. *Genes Dev.* 18, 35–47. doi: 10.1101/gad.1159204

Ho, S., Clipstone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, D., et al. (1996). The mechanism of action of cyclosporin A and FK506. *Clin. Immunol. Immunopathol.* 80, S40–S45. doi: 10.1006/clin.1996.0140

Juvvadi, P. R., Gehrke, C., Fortwendel, J. R., Lamoth, F., Soderblom, E. J., Cook, E. C., et al. (2013). Phosphorylation of calcineurin at a novel serine-proline rich region orchestrates hyphal growth and virulence in *Aspergillus fumigatus*. *PLoS Pathog.* 9:e1003564. doi: 10.1371/journal.ppat.1003564

Kashishian, A., Howard, M., Loh, C., Gallatin, W. M., Hoekstra, M. F., and Lai, Y. (1998). AKAP79 Inhibits calcineurin through a site distinct from the immunophilin-binding region. *J. Biol. Chem.* 273, 27412–27419. doi: 10.1074/jbc.273.42.27412

Kingsbury, T. J., and Cunningham, K. W. (2000). A conserved family of calcineurin regulators. *Genes Dev.* 14, 1595–1604. doi: 10.1101/gad.14.13.1595

Kishi, T., Ikeda, A., Nagao, R., and Koyama, N. (2007). The SCFCdc4 ubiquitin ligase regulates calcineurin signaling through degradation of phosphorylated Rcn1, an inhibitor of calcineurin. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17418–17423. doi: 10.1073/pnas.0704951104

Lai, M. M., Burnett, P. E., Wolosker, H., Blackshaw, S., and Snyder, S. H. (1998). Cain, a novel physiologic protein inhibitor of calcineurin. *J. Biol. Chem.* 273, 18325–18331. doi: 10.1074/jbc.273.29.18325

Langfelder, K., Philippe, B., Jahn, B., Latgé, J.-P., and Brakhage, A. A. (2001). Differential expression of the *Aspergillus fumigatus* pksP gene detected in vitro and in vivo with green fluorescent protein. *Infect. Immun.* 69, 6411–6418. doi: 10.1128/iai.69.10.6411-6418.2001

Leahy, K. P., and Crawford, D. R. (2000). Adapt78 protects cells against stress damage and suppresses cell growth. *Arch. Biochem. Biophys.* 379, 221–228. doi: 10.1006/abbi.2000.1897

Martínez-Høyer, S., Aranguren-Ibáñez, Á., García-García, J., Serrano-Candelas, E., Vilardell, J., Nunes, V., et al. (2013). Protein kinase CK2-dependent phosphorylation of the human regulators of calcineurin reveals a novel mechanism regulating the calcineurin–NFATc signaling pathway. *Biochim. Biophys. Acta* 1833, 2311–2321. doi: 10.1016/j.bbamcr.2013.05.021

Martínez-Martínez, S., Genescà, L., Rodríguez, A., Raya, A., Salichs, E., Were, F., et al. (2009). The RCAN carboxyl end mediates calcineurin docking-dependent inhibition via a site that dictates binding to substrates and regulators. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6117–6122. doi: 10.1073/pnas.0812544106

Mehta, S., Li, H., Hogan, P. G., and Cunningham, K. W. (2009). Domain architecture of the regulators of calcineurin (RCANs) and identification of a divergent RCAN in yeast. *Mol. Cell. Biol.* 29, 2777–2793. doi: 10.1128/mcb.01197-08

Mulero, M. C., Aubareda, A., Orzáez, M., Messeguer, J., Serrano-Candelas, E., Martínez-Hoyer, S., et al. (2009). Inhibiting the calcineurin-NFAT (nuclear factor of activated T cells) signaling pathway with a regulator of calcineurin-derived peptide without affecting general calcineurin phosphatase activity. *J. Biol. Chem.* 284, 9394–9401. doi: 10.1074/jbc.M805889200

Mulero, M. C., Aubareda, A., Schlüter, A., and Pérez-Riba, M. (2007). RCAN3, a novel calcineurin inhibitor that down-regulates NFAT-dependent cytokine gene expression. *Biochim. Biophys. Acta* 1773, 330–341. doi: 10.1016/j.bbamcr.2006.12.007

Pinchai, N., Perfect, B. Z., Juvvadi, P. R., Fortwendel, J. R., Cramer, R. A., Asfaw, Y. G., et al. (2009). *Aspergillus fumigatus* calcipressin cbpA is involved in hyphal growth and calcium homeostasis. *Eukaryot. Cell* 8, 511–519. doi: 10.1128/ec.00336-08

Rothermel, B., Vega, R. B., Yang, J., Wu, H., Bassel-Duby, R., and Williams, R. S. (2000). A protein encoded within the down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. *J. Biol. Chem.* 275, 8719–8725. doi: 10.1074/jbc.275.12.8719

Rothermel, B. A., Vega, R. B., and Williams, R. S. (2003). The role of modulatory calcineurin-interacting proteins in calcineurin signaling. *Trends Cardiovasc. Med.* 13, 15–21. doi: 10.1016/S1050-1738(02)00188-3

Sanna, B., Brandt, E. B., Kaiser, R. A., Pfluger, P., Witt, S. A., Kimball, T. R., et al. (2006). Modulatory calcineurin-interacting proteins 1 and 2 function as calcineurin facilitators in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7327–7332. doi: 10.1073/pnas.0509340103

Shin, S.-Y., Yang, H. W., Kim, J.-R., Do Heo, W., and Cho, K.-H. (2011). A hidden incoherent switch regulates RCAN1 in the calcineurin–NFAT signaling network. *J. Cell Sci.* 124, 82–90. doi: 10.1242/jcs.076034

Steinbach, W. J., Cramer, R. A. Jr., Perfect, B. Z., Asfaw, Y. G., Sauer, T. C., Najvar, L. K., et al. (2006). Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. *Eukaryot. Cell* 5, 1091–1103. doi: 10.1128/EC.00139-06

Steinbach, W. J., Reedy, J. L., Cramer, R. A., Perfect, J. R., and Heitman, J. (2007). Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nat. Rev. Micro.* 5, 418–430. doi: 10.1038/nrmicro1680

Vega, R. B., Yang, J., Rothermel, B. A., Bassel-Duby, R., and Williams, R. S. (2002). Multiple domains of MCIP1 contribute to inhibition of calcineurin activity. *J. Biol. Chem.* 277, 30401–30407. doi: 10.1074/jbc.M200123200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Juvvadi, Ma, Richards, Soderblom, Moseley, Lamoth and Steinbach. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 16 February 2015
doi: 10.3389/fmicb.2015.00096

Histone deacetylase inhibition as an alternative strategy against invasive aspergillosis

Frédéric Lamoth[1,2,3]*, Praveen R. Juvvadi[1] and William J. Steinbach[1,4]

[1] *Division of Pediatric Infectious Diseases, Department of Pediatrics, Duke University Medical Center, Durham, NC, USA*
[2] *Infectious Diseases Service, Department of Medicine, Lausanne University Hospital, Lausanne, Switzerland*
[3] *Institute of Microbiology, Lausanne University Hospital, Lausanne, Switzerland*
[4] *Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA*

Edited by:
Helio K. Takahashi, Universidade Federal de São Paulo, Brazil

Reviewed by:
Marcelo Tolmasky, California State University at Fullerton, USA
Olaf Kniemeyer, Leibniz Institute for Natural Product Research and Infection Biology - Hans-Knoell-Institute, Germany

****Correspondence:***
Frédéric Lamoth, Division of Pediatric Infectious Diseases, Department of Pediatrics, Duke University Medical Center, 427 Jones Building, Research Drive, Durham, NC 27710, USA
e-mail: fredlamoth@gmail.com

Invasive aspergillosis (IA) is a life-threatening infection due to Aspergillus fumigatus and other Aspergillus spp. Drugs targeting the fungal cell membrane (triazoles, amphotericin B) or cell wall (echinocandins) are currently the sole therapeutic options against IA. Their limited efficacy and the emergence of resistance warrant the identification of new antifungal targets. Histone deacetylases (HDACs) are enzymes responsible of the deacetylation of lysine residues of core histones, thus controlling chromatin remodeling and transcriptional activation. HDACs also control the acetylation and activation status of multiple non-histone proteins, including the heat shock protein 90 (Hsp90), an essential molecular chaperone for fungal virulence and antifungal resistance. This review provides an overview of the different HDACs in *Aspergillus* spp. as well as their respective contribution to total HDAC activity, fungal growth, stress responses, and virulence. The potential of HDAC inhibitors, currently under development for cancer therapy, as novel alternative antifungal agents against IA is discussed.

Keywords: lysine deacetylases, *Aspergillus fumigatus*, trichostatin A, heat shock protein 90, antifungal therapy, antifungal resistance

INTRODUCTION

The filamentous fungus *Aspergillus fumigatus* is the primary cause of invasive aspergillosis (IA), a frequent and life-threatening infection in immunosuppressed patients. Novel therapeutic approaches of IA are needed to overcome emerging resistance to azoles, used as first-line therapy, and the toxicity or limited efficacy of second-line treatments such as amphotericin B and echinocandins. Moreover, other *Aspergillus* spp. with less susceptibility to current antifungal drugs (*A. flavus*, *A. terreus*, *A. ustus*) account for a substantial proportion of IA.

The pathogenesis of IA relies on multiple microbial and host factors. At the pathogen level, morphogenetic changes (germination, hyphal growth), thermal and metabolic adaptation to the human body, metabolite production and resistance to antifungal drugs are all determinants contributing to the virulence of *A. fumigatus* (Tekaia and Latge, 2005; Kwon-Chung and Sugui, 2013). Adaptation to environmental conditions involves processes of chromatin remodeling and transcriptional regulation. The modulation of gene expression depends on the packaging of DNA by core histones constituting the dynamic structure of chromatin. Direct DNA methylation and post-translational modifications of histones (such as acetylation and methylation) are important for conformational changes and transcriptional regulation (Brosch et al., 2008). Histone acetyltransferases (HATs) and histone deacetylases (HDACs, also referred to as lysine deacetylases, KDACs) are the enzymes responsible for the reversible process of acetylation (i.e., addition of an acetyl group to the ε-amino group of a lysine residue) and deacetylation of core histones, respectively. Moreover, these enzymes are also involved in the functional regulation of proteins other than core histones, including the heat shock protein 90 (Hsp90), an essential molecular chaperone for proper protein folding and maturation (Yu et al., 2002). In fungi, Hsp90 was shown to play a crucial role in morphogenetic changes, stress adaptation, virulence, and antifungal resistance, and thus represents an attractive antifungal target (Cowen, 2013; Lamoth et al., 2014a). Acetylation of Hsp90 results in impaired Hsp90 function, while HDACs reverse this process and activate the chaperone (Robbins et al., 2012; Lamoth et al., 2014c). Thus, HDACs play a role in fungal virulence by controlling the expression and function of multiple proteins, including chaperones and secondary metabolites that are important for basal growth or stress adaptation, either at the transcriptional level (by deacetylation of core histones and regulation of their expression) or at the post-translational level (by deacetylation and activation of the protein; **Figure 1**). This review will focus on HDACs and their link with Hsp90. The potential of HDAC inhibitors as novel antifungal therapies of IA will be discussed.

HDACs IN *Aspergillus*

Histone deacetylases are categorized in three families: (1) the zinc-dependent or "classical" HDACs (including classes 1 and 2, as well as HOS3-like HDACs in fungi and class 4 in other eukaryotes), (2) the NAD^+-dependent SIR2-like HDACs or sirtuins

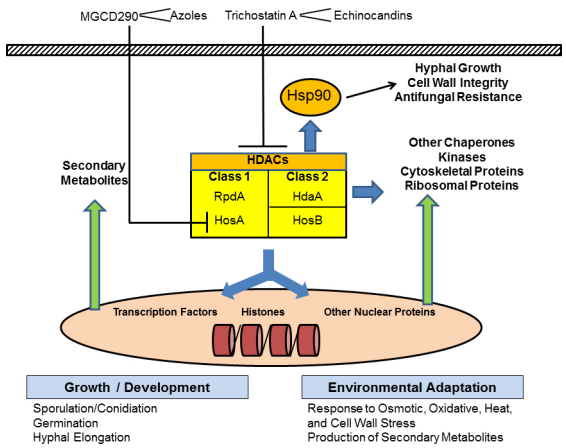


FIGURE 1 | Schematic representation of the role of the classical histone deacetylases (HDACs) in *Aspergillus fumigatus*. Classical HDACs in *Aspergillus* spp. include RpdA and HdaA, contributing to the major part of total HDAC activity, and HosA and HosB, contributing to a minor part. RpdA and HosA belong to class 1. HdaA belongs to class 2. HosB was proposed as the only member of a distinct class. In the nucleus, HDACs deacetylate core histones, which results in chromatin remodeling and transcriptional regulation of the expression of secondary metabolites and multiple proteins. They also regulate the function of other nuclear proteins involved in DNA replication, DNA repair, nuclear transport, or cell cycle. HDACs also deacetylate and control the activation of multiple cytosolic proteins, including the heat shock protein 90 (Hsp90), which is essential for fungal growth and stress responses. HDACs thus contribute to fungal development and environmental adaptation in multiple ways. Trichostatin A and other hydroxamate analogs inhibit both class 1 and class 2 HDACs (with the exception of HosB) displaying antifungal activity against *A. fumigatus* and potentiating (<) the effect of cell wall inhibitors such as the echinocandins. MGCD290 is a specific inhibitor of HosA with poor intrinsic antifungal activity, but potentiates the effect of cell membrane inhibitors such as the azoles.

(also referred as class 3), and (3) the HD2-like enzymes (found exclusively in plants; Trojer et al., 2003; Brosch et al., 2008).

CLASSICAL HDACs

Five classical fungal HDACs have been characterized in the model yeast *Saccharomyces cerevisiae* and divided into two classes, the class 1 or RPD3-type HDACs (including RPD3, HOS1, and HOS2) and the class 2 or HDA1-type HDACs (HDA1; Rundlett et al., 1996; Kurdistani and Grunstein, 2003). HOS3 is a fungal-specific HDAC, representing a third group distinct from the other ones, albeit initially classified within class 2 (Carmen et al., 1999). Our knowledge of classical HDACs in filamentous fungi is essentially derived from studies in *A. nidulans*, *A. oryzae*, and in plant pathogenic fungi (Brosch et al., 2008). In *A. nidulans*, two class 1 enzymes have been identified, RpdA and HosA, with high sequence similarity to yeast RPD3 and HOS2, respectively, except for a 200-amino acid C-terminal extension specific to *A. nidulans* RpdA (Graessle et al., 2000). There is no HOS1 *Aspergillus* ortholog. HdaA was characterized as the yeast HDA1 (class 2) ortholog (Trojer et al., 2003). A HOS3 ortholog, HosB, was also identified (Trojer et al., 2003). Putative orthologs for all these enzymes were identified in *A. oryzae* and *A. fumigatus* (Trojer et al., 2003; Kawauchi et al., 2013).

Both RpdA (class 1) and HdaA (class 2), acting in multiprotein complexes, were shown to contribute to the majority of total HDAC activity in *A. nidulans*, with HdaA being the predominant one (Trojer et al., 2003; Tribus et al., 2005). In contrast, HosA and HosB contribute to a negligible portion of total HDAC activity.

SIRTUINS

The sirtuins are conserved eukaryotic enzymes requiring nicotinamide adenine dinucleotide (NAD^+) for their activity. Their role in promoting longevity has generated great interest. In *S. cerevisiae*, SIR2 silences transcription at the silent mating type loci and reduces ribosomal DNA recombination and rDNA extrachromosomal circles production, resulting in an extended life span (Wierman and Smith, 2014). *S. cerevisiae* has four additional SIR2-like sirtuins, HST1-4 (Wierman and Smith, 2014). Little is known about sirtuins in filamentous fungi. Putative SIR2-type and HST-type orthologs were identified by sequence alignment (Brosch et al., 2008). In *A. nidulans*, it is evident that a proportion of the total HDAC activity, albeit less important than that attributed to classical HDACs, is NAD^+-dependent and thus attributable to sirtuins (Trojer et al., 2003).

ROLE OF HDACs IN *Aspergillus* GROWTH AND ENVIRONMENTAL ADAPTATION

HdaA

Genetic deletion of *hdaA*, the major contributor to total HDAC activity, was performed in *A. nidulans*, *A. oryzae* and *A. fumigatus* (Tribus et al., 2005; Lee et al., 2009; Kawauchi et al., 2013). A growth defect was only observed in the *A. fumigatus ΔhdaA* strain, but did not result in decreased virulence in a murine model of IA (Lee et al., 2009). Altered responses to osmotic or oxidative stress were observed in *A. nidulans* and *A. oryzae* (Tribus et al., 2005; Kawauchi and Iwashita, 2014). HdaA was also found to have a role in the production of secondary metabolites, which was associated with the transcriptional regulation of two telomere-proximal secondary metabolic gene clusters (the sterigmatocystin and penicillin clusters; Shwab et al., 2007; Lee et al., 2009).

RpdA

While RpdA was found to be less important than HdaA for total HDAC activity (Trojer et al., 2003) and could be deleted in yeasts (Robbins et al., 2012), it was found to be essential in *A. nidulans* and *A. oryzae*, as well as in the plant pathogenic filamentous fungus *Magnaporthe oryzae* (Izawa et al., 2009; Tribus et al., 2010; Kawauchi et al., 2013). Genetic repression was achieved in *A. nidulans* by substitution of the native *rpdA* promoter by the alcohol dehydrogenase or xylanase inducible promoters and resulted in an important growth and sporulation defect (Tribus et al., 2010). Truncations in the C-terminal portion of *A. nidulans* RpdA showed that a fungal-specific conserved motif was essential for the catalytic activity of the enzyme and for fungal viability (Tribus et al., 2010). Heterologous expression of the C-terminal motif of *A. fumigatus* RpdA was able to restore RpdA functionality in *A. nidulans*, suggesting a common crucial role of this conserved region among *Aspergillus* spp and possibly other filamentous fungi.

HosA AND HosB

Despite their minor contribution to total HDAC activity in *Aspergillus* spp, functional analyses suggest that HosA (yeast HOS2) is important for growth and virulence in filamentous fungi. Its deletion in *A. oryzae* had more impact on radial growth and sporulation than the *hdaA* deletion and also resulted in altered stress responses (Kawauchi et al., 2013; Kawauchi and Iwashita, 2014). In plant pathogenic fungi (*Fusarium graminearum*, *M. oryzae*, *Cochliobolus carbonum*), HOS2-related enzymes appear as the most important class 2 HDAC for vegetative growth, sporulation and virulence (Baidyaroy et al., 2001; Izawa et al., 2009; Ding et al., 2010; Li et al., 2011). Indeed, Hos2 (HosA ortholog) was found to contribute to an important part of total HDAC activity in *M. oryzae* (Ding et al., 2010), which was not the case for *A. nidulans* (Trojer et al., 2003).

Genetic deletion of *hosB* (the HOS3 type HDAC) did not result in significant phenotypic consequences in *A. nidulans* and *A. oryzae*, confirming its minor contribution to total HDAC activity although this HDAC seems to have some additive role in metabolite production (Tribus et al., 2005; Shwab et al., 2007; Kawauchi et al., 2013; Kawauchi and Iwashita, 2014).

OTHER HDACs

Genetic deletion of 6 sirtuins of *A. oryzae* was performed and only HstD/AoHst4 was associated with a significant role in fungal growth, sporulation, stress responses and production of secondary metabolites via the regulation of LaeA (Kawauchi et al., 2013; Kawauchi and Iwashita, 2014).

THE KEY ROLE OF HDACs IN REGULATING HEAT SHOCK PROTEIN 90

In addition to histones, an increasing number of proteins have been identified as substrates of HDACs, leading to the use of the more general terminology of lysine deacetylases (KDAC; Glozak et al., 2005; Choudhary et al., 2009). Many of these proteins belong to categories that are important for fungal growth and virulence, such as transcription factors, cytoskeletal proteins, and molecular chaperones (Choudhary et al., 2009). Among them, Hsp90 emerges as a key player for which the relationship to HDACs has been recently described. Hsp90 was shown to be essential for fungal survival and to be an important trigger of antifungal resistance to both azole and echinocandin classes and possibly to amphotericin B (Cowen and Lindquist, 2005; Cowen et al., 2009; Singh et al., 2009; Lamoth et al., 2012, 2014b; Blum et al., 2013). In *A. fumigatus*, Hsp90 governs the basal resistance to echinocandins including the paradoxical effect of caspofungin, a compensatory mechanism of the cell wall resulting in decreased caspofungin antifungal activity at increased concentrations (Wiederhold, 2007; Lamoth et al., 2014b). Pan-HDAC inhibitors (depsipeptide, hydroxamic acid analogs) induced acetylation of Hsp90 in human cancer cells, which prevented the binding of Hsp90 to client proteins (Yu et al., 2002; Nimmanapalli et al., 2003). Human HDAC6, a class 2b HDAC related to fungal HDA1, deacetylates Hsp90 and modulates its activity (Bali et al., 2005; Kovacs et al., 2005). Other HDACs of class 2, such as HDAC1 and HDAC10, are also involved in the acetylation of Hsp90 in human (Park et al., 2008; Zhou et al., 2008). Lysine 294 (K294) of human Hsp90 was identified as a key acetylation site for Hsp90 function (Scroggins et al., 2007). Several additional acetylation sites have been recovered in human Hsp90 (Yang et al., 2008; Mollapour and Neckers, 2012).

The role of HDACs in the control of fungal Hsp90 was first demonstrated in *S. cerevisiae* by genetic or pharmacologic inhibition of RPD3 and HDA1, which resulted in the abrogation of Hsp90-dependent azole resistance and impaired interaction of Hsp90 with multiple client proteins (Robbins et al., 2012). Lysine 27 (K27) was found to be acetylated in yeast Hsp90 after deletion of both HDA1 and RPD3 (Robbins et al., 2012). We detected acetylation at K271 (K294 in human) in *A. fumigatus* Hsp90 after treatment with the HDAC inhibitor trichostatin A (TSA; Lamoth et al., 2014c). Mutational analyses actually suggest that both K27 and K271 (K270 in yeast) are important for Hsp90 function. Mutations of both sites resulted in impaired Hsp90 function in *S. cerevisiae* and *A. fumigatus* and were associated with decreased virulence in a murine model of IA (Robbins et al., 2012; Lamoth et al., 2014c). However, while K270 appears to be the predominant site in yeast, K27 seems to be more important in *A. fumigatus*. An acetylation-mimetic mutation (lysine to alanine) of this residue was sufficient to affect Hsp90 function. This effect could be reversed by a deacetylation-mimetic mutation (lysine to arginine) of K27, suggesting that this site must be deacetylated for proper Hsp90 function (Lamoth et al., 2014c). Mutation of K27 alone also resulted in increased susceptibility to both caspofungin and voriconazole in *A. fumigatus* (Lamoth et al., 2014c). However, neither K27 nor K270 mutations were able to decrease azole resistance of an *erg3* mutant of *S. cerevisiae*, while this effect could be achieved by treatment with TSA and by genetic deletion of both RPD3 and HDA1 (Robbins et al., 2012). Thus, the role of HDACs in governing Hsp90 function in antifungal resistance may differ among fungi.

THE ANTIFUNGAL ACTIVITY OF HDAC INHIBITORS

The fact that TSA, an organic antibiotic produced by *Streptomyces hygroscopicus*, and its hydroxamate analogs display some antifungal activity is well-known (Tsuji et al., 1976). Although these compounds are known to be broad-spectrum inhibitors of both class 1 and 2 HDACs (with the exception of HOS3-like HDAC), their precise mode of action against fungi remains poorly elucidated. HDAC inhibitors were shown to considerably modify the profile of secondary metabolites produced by *Aspergillus* spp., although the impact of this effect on virulence is unclear (Henrikson et al., 2009; Konig et al., 2013). Recent studies suggest that their antifungal effect mainly results from the acetylation and inhibition of Hsp90 (Robbins et al., 2012; Lamoth et al., 2014c). HDACs inhibitors have raised considerable interest because of their potential as anticancer therapy and are undergoing rapid development in the pharmaceutical industry (West and Johnstone, 2014). Some of them also display antiparasitic activity against *Plasmodium falciparum* and *Toxoplasma gondii* (Strobl et al., 2007; Trenholme et al., 2014).

Trichostatin A has minimal antifungal activity against *C. albicans*, although it potentiates the activity of azoles by a mechanism which is supposed to be essentially mediated via Hsp90 inhibition and associated with loss in the upregulation of *ERG* and *CDR*

genes in response to azoles (Smith and Edlind, 2002; Robbins et al., 2012). TSA alone appears to be more active against molds. We have observed a 50 and >90% growth inhibition of the wild-type *A. fumigatus* strain AF293 at TSA concentrations of 1 and 4 μg/ml, respectively (Lamoth et al., 2014c). TSA also had variable antifungal activity against clinical isolates of *Aspergillus* spp. and other pathogenic non-*Aspergillus* molds, and was particularly active against the azole-resistant *A. ustus* and the multi-resistant *Scedosporium prolificans* isolates (Lamoth et al., 2015). TSA also exhibited synergistic activity with caspofungin against some *Aspergillus* spp. and with the Hsp90 inhibitor geldanamycin against *Rhizopus* spp. (Lamoth et al., 2014c, 2015). The differential effect of TSA against *C. albicans* and *A. fumigatus* may be related to different patterns of HDAC activity in these fungi. The better antifungal activity of TSA against *A. fumigatus* is consistent with the finding that genetic compromise of *hdaA* and *rpdA* had a much greater impact on fungal growth and survival in *Aspergillus* spp. compared to deletion of their orthologs in yeast (Lee et al., 2009; Tribus et al., 2010; Robbins et al., 2012).

The effect of TSA against *A. fumigatus* had some similitude with that observed after acetylation-mimetic mutations or genetic repression of *hsp90*, including a growth and conidiation defect and hypersensitivity to geldanamycin (Lamoth et al., 2014c). Moreover, TSA enhanced the antifungal activity of caspofungin and abolished the paradoxical effect, which is a hallmark of Hsp90 inhibition. However, TSA did not potentiate the effect of voriconazole against *A. fumigatus*, which contrasts with previous reports in yeasts showing a positive interaction of TSA with azoles but not with echinocandins (Smith and Edlind, 2002; Robbins et al., 2012). Indeed, differences in Hsp90-dependent pathways of azole and echinocandin resistance between *C. albicans* and *A. fumigatus* have been previously outlined (Lamoth et al., 2014a). Overall, these data support that the antifungal effect of TSA is largely mediated via indirect inhibition of Hsp90. However, considering the role of HDACs in transcriptional regulation, metabolite production, and activation of multiple non-histone proteins, it is probable that other mechanisms are involved.

Trichostatin A was well tolerated in pharmacokinetic murine models, but is rapidly metabolized (half-life of 5–10 min; Sanderson et al., 2004). Numerous novel HDAC inhibitors are currently under investigation for the treatment of cancer in clinical and preclinical trials (West and Johnstone, 2014). Vorinostat (suberoylanilide hydroxamic acid, SAHA) is a TSA analog which has an extended half-life and has been approved by the food and drug administration (FDA) for the treatment of cutaneous T cell lymphoma (Duvic et al., 2007). The antifungal activity of SAHA and other hydroxamate analogs remains to be investigated. Although these compounds have been well tolerated in clinical trials (Duvic et al., 2009; Fouladi et al., 2010), some rare and possibly dose-dependent hematologic adverse events (granulocytopenia, thrombocytopenia) may counterbalance their potential benefit in the setting of invasive fungal diseases.

A selective HDAC inhibitor of fungal HOS2, MGCD290 (MethylGene Inc., Montreal, QC, Canada) was tested against clinical isolates of yeasts and molds (Pfaller et al., 2009). MGCD290 alone displayed some *in vitro* antifungal activity against *Candida* spp. and other yeasts, but was inactive against filamentous fungi. Most importantly, MGCD290 exhibited synergism with azoles (fluconazole, voriconazole and posaconazole) against both yeast and mold species. The combination of MGCD290 and fluconazole was recently tested for the treatment of vulvovaginal candidiasis in a randomized phase II study but did not demonstrate a benefit compared to fluconazole monotherapy (Augenbraun et al., 2013).

CONCLUSION AND PERSPECTIVES

In filamentous fungi, HDACs are involved in multiple processes contributing to virulence via transcription control and functional regulation of important proteins (**Figure 1**). HDAC inhibition thus represents an interesting alternative antifungal approach to current strategies targeting the cell wall or membrane. The antifungal effect of the HDAC inhibitor TSA and its analogs against *A. fumigatus* seems to be mainly achieved via the acetylation and inhibition of Hsp90 (Lamoth et al., 2014c). This molecular chaperone is essential for virulence and triggers stress responses and resistance to the most important antifungal classes (Lamoth et al., 2014a). HDAC inhibitors have demonstrated a potential as antifungal monotherapy *in vitro*, as well as adjunctive therapies to enhance the effect of existing drugs against *A. fumigatus* (Pfaller et al., 2009; Lamoth et al., 2014c, 2015). Their potential as anti-cancer therapy is generating an intense research activity in drug development with several compounds currently investigated in clinical and pre-clinical trials (West and Johnstone, 2014). There is a great opportunity to harness these recent advances, and gain in-depth understanding of fungal HDACs, to pursue novel and more effective antifungal strategies against IA.

ACKNOWLEDGMENTS

FL is supported by the Swiss National Science Foundation and the Swiss Foundation for Medical-Biological Grants (P3SMP3-151742).

REFERENCES

Augenbraun, M., Livingston, J., Parker, R., Lederman, S., Chavoustie, S., Morgan, F., et al. (2013). "Fluconazole and MGCD290 in vulvo vaginal candidiasis (VVC): results from a randomized phase II study (1330)," in *IDWeek 2013*, October 2–6, 2013, San Francisco, CA.

Baidyaroy, D., Brosch, G., Ahn, J. H., Graessle, S., Wegener, S., Tonukari, N. J., et al. (2001). A gene related to yeast HOS2 histone deacetylase affects extracellular depolymerase expression and virulence in a plant pathogenic fungus. *Plant Cell* 13, 1609–1624. doi: 10.1105/tpc.13.7.1609

Bali, P., Pranpat, M., Bradner, J., Balasis, M., Fiskus, W., Guo, F., et al. (2005). Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. *J. Biol. Chem.* 280, 26729–26734. doi: 10.1074/jbc.C500186200

Blum, G., Kainzner, B., Grif, K., Dietrich, H., Zelger, B., Sonnweber, T., et al. (2013). In vitro and in vivo role of heat shock protein 90 in Amphotericin B resistance of *Aspergillus terreus*. *Clin. Microbiol. Infect.* 19, 50–55. doi: 10.1111/j.1469-0691.2012.03848.x

Brosch, G., Loidl, P., and Graessle, S. (2008). Histone modifications and chromatin dynamics: a focus on filamentous fungi. *FEMS Microbiol. Rev.* 32, 409–439. doi: 10.1111/j.1574-6976.2007.00100.x

Carmen, A. A., Griffin, P. R., Calaycay, J. R., Rundlett, S. E., Suka, Y., and Grunstein, M. (1999). Yeast HOS3 forms a novel trichostatin A-insensitive homodimer with intrinsic histone deacetylase activity. *Proc. Natl. Acad. Sci. U.S.A.* 96, 12356–12361. doi: 10.1073/pnas.96.22.12356

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., et al. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 325, 834–840. doi: 10.1126/science.1175371

Cowen, L. E. (2013). The fungal Achilles' heel: targeting Hsp90 to cripple fungal pathogens. *Curr. Opin. Microbiol.* 16, 377–384. doi: 10.1016/j.mib.2013.03.005

Cowen, L. E., and Lindquist, S. (2005). Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science* 309, 2185–2189. doi: 10.1126/science.1118370

Cowen, L. E., Singh, S. D., Kohler, J. R., Collins, C., Zaas, A. K., Schell, W. A., et al. (2009). Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2818–2823. doi: 10.1073/pnas.0813394106

Ding, S. L., Liu, W., Iliuk, A., Ribot, C., Vallet, J., Tao, A., et al. (2010). The tig1 histone deacetylase complex regulates infectious growth in the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* 22, 2495–2508. doi: 10.1105/tpc.110.074302

Duvic, M., Talpur, R., Ni, X., Zhang, C., Hazarika, P., Kelly, C., et al. (2007). Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood* 109, 31–39. doi: 10.1182/blood-2006-06-025999

Duvic, M., Olsen, E. A., Breneman, D., Pacheco, T. R., Parker, S., Vonderheid, E. C., et al. (2009). Evaluation of the long-term tolerability and clinical benefit of vorinostat in patients with advanced cutaneous T-cell lymphoma. *Clin. Lymphoma Myeloma* 9, 412–416. doi: 10.3816/CLM.2009.n.082

Fouladi, M., Park, J. R., Stewart, C. F., Gilbertson, R. J., Schaiquevich, P., Sun, J., et al. (2010). Pediatric phase I trial and pharmacokinetic study of vorinostat: a Children's Oncology Group phase I consortium report. *J. Clin. Oncol.* 28, 3623–3629. doi: 10.1200/JCO.2009.25.9119

Glozak, M. A., Sengupta, N., Zhang, X., and Seto, E. (2005). Acetylation and deacetylation of non-histone proteins. *Gene* 363, 15–23. doi: 10.1016/j.gene.2005.09.010

Graessle, S., Dangl, M., Haas, H., Mair, K., Trojer, P., Brandtner, E. M., et al. (2000). Characterization of two putative histone deacetylase genes from *Aspergillus nidulans*. *Biochim. Biophys. Acta* 1492, 120–126. doi: 10.1016/S0167-4781(00)00093-2

Henrikson, J. C., Hoover, A. R., Joyner, P. M., and Cichewicz, R. H. (2009). A chemical epigenetics approach for engineering the in situ biosynthesis of a cryptic natural product from *Aspergillus niger*. *Org. Biomol. Chem.* 7, 435–438. doi: 10.1039/b819208a

Izawa, M., Takekawa, O., Arie, T., Teraoka, T., Yoshida, M., Kimura, M., et al. (2009). Inhibition of histone deacetylase causes reduction of appressorium formation in the rice blast fungus *Magnaporthe oryzae*. *J. Gen. Appl. Microbiol.* 55, 489–498. doi: 10.2323/jgam.55.489

Kawauchi, M., and Iwashita, K. (2014). Functional analysis of histone deacetylase and its role in stress response, drug resistance and solid-state cultivation in *Aspergillus oryzae*. *J. Biosci. Bioeng* 118, 172–176. doi: 10.1016/j.jbiosc.2014.02.004

Kawauchi, M., Nishiura, M., and Iwashita, K. (2013). Fungus-specific sirtuin HstD coordinates secondary metabolism and development through control of LaeA. *Eukaryot. Cell* 12, 1087–1096. doi: 10.1128/EC.00003-13

Konig, C. C., Scherlach, K., Schroeckh, V., Horn, F., Nietzsche, S., Brakhage, A. A., et al. (2013). Bacterium induces cryptic meroterpenoid pathway in the pathogenic fungus *Aspergillus fumigatus*. *Chembiochem* 14, 938–942. doi: 10.1002/cbic.201300070

Kovacs, J. J., Murphy, P. J., Gaillard, S., Zhao, X., Wu, J. T., Nicchitta, C. V., et al. (2005). HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Mol. Cell* 18, 601–607. doi: 10.1016/j.molcel.2005.04.021

Kurdistani, S. K., and Grunstein, M. (2003). Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell Biol.* 4, 276–284. doi: 10.1038/nrm1075

Kwon-Chung, K. J., and Sugui, J. A. (2013). *Aspergillus fumigatus*—what makes the species a ubiquitous human fungal pathogen? *PLoS Pathog.* 9:e1003743. doi: 10.1371/journal.ppat.1003743

Lamoth, F., Alexander, B. D., Juvvadi, P. R., and Steinbach, W. J. (2015). Antifungal activity of compounds targeting the Hsp90-calcineurin pathway against various mould species. *J. Antimicrob. Chemother.* doi: 10.1093/jac/dku549 [Epub ahead of print].

Lamoth, F., Juvvadi, P. R., Fortwendel, J. R., and Steinbach, W. J. (2012). Heat shock protein 90 is required for conidiation and cell wall integrity in *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 1324–1332. doi: 10.1128/EC.00032-12

Lamoth, F., Juvvadi, P. R., and Steinbach, W. J. (2014a). Heat shock protein 90 (Hsp90): a novel antifungal target against *Aspergillus fumigatus*. *Crit. Rev. Microbiol.* doi: 10.3109/1040841X.2014.947239 [Epub ahead of print].

Lamoth, F., Juvvadi, P. R., Gehrke, C., Asfaw, Y. G., and Steinbach, W. J. (2014b). Transcriptional activation of heat shock protein 90 mediated via a proximal promoter region as trigger of caspofungin resistance in *Aspergillus fumigatus*. *J. Infect. Dis.* 209, 473–481. doi: 10.1093/infdis/jit530

Lamoth, F., Juvvadi, P. R., Soderblom, E. J., Moseley, M. A., Asfaw, Y. G., and Steinbach, W. J. (2014c). Identification of a key lysine residue in heat shock protein 90 required for azole and echinocandin resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 58, 1889–1896. doi: 10.1128/AAC.02286-13

Lee, I., Oh, J. H., Shwab, E. K., Dagenais, T. R., Andes, D., and Keller, N. P. (2009). HdaA, a class 2 histone deacetylase of *Aspergillus fumigatus*, affects germination and secondary metabolite production. *Fungal Genet. Biol.* 46, 782–790. doi: 10.1016/j.fgb.2009.06.007

Li, Y., Wang, C., Liu, W., Wang, G., Kang, Z., Kistler, H. C., et al. (2011). The HDF1 histone deacetylase gene is important for conidiation, sexual reproduction, and pathogenesis in Fusarium graminearum. *Mol. Plant Microbe Interact.* 24, 487–496. doi: 10.1094/MPMI-10-10-0233

Mollapour, M., and Neckers, L. (2012). Post-translational modifications of Hsp90 and their contributions to chaperone regulation. *Biochim. Biophys. Acta* 1823, 648–655. doi: 10.1016/j.bbamcr.2011.07.018

Nimmanapalli, R., Fuino, L., Bali, P., Gasparetto, M., Glozak, M., Tao, J., et al. (2003). Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. *Cancer Res.* 63, 5126–5135.

Park, J. H., Kim, S. H., Choi, M. C., Lee, J., Oh, D. Y., Im, S. A., et al. (2008). Class II histone deacetylases play pivotal roles in heat shock protein 90-mediated proteasomal degradation of vascular endothelial growth factor receptors. *Biochem. Biophys. Res. Commun.* 368, 318–322. doi: 10.1016/j.bbrc.2008.01.056

Pfaller, M. A., Messer, S. A., Georgopapadakou, N., Martell, L. A., Besterman, J. M., and Diekema, D. J. (2009). Activity of MGCD290, a Hos2 histone deacetylase inhibitor, in combination with azole antifungals against opportunistic fungal pathogens. *J. Clin. Microbiol.* 47, 3797–3804. doi: 10.1128/JCM.00618-09

Robbins, N., Leach, M. D., and Cowen, L. E. (2012). Lysine deacetylases Hda1 and Rpd3 regulate Hsp90 function thereby governing fungal drug resistance. *Cell Rep.* 2, 878–888. doi: 10.1016/j.celrep.2012.08.035

Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14503–14508. doi: 10.1073/pnas.93.25.14503

Sanderson, L., Taylor, G. W., Aboagye, E. O., Alao, J. P., Latigo, J. R., Coombes, R. C., et al. (2004). Plasma pharmacokinetics and metabolism of the histone deacetylase inhibitor trichostatin a after intraperitoneal administration to mice. *Drug Metab. Dispos.* 32, 1132–1138. doi: 10.1124/dmd.104.000638

Scroggins, B. T., Robzyk, K., Wang, D., Marcu, M. G., Tsutsumi, S., Beebe, K., et al. (2007). An acetylation site in the middle domain of Hsp90 regulates chaperone function. *Mol. Cell* 25, 151–159. doi: 10.1016/j.molcel.2006.12.008

Shwab, E. K., Bok, J. W., Tribus, M., Galehr, J., Graessle, S., and Keller, N. P. (2007). Histone deacetylase activity regulates chemical diversity in *Aspergillus*. *Eukaryot. Cell* 6, 1656–1664. doi: 10.1128/EC.00186-07

Singh, S. D., Robbins, N., Zaas, A. K., Schell, W. A., Perfect, J. R., and Cowen, L. E. (2009). Hsp90 governs echinocandin resistance in the pathogenic yeast Candida albicans via calcineurin. *PLoS Pathog.* 5:e1000532. doi: 10.1371/journal.ppat.1000532

Smith, W. L., and Edlind, T. D. (2002). Histone deacetylase inhibitors enhance *Candida albicans* sensitivity to azoles and related antifungals: correlation with reduction in CDR and ERG upregulation. *Antimicrob. Agents Chemother.* 46, 3532–3539. doi: 10.1128/AAC.46.11.3532-3539.2002

Strobl, J. S., Cassell, M., Mitchell, S. M., Reilly, C. M., and Lindsay, D. S. (2007). Scriptaid and suberoylanilide hydroxamic acid are histone deacetylase inhibitors with potent anti-*Toxoplasma gondii* activity in vitro. *J. Parasitol.* 93, 694–700. doi: 10.1645/GE-1043R.1

Tekaia, F., and Latge, J. P. (2005). *Aspergillus fumigatus*: saprophyte or pathogen? *Curr. Opin. Microbiol.* 8, 385–392. doi: 10.1016/j.mib.2005.06.017

Trenholme, K., Marek, L., Duffy, S., Pradel, G., Fisher, G., Hansen, F. K., et al. (2014). Lysine acetylation in sexual stage malaria parasites is a target for

antimalarial small molecules. *Antimicrob. Agents Chemother.* 58, 3666–3678. doi: 10.1128/AAC.02721-13

Tribus, M., Bauer, I., Galehr, J., Rieser, G., Trojer, P., Brosch, G., et al. (2010). A novel motif in fungal class 1 histone deacetylases is essential for growth and development of *Aspergillus*. *Mol. Biol. Cell* 21, 345–353. doi: 10.1091/mbc.E09-08-0750

Tribus, M., Galehr, J., Trojer, P., Brosch, G., Loidl, P., Marx, F., et al. (2005). HdaA, a major class 2 histone deacetylase of *Aspergillus nidulans*, affects growth under conditions of oxidative stress. *Eukaryot. Cell* 4, 1736–1745. doi: 10.1128/EC.4.10.1736-1745.2005

Trojer, P., Brandtner, E. M., Brosch, G., Loidl, P., Galehr, J., Linzmaier, R., et al. (2003). Histone deacetylases in fungi: novel members, new facts. *Nucleic Acids Res.* 31, 3971–3981. doi: 10.1093/nar/gkg473

Tsuji, N., Kobayashi, M., Nagashima, K., Wakisaka, Y., and Koizumi, K. (1976). A new antifungal antibiotic, trichostatin. *J. Antibiot.* (*Tokyo*) 29, 1–6. doi: 10.7164/antibiotics.29.1

West, A. C., and Johnstone, R. W. (2014). New and emerging HDAC inhibitors for cancer treatment. *J. Clin. Invest.* 124, 30–39. doi: 10.1172/JCI69738

Wiederhold, N. P. (2007). Attenuation of echinocandin activity at elevated concentrations: a review of the paradoxical effect. *Curr. Opin. Infect. Dis.* 20, 574–578. doi: 10.1097/QCO.0b013e3282f1be7f

Wierman, M. B., and Smith, J. S. (2014). Yeast sirtuins and the regulation of aging. *FEMS Yeast Res.* 14, 73–88. doi: 10.1111/1567-1364.12115

Yang, Y., Rao, R., Shen, J., Tang, Y., Fiskus, W., Nechtman, J., et al. (2008). Role of acetylation and extracellular location of heat shock protein 90alpha in tumor cell invasion. *Cancer Res.* 68, 4833–4842. doi: 10.1158/0008-5472.CAN-08-0644

Yu, X., Guo, Z. S., Marcu, M. G., Neckers, L., Nguyen, D. M., Chen, G. A., et al. (2002). Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. *J. Natl. Cancer Inst.* 94, 504–513. doi: 10.1093/jnci/94.7.504

Zhou, Q., Agoston, A. T., Atadja, P., Nelson, W. G., and Davidson, N. E. (2008). Inhibition of histone deacetylases promotes ubiquitin-dependent proteasomal degradation of DNA methyltransferase 1 in human breast cancer cells. *Mol. Cancer Res.* 6, 873–883. doi: 10.1158/1541-7786.MCR-07-0330

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 December 2014; accepted: 26 January 2015; published online: 16 February 2015.

Citation: Lamoth F, Juvvadi PR and Steinbach WJ (2015) Histone deacetylase inhibition as an alternative strategy against invasive aspergillosis. Front. Microbiol. ***6****:96. doi: 10.3389/fmicb.2015.00096*

This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology.

Copyright © 2015 Lamoth, Juvvadi and Steinbach. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 27 February 2015
doi: 10.3389/fmicb.2015.00160

Targeting zinc homeostasis to combat *Aspergillus fumigatus* infections

Rocío Vicentefranqueira[1,2], Jorge Amich[1,2†], Paris Laskaris[3], Oumaima Ibrahim-Granet[3], Jean P. Latgé[4], Héctor Toledo[1,2], Fernando Leal[1,2] and José A. Calera[1,2]*

[1] Instituto de Biología Funcional y Genómica, Centro Mixto del Consejo Superior de Investigaciones Científicas y Universidad de Salamanca, Salamanca, Spain
[2] Departamento de Microbiología y Genética, Universidad de Salamanca, Salamanca, Spain
[3] Unité de Recherche Cytokines and Inflammation, Institut Pasteur, Paris, France
[4] Unité des Aspergillus, Institut Pasteur, Paris, France

Edited by:
Frederic Lamoth, Duke University, USA

Reviewed by:
Sven Krappmann, Friedrich-Alexander-Universität Erlangen-Nürnberg, University Hospital Erlangen, Germany
Karthik M. Krishnan, University of Cincinnati, USA

***Correspondence:**
José A. Calera, Instituto de Biología Funcional y Genómica, Centro Mixto del Consejo Superior de Investigaciones Científicas y Universidad de Salamanca, Lab. P1.10. C/Zacarías González no. 2, 37007 Salamanca, Spain
e-mail: jacalera@usal.es

† Present address:
Jorge Amich, IZKF Forschergruppe für Experimentelle Stammzelltransplantation, Medizinische Klinik und Poliklinik II and Universitäts-Kinderklinik, ZEMM-Zinklesweg 10, 97078 Würzburg, Germany

Aspergillus fumigatus is able to invade and grow in the lungs of immunosuppressed individuals and causes invasive pulmonary aspergillosis. The concentration of free zinc in living tissues is much lower than that required for optimal fungal growth *in vitro* because most of it is tightly bound to proteins. To obtain efficiently zinc from a living host *A. fumigatus* uses the zinc transporters ZrfA, ZrfB, and ZrfC. The ZafA transcriptional regulator induces the expression of all these transporters and is essential for virulence. Thus, ZafA could be targeted therapeutically to inhibit fungal growth. The ZrfC transporter plays the major role in zinc acquisition from the host whereas ZrfA and ZrfB rather have a supplementary role to that of ZrfC. In addition, only ZrfC enables *A. fumigatus* to overcome the inhibitory effect of calprotectin, which is an antimicrobial Zn/Mn-chelating protein synthesized and released by neutrophils within the fungal abscesses of immunosuppressed non-leucopenic animals. Hence, fungal survival in these animals would be undermined upon blocking therapeutically the function of ZrfC. Therefore, both ZafA and ZrfC have emerged as promising targets for the discovery of new antifungals to treat *Aspergillus* infections.

Keywords: *Aspergillus fumigatus*, zinc homeostasis, fungal pathogenesis, zinc transporters, transcription factors, drug discovery

INTRODUCTION

Zinc is, after magnesium, the second most widespread metal present in enzymes belonging to all six major functional classes (Andreini et al., 2008). In addition, zinc ions are structural components of DNA binding domains of many transcription regulators and are required for their proper folding and binding to DNA. Consequently, zinc is essential for a wide variety of biochemical processes, for the adequate regulation of gene expression, and for cellular growth and development. Thus, when the cellular zinc content is lower than the "zinc quota," i.e., the total amount of zinc required for a cell to grow optimally (Outten and O'Halloran, 2001), cell growth stops. In contrast, cells become intoxicated when the cellular zinc content exceeds an upper threshold. Like all organisms, the filamentous fungus *Aspergillus fumigatus* regulates tightly zinc homeostasis to maintain its own zinc quota. However, unlike most saprophytic fungi, *A. fumigatus* has different biological traits that turn it into an opportunistic pathogen (Tekaia and Latge, 2005), including its capacity to uptake zinc from host tissues (Amich et al., 2014). Thus, it is able to invade the lungs of susceptible individuals and causes invasive pulmonary aspergillosis (IPA), whose mortality rate may reach up to 90% depending on the host's immune status (Kousha et al., 2011). One of the reasons for this high mortality rate is the low efficiency of the antifungal drugs currently in use to stop rapidly fungal growth. In this regard, we propose both regulation of zinc homeostasis and zinc acquisition as ideal therapeutic targets for the development of a next generation of antifungals, as an alternative to the classical antifungals that target either cell wall or ergosterol biosynthesis.

ZINC AVAILABILITY AND MYCELIAL GROWTH OF *Aspergillus fumigatus*

The primary ecological niche of *A. fumigatus* is soil, where it grows as a saprophyte on organic decaying matter. In soils zinc is found in solution as free ions (Zn^{2+} and $ZnOH^{+}$) and/or forming

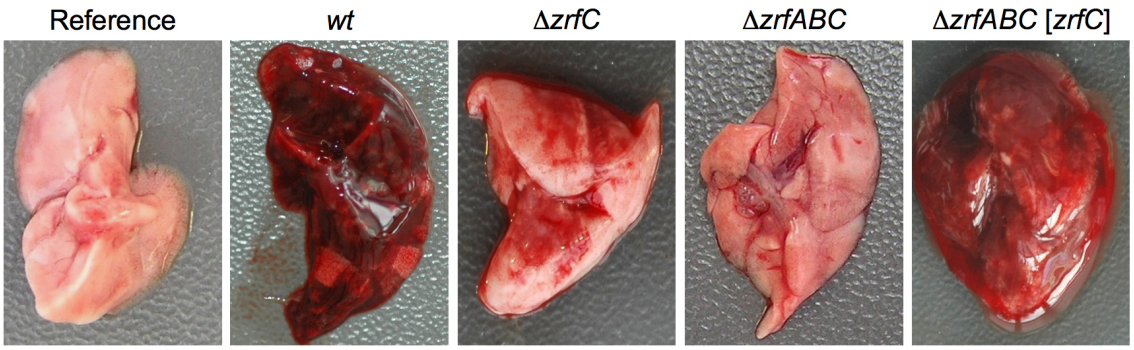


FIGURE 1 | Lungs from immunosuppressed mice infected with different *A. fumigatus* strains. The mice were immunosuppressed using a leucopenic regime and inoculated intranasally with 10^5 conidia of the AF14 (wild-type), AF54 (Δ*zrfC*), AF721 (Δ*zrfA*Δ*zrfB*Δ*zrfC*), and AF731 (Δ*zrfA*Δ*zrfB*Δ*zrfC*[*zrfC*]) strains, as described in Amich et al. (2014). Mice were sacrificed after 4 days post-inoculation and the left lung was excised and photographed. The lungs from mice infected with the wild-type and Δ*zrfABC*[*zrfC*] strains showed the greatest signs of pulmonary infarction. In contrast, the lungs from mice inoculated with the Δ*zrfC* strain showed a very low degree of infarction. The lungs from mice inoculated with the Δ*zrfABC* strain appeared healthy similar to that from a non-inoculated mouse used as reference.

organic zinc complexes, as exchangeable zinc adsorbed to solid surfaces and forming insoluble complexes with other minerals (Alloway, 2008). The concentration of zinc in the soil liquid phase depends upon several factors but the ones that most strongly influence zinc solubility in soils are pH and moisture. Thus, although the concentration of soluble zinc in most soils ranges from 0.06 to 4.2 μM, in very acid soils it may exceed 110 μM (Alloway, 2008). Only the exchangeable zinc pool (i.e., the soluble zinc pool and the one that can be easily desorbed or released from soil particles) is available for plants and microorganisms. In addition, soils are dynamic environments that undergo wide fluctuations in many of their chemical and physical parameters that influence zinc availability. This explains why saprophytic soil inhabitants, particularly those adapted to grow in a wide range of pH values (e.g., *A. fumigatus*), are well equipped to face variations in soil zinc availability.

Aspergillus fumigatus has many genes encoding both zinc importers and exporters. The predicted role in zinc homeostasis of most of these transporters is based on what is known about similar proteins in other fungi, plants, and bacteria (Amich and Calera, 2014). The regulation of zinc homeostasis in *A. fumigatus* is mediated by the master zinc-responsive transcription factor ZafA (Moreno et al., 2007). In addition, the ZafA activity is further modulated in a pH-dependent manner likely influenced by the PacC transcriptional regulator (Amich et al., 2009, 2010). Thus, ZafA induces the transcription of *zrfA* and *zrfB* more strongly in acidic than in alkaline zinc-limiting media. In contrast, ZafA induces much more strongly the transcription of *zrfC* in alkaline than in acidic zinc-limiting media (Vicentefranqueira et al., 2005; Amich et al., 2010).

Although *A. fumigatus* is primarily a saprophytic fungus, it can grow also as a parasite within a susceptible, immunosuppressed animal host. Unlike soils, where zinc availability may change dramatically as a result of weather, leaching, animal activities, and/or human interventions, living environments provide homeostatic conditions in which zinc is tightly bound to zinc-binding proteins, such that the concentration of labile zinc in host tissues (i.e., the pool of free Zn^{2+} ions in both soluble and exchangeable form) is in the nanomolar range (Iyengar and Woittiez, 1988). Thus, zinc availability in living tissues is maintained constantly too low to support a sustained growth of most microorganisms, unless they have evolved mechanisms to overcome zinc starvation during their saprophytic growth that could be adapted for parasitic growth. In this regard, *A. fumigatus* takes advantage of the zinc transporters ZrfA, ZrfB, and ZrfC to grow in the slightly alkaline and extreme zinc-limiting environment provided by the host.

ZINC ACQUISITION AND VIRULENCE IN *A. fumigatus* AND OTHER FUNGAL PATHOGENS

The relationship between zinc homeostasis and virulence of human fungal pathogens was shown for the first time in *A. fumigatus*. The ZafA-mediated regulation of zinc homeostasis is essential for *A. fumigatus* growth within a host but dispensable for it to grow as a saprophyte in zinc-replete media (Moreno et al., 2007). The avirulence of a Δ*zafA* mutant of *A. fumigatus* primarily resides in its inability to obtain zinc from the host, which results in a zinc shortage that stops germ tube elongation. This is consistent with the avirulence of a Δ*zrfA*Δ*zrfB*Δ*zrfC* strain (Amich et al., 2014), which lacks the three major downstream targets of ZafA. However, the ZrfA and ZrfB acidic zinc transporters and the ZrfC alkaline zinc transporter contribute differentially to fungal virulence as shown by the appearance of lungs excised from mice infected with the Δ*zrfA*Δ*zrfB* and Δ*zrfC* mutants (**Figure 1**). The ZrfC protein plays a major role in obtaining zinc from living tissues and sustaining fungal growth within them, whereas the acidic transporters ZrfA/B have a supplementary function in zinc acquisition from the host. The importance of ZrfC for fungal virulence relies on its long extracellular N-terminus, which has four putative zinc-binding motifs and that is absent in the acidic transporters (Amich et al., 2010, 2014). Indeed, in terms of fungal virulence the zinc uptake activity of the ZrfC protein without its N-terminus equals to that of ZrfA and ZrfB together (Amich et al., 2014). ZafA-like regulatory systems have also been described

in and related to the virulence of the pathogenic fungi *Cryptococcus gattii*, *Candida albicans*, and *Paracoccidioides brasiliensis*, although in these latter two microorganisms this has not yet been experimentally confirmed (Nobile et al., 2009; Schneider et al., 2012; Parente et al., 2013). These fungal pathogens have genes encoding proteins similar to ZrfA/B and ZrfC, although the N-terminus of the *C. gatti* ZrfC-like protein (XP_003195293) is very different from that of ZrfC and the *C. albicans* (XP_715421) and *P. brasiliensis* (XP_010761875) ZrfC-like proteins. In either case, the roles of these transporters for virulence have not been investigated.

ZINC-BASED STRATEGIES DEPLOYED BY PHAGOCYTES TO INHIBIT MICROBIAL GROWTH

The regulation of zinc homeostasis is especially relevant for pathogens because the amount of labile zinc in host tissues is very low. Accordingly, mammals have evolved the capacity to inhibit microbial growth in their tissues by zinc starvation, as part of a broader defense mechanism termed "nutritional immunity" (Hood and Skaar, 2012). Two different strategies to sequester labile zinc have been described in mammals thus far. One of them is directed to inhibit the growth of extracellular pathogens and relies upon releasing of calprotectin (CP) by neutrophils in abscesses (Corbin et al., 2008). The other one operates in activated macrophages infected with intracellular pathogens and involves the binding of zinc to metallothioneins (MTs; Subramanian Vignesh et al., 2013). However, an excess of zinc is also noxious for pathogens, probably because it reacts with the −SH groups of proteins. Thus, it is not surprising that mammals had evolved also an antimicrobial strategy based upon zinc poisoning of microbes enclosed in macrophage endosomes (Botella et al., 2011). These defense strategies against pathogens deployed by mammals are consistent with the notion that zinc acquisition and zinc detoxification are virulence attributes of bacterial and fungal pathogens. It might be possible that both MTs and zinc poisoning play a role in macrophages against *A. fumigatus*, as they do respectively against *Histoplasma capsulatum* (Subramanian Vignesh et al., 2013) and *Mycobacterium tuberculosis* (Botella et al., 2011). However, the effects of MTs and zinc poisoning on conidia germination inside the macrophages have not been investigated. In contrast, it has been reported that CP is the major component of the extracellular traps released by neutrophils (NETs) as a defense against *C. albicans* (Urban et al., 2009). NETs are also produced against *A. fumigatus* both *in vitro* and *in vivo* (Bruns et al., 2010; McCormick et al., 2010). More recently it has been shown that CP can reduce the growth capacity *in vitro* of *A. fumigatus* and that the ability to grow of this fungus in the presence of CP under alkaline Zn/Mn-limiting conditions relies on the function of ZrfC (Amich et al., 2014). The only leukocytes infiltrated into *A. fumigatus* abscesses in immunosuppressed non-leucopenic mice that are able to produce CP are the polymorphonuclear neutrophils (PMNs) and plasmacytoid dendritic cells (pDCs; a subtype of DCs). PMNs produce huge amounts of CP that is released in NETs following their lysis (Urban et al., 2009) whereas pDCs synthesize and transport CP to their surface upon activation (Ramirez-Ortiz et al., 2011). In fungal abscesses heavily infiltrated with leukocytes we have observed patches and thread-like structures stained intensely with hematoxylin (**Figure 2A**). These structures, which might correspond to chromatin present in DCs, monocytes, PMNs, and/or NETs, are either spread out on the surface of hyphae or enveloping them. Interestingly, the observation at high magnification of infiltrated abscesses immunostained for CP indicated that this was most frequently detected in leukocytes located in close proximity to hyphae or attached to them (**Figure 2B**). This suggests that CP may create a Zn/Mn-deprived microenvironment around fungal cells to restrict their growth at the time that *A. fumigatus* manages to overcome the inhibitory effect of CP through the action of the ZrfC. Thus, ZrfC has a dual role in fungal virulence: it is needed to mediate zinc uptake and to counteract the inhibitory effect of CP in abscesses.

THE REGULATION OF ZINC HOMEOSTASIS AND ZINC ACQUISITION AS IDEAL TARGETS FOR ANTIFUNGAL DISCOVERY AND DEVELOPMENT

The fact that host mammals are able to inhibit microbial growth either by intoxicating pathogens with zinc or by limiting the access of pathogens to the host zinc pool have led us to presume that these strategies could be imitated to control therapeutically the growth of pathogens in a host.

The lack of knowledge about whether the host immune cells use zinc poisoning to prevent or combat *A. fumigatus* infections and, in this case, whether the fungus has countermeasures to protect itself from it, does not allow us to envision to date a specific therapeutic approach to mimic it. In contrast, what is known to date about zinc homeostasis is enough for us to propose that a therapeutic approach based on preventing the access of the fungus to host zinc would be deleterious for fungal cells because fungal growth depends on a constant intake of zinc and fungal zinc depletion increases the synthesis of harmful reactive oxygen species (reviewed in Eide, 2011). Thus, any compound that interfere intra or extracellularly with zinc homeostasis would predictably inhibit fast and efficiently the fungal growth within host tissues.

The major challenge to treat IPA by targeting zinc homeostasis relies on the identification of the targets and the discovery and development of specific antifungal compounds. Regarding the first point, we propose the ZrfC transporter and ZafA transcription factor as targets. Nutrient transporters are currently considered as new promising therapeutic targets (Slavic et al., 2011; Prati et al., 2014). ZrfC has a long N-terminus that has been predicted to be located toward the extracellular side of the plasma membrane and, hence, that would be readily accessible to specific inhibitors (Amich et al., 2010). In addition, it would be expected that any interference with the function of the N-terminus of ZrfC had the same effect as inactivation of the whole protein in terms of both fungal growth and virulence (Amich et al., 2014). However, the inactivation of ZrfC is partially compensated by an increase in the expression level of the acidic transporters (ZrfA and ZrfB; Amich et al., 2014), which is most likely mediated by ZafA. This indicates that to block efficiently fungal growth it would be required to inhibit either ZrfA/B or ZafA in addition to ZrfC. By taking advantage of the key role of ZafA in the homeostatic and adaptive responses to zinc deprivation, we consider that ZafA

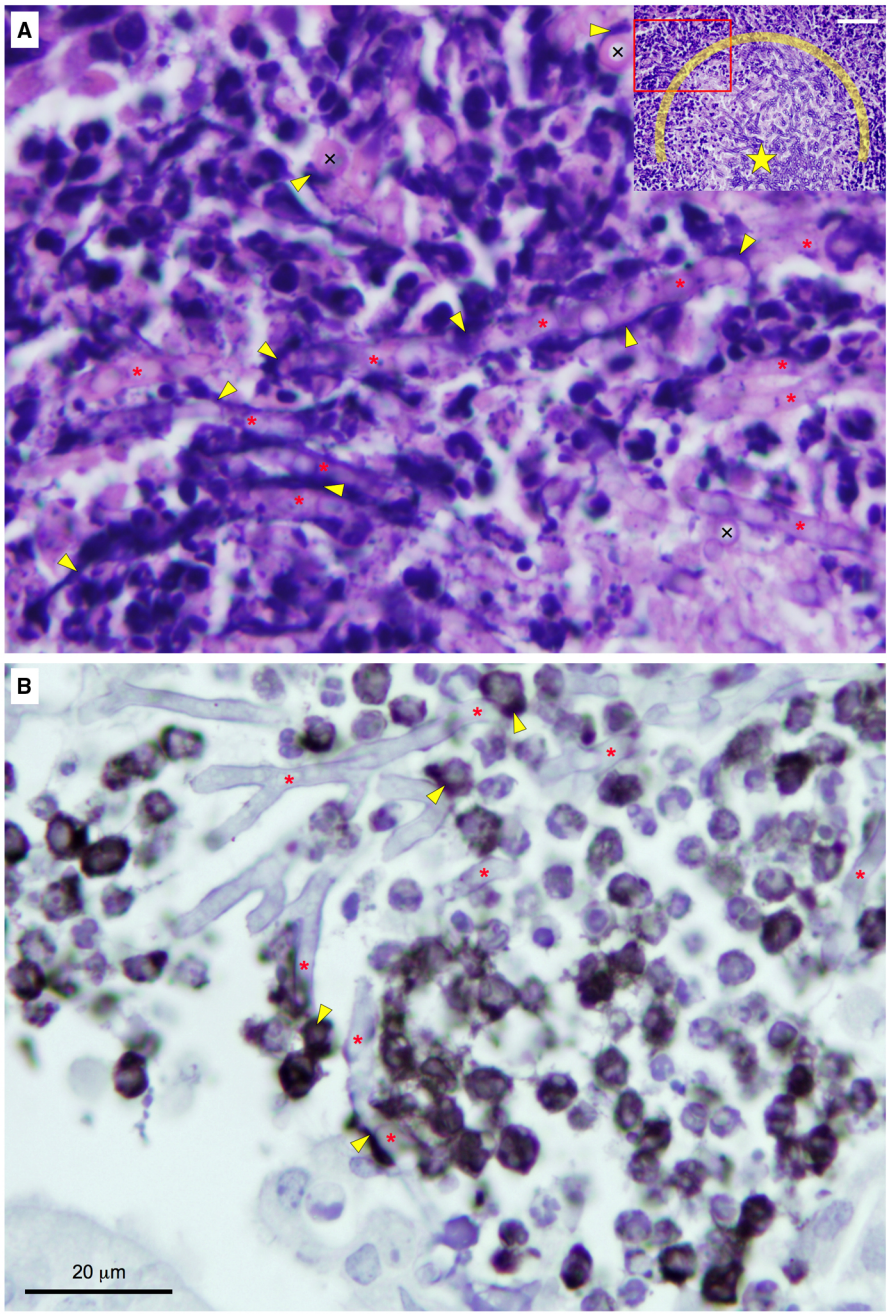


FIGURE 2 | Detailed observation of the edge of *A. fumigatus* abscesses heavily infiltrated with immune cells in lung sections from non-leucopenic mice infected with a wild-type strain. The mice were euthanized after 4 days post-inoculation. The lungs were fixed and processed for histology and immunocytochemistry, as described in Amich et al. (2014). The slides were stained with H&E **(A)** or immunostained using an antibody against the S100A8 component (calgranulin A) of calprotectin **(B)** and observed at the highest magnification using with a 100× objective. In H&E-stained slides of lungs infected with filamentous fungi is usually hard to distinguish between scattered hyphae and the surrounding lung tissue. However, in abscesses with a high fungal load, hyphae are easily recognized from the surrounding lung tissue in H&E-stained slides. In panel **(A)** is shown in great detail the edge of an abscess with a heavy fungal load. Lengthwise hyphae are indicated with red asterisks and cross-sectioned hyphae are indicated with the "×" symbol. Yellow arrowheads indicate haematoxylin-positive thread-like structures and patches. The hyphae more heavily covered with haematoxylin-positive material were typically located at the outlying region of the infectious foci (see the inset image as a reference) and showed both a degree of vacuolization higher and a diameter wider than those of hyphae located toward the middle of the infectious foci (indicated with a star in the inset), which probably anticipates their lysis. In the inset is shown about half of the infectious focus chosen to take the picture (inset bar = 50 μm). The red rectangle in the inset delimits the enlarged image showed in panel **(A)**. The area enclosed by the semicircle around the star delimits the part of the infectious focus with the heaviest fungal load. In panel **(B)** calprotectin was immunodetected either associated to immune cells (most likely pDCs and/or neutrophils) or laid out over hyphae.

would be an alternative target to ZrfA/B. However, to inhibit or reduce noticeably the function of any transcription factor it would be required to disrupt specifically either a DNA–protein or a protein–protein interaction. To disrupt the interaction of ZafA with DNA we should target their C_2H_2 zinc fingers. However, given the highly conserved C_2H_2 structure of the zinc fingers in many transcription factors from fungi to humans have led us to think that is very unlikely to find a drug able to bind selectively to the zinc fingers of ZafA without interfering with human transcription factors. Thus, we propose to target the ZafA transactivation domains to disable the specific interaction of ZafA with the transcriptional machinery. For instance, this mechanism of action has been reported for triptolide, which is a natural compound that inhibits the transactivating function of the human HSF1 transcription factor (Westerheide et al., 2006).

Importantly, the ZafA- and ZrfC-like proteins are distributed exclusively among fungi and no orthologs have been found in mammals. In fact, the human MTF-1 protein (reviewed in Gunther et al., 2012), which might be considered functionally analogous to ZafA, is structurally unrelated to ZafA. Moreover, the hMTF-1 regulator functions in an opposite manner to that of ZafA. Thus, hMTF-1 induces gene expression under zinc-replete conditions whereas ZafA induces gene expression under zinc-limiting conditions. On the other hand, ZrfC is very distantly related to each human ZIP protein. Indeed, it is very difficult to infer a functional similarity of ZrfC with any of the 14 human ZIP proteins (reviewed in Cousins et al., 2006), which suggests that the mechanism of action of ZrfC might be disrupted without disturbing the action of any hZIP.

The chemical compounds able to disrupt zinc homeostasis in the fungus growing within the host could exert their effect by either chelating zinc or by inhibiting selectively the function of specific proteins that are essential to maintain zinc homeostasis. The use of chelating agents has been explored as an option for anticancer (Huesca et al., 2009; Lui et al., 2012; Zuo et al., 2012; Fatfat et al., 2014) and antimicrobial therapies (reviewed in Santos et al., 2012). Several studies have shown that the treatment with different zinc chelators kills different types of cancer cells, which suggest that zinc chelators may be theoretically useful for the treatment of different types of cancer (Torti et al., 1998; Hashemi et al., 2007; Huesca et al., 2009; Zuo et al., 2012). In contrast, most evidences about the reliability of using metal chelators for the treatment of *A. fumigatus* infections have arisen from the use of iron chelators either alone or combined with classical antifungal drugs (Zarember et al., 2009; Ibrahim et al., 2010; Leal et al., 2013). The only study to support the notion that zinc chelation might be useful to combat *A. fumigatus* arose from the finding that EDTA is an adjunct antifungal agent in a rodent model of IPA (Hachem et al., 2006). However, EDTA is a broad-spectrum chelator, so whether its antifungal effect is due to its capacity to chelate zinc and/or other metals is unknown. Nevertheless, the survival rate of mice with IPA increases significantly when they are treated with the zinc-specific chelator TPEN (unpublished data), which demonstrates that a compound able to interfere with zinc homeostasis can be useful as antifungal drug. However, available scientific evidences do not support claims that chelation therapies provide a safe treatment for either cancer or infectious diseases. In this regard, it has been reported that iron chelation has the potential risk of producing undesired side effects in human patients by altering the normal iron homeostasis (Kontoghiorghes et al., 2010). Likewise, the American Cancer Society has warned about the harmful side effects of chelation therapy. Moreover, loss of zinc can also lead to mutations in cells, which may actually increase the risk of cancer (Song et al., 2009). In summary, the reliability of chelation therapies remains too controversial as to be considered the best option (Nissen, 2013). Instead, the discovery of compounds that specifically block the function of fungal proteins required for counteracting host defense mechanisms and/or zinc acquisition (e.g., ZafA and ZrfC) appears more promising. For instance, it has been found recently an anti-mycobacterial compound that interferes with zinc homeostasis by inactivating the countermeasures used by *Mycobacterium* to protect itself of the zinc-mediated intoxication deployed by host phagocytes to kill it inside the phagosomes (Rybniker et al., 2014). We await that a screening to identify anti-*Aspergillus* compounds lead us to the discovery of new drugs to block the regulation of zinc homeostasis and zinc acquisition.

CONCLUSIONS AND OUTLOOK

The regulation of zinc homeostasis by ZafA and zinc acquisition by ZrfC constitute highly promising therapeutic targets to combat *Aspergillus* infections and perhaps also other infections caused by fungal pathogens bearing similar targets. Furthermore, by targeting two different steps (regulation of zinc homeostasis and zinc acquisition) of the same biological process (zinc metabolism) with a specific combination of two drugs we could get two of the major achievements in the development of emerging antimicrobial therapies: (i) a synergistic interaction and, (ii) a significant reduction in the probability of evolving mutations that enhance the resistance of the fungus to any of these compounds (Cottarel and Wierzbowski, 2007). Finally, to prevent the unavoidable pleiotropic effect of chelators on host cells we propose that future efforts to develop inhibitors for homeostasis of specific metals in pathogens, including *A. fumigatus*, should be directed to the discovery of compounds that specifically block the function of microbial proteins required to regulate the homeostasis and/or acquisition of metals.

ACKNOWLEDGMENTS

We wish to thank Emilia Mellado (Instituto de Salud Carlos III, Madrid, Spain) for her assistance with animal experiments and Mercedes Garayoa (Centro de Investigación del Cancer, Salamanca, Spain) for the meticulous examination of slices under the light microscope. We thank the Spanish Ministry of Economy and Competitiveness for financial support through grants BFU2010-22172 and SAF2013-48382-R.

REFERENCES

Alloway, B. J. (2008). *Zinc in Soils and Crop Nutrition*. Brussels and Paris: International Zinc Association and the International Fertilizer Industry Association.

Amich, J., and Calera, J. A. (2014). Zinc acquisition: a key aspect in *Aspergillus fumigatus* virulence. *Mycopathologia* 178, 379–385. doi: 10.1007/s11046-014-9764-2

Amich, J., Leal, F., and Calera, J. A. (2009). Repression of the acid ZrfA/ZrfB zinc-uptake system of *Aspergillus fumigatus* mediated by PacC under neutral, zinc-limiting conditions. *Int. Microbiol.* 12, 39–47. doi: 10.2436/20.1501.01.80

Amich, J., Vicentefranqueira, R., Leal, F., and Calera, J. A. (2010). *Aspergillus fumigatus* survival in alkaline and extreme zinc-limiting environments relies on the induction of a zinc homeostasis system encoded by the zrfC and aspf2 genes. *Eukaryot. Cell* 9, 424–437. doi: 10.1128/EC.00348-09

Amich, J., Vicentefranqueira, R., Mellado, E., Ruiz-Carmuega, A., Leal, F., and Calera, J. A. (2014). The ZrfC alkaline zinc transporter is required for *Aspergillus fumigatus* virulence and its growth in the presence of the Zn/Mn-chelating protein calprotectin. *Cell. Microbiol.* 16, 548–564. doi: 10.1111/cmi.12238

Andreini, C., Bertini, I., Cavallaro, G., Holliday, G. L., and Thornton, J. M. (2008). Metal ions in biological catalysis: from enzyme databases to general principles. *J. Biol. Inorg. Chem.* 13, 1205–1218. doi: 10.1007/s00775-008-0404-5

Botella, H., Peyron, P., Levillain, F., Poincloux, R., Poquet, Y., Brandli, I., et al. (2011). Mycobacterial p_1-type ATPases mediate resistance to zinc poisoning in human macrophages. *Cell Host Microbe* 10, 248–259. doi: 10.1016/j.chom.2011.08.006

Bruns, S., Kniemeyer, O., Hasenberg, M., Aimanianda, V., Nietzsche, S., Thywissen, A., et al. (2010). Production of extracellular traps against *Aspergillus fumigatus in vitro* and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog.* 6:e1000873. doi: 10.1371/journal.ppat.1000873

Corbin, B. D., Seeley, E. H., Raab, A., Feldmann, J., Miller, M. R., Torres, V. J., et al. (2008). Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science* 319, 962–965. doi: 10.1126/science.1152449

Cottarel, G., and Wierzbowski, J. (2007). Combination drugs, an emerging option for antibacterial therapy. *Trends Biotechnol.* 25, 547–555. doi: 10.1016/j.tibtech.2007.09.004

Cousins, R. J., Liuzzi, J. P., and Lichten, L. A. (2006). Mammalian zinc transport, trafficking, and signals. *J. Biol. Chem.* 281, 24085–24089. doi: 10.1074/jbc.R600011200

Eide, D. J. (2011). The oxidative stress of zinc deficiency. *Metallomics* 3, 1124–1129. doi: 10.1039/c1mt00064k

Fatfat, M., Merhi, R. A., Rahal, O., Stoyanovsky, D. A., Zaki, A., Haidar, H., et al. (2014). Copper chelation selectively kills colon cancer cells through redox cycling and generation of reactive oxygen species. *BMC Cancer* 14:527. doi: 10.1186/1471-2407-14-527

Gunther, V., Lindert, U., and Schaffner, W. (2012). The taste of heavy metals: gene regulation by MTF-1. *Biochim. Biophys. Acta* 1823, 1416–1425. doi: 10.1016/j.bbamcr.2012.01.005

Hachem, R., Bahna, P., Hanna, H., Stephens, L. C., and Raad, I. (2006). EDTA as an adjunct antifungal agent for invasive pulmonary aspergillosis in a rodent model. *Antimicrob. Agents Chemother.* 50, 1823–1827. doi: 10.1128/AAC.50.5.1823-1827.2006

Hashemi, M., Ghavami, S., Eshraghi, M., Booy, E. P., and Los, M. (2007). Cytotoxic effects of intra and extracellular zinc chelation on human breast cancer cells. *Eur. J. Pharmacol.* 557, 9–19. doi: 10.1016/j.ejphar.2006.11.010

Hood, M. I., and Skaar, E. P. (2012). Nutritional immunity: transition metals at the pathogen–host interface. *Nat. Rev. Microbiol.* 10, 525–537. doi: 10.1038/nrmicro2836

Huesca, M., Lock, L. S., Khine, A. A., Viau, S., Peralta, R., Cukier, I. H., et al. (2009). A novel small molecule with potent anticancer activity inhibits cell growth by modulating intracellular labile zinc homeostasis. *Mol. Cancer Ther.* 8, 2586–2596. doi: 10.1158/1535-7163.MCT-08-1104

Ibrahim, A. S., Gebremariam, T., French, S. W., Edwards, J. E. Jr., and Spellberg, B. (2010). The iron chelator deferasirox enhances liposomal amphotericin B efficacy in treating murine invasive pulmonary aspergillosis. *J. Antimicrob. Chemother.* 65, 289–292. doi: 10.1093/jac/dkp426

Iyengar, V., and Woittiez, J. (1988). Trace elements in human clinical specimens: evaluation of literature data to identify reference values. *Clin. Chem.* 34, 474–481.

Kontoghiorghes, G. J., Kolnagou, A., Peng, C. T., Shah, S. V., and Aessopos, A. (2010). Safety issues of iron chelation therapy in patients with normal range iron stores including thalassaemia, neurodegenerative, renal and infectious diseases. *Expert Opin. Drug Saf.* 9, 201–206. doi: 10.1517/14740330903535845

Kousha, M., Tadi, R., and Soubani, A. O. (2011). Pulmonary aspergillosis: a clinical review. *Eur. Respir. Rev.* 20, 156–174. doi: 10.1183/09059180.00001011

Leal, S. M. Jr., Roy, S., Vareechon, C., Carrion, S., Clark, H., Lopez-Berges, M. S., et al. (2013). Targeting iron acquisition blocks infection with the fungal pathogens *Aspergillus fumigatus* and *Fusarium oxysporum*. *PLoS Pathog.* 9:e1003436. doi: 10.1371/journal.ppat.1003436

Lui, G. Y., Obeidy, P., Ford, S. J., Tselepis, C., Sharp, D. M., Jansson, P. J., et al. (2012). The iron chelator, deferasirox, as a novel strategy for cancer treatment: oral activity against human lung tumor xenografts and molecular mechanism of action. *Mol. Pharmacol.* 83, 179–190. doi: 10.1124/mol.112.081893

McCormick, A., Heesemann, L., Wagener, J., Marcos, V., Hartl, D., Loeffler, J., et al. (2010). NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbes Infect.* 12, 928–936. doi: 10.1016/j.micinf.2010.06.009

Moreno, M. A., Ibrahim-Granet, O., Vicentefranqueira, R., Amich, J., Ave, P., Leal, F., et al. (2007). The regulation of zinc homeostasis by the ZafA transcriptional activator is essential for *Aspergillus fumigatus* virulence. *Mol. Microbiol.* 64, 1182–1197. doi: 10.1111/j.1365-2958.2007.05726.x

Nissen, S. E. (2013). Concerns about reliability in the trial to assess chelation therapy (TACT). *JAMA* 309, 1293–1294. doi: 10.1001/jama.2013.2778

Nobile, C. J., Nett, J. E., Hernday, A. D., Homann, O. R., Deneault, J. S., Nantel, A., et al. (2009). Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol.* 7:e1000133. doi: 10.1371/journal.pbio.1000133

Outten, C. E., and O'Halloran, T. V. (2001). Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* 292, 2488–2492. doi: 10.1126/science.1060331

Parente, A. F., de Rezende, T. C., de Castro, K. P., Bailao, A. M., Parente, J. A., Borges, C. L., et al. (2013). A proteomic view of the response of Paracoccidioides yeast cells to zinc deprivation. *Fungal Biol.* 117, 399–410. doi: 10.1016/j.funbio.2013.04.004

Prati, F., Goldman-Pinkovich, A., Lizzi, F., Belluti, F., Koren, R., Zilberstein, D., et al. (2014). Quinone-amino acid conjugates targeting *Leishmania* amino acid transporters. *PLoS ONE* 9:e107994. doi: 10.1371/journal.pone.0107994

Ramirez-Ortiz, Z. G., Lee, C. K., Wang, J. P., Boon, L., Specht, C. A., and Levitz, S. M. (2011). A nonredundant role for plasmacytoid dendritic cells in host defense against the human fungal pathogen *Aspergillus fumigatus*. *Cell Host Microbe* 9, 415–424. doi: 10.1016/j.chom.2011.04.007

Rybniker, J., Chen, J. M., Sala, C., Hartkoorn, R. C., Vocat, A., Benjak, A., et al. (2014). Anticytolytic screen identifies inhibitors of mycobacterial virulence protein secretion. *Cell Host Microbe* 16, 538–548. doi: 10.1016/j.chom.2014.09.008

Santos, A. L., Sodre, C. L., Valle, R. S., Silva, B. A., Abi-Chacra, E. A., Silva, L. V., et al. (2012). Antimicrobial action of chelating agents: repercussions on the microorganism development, virulence and pathogenesis. *Curr. Med. Chem.* 19, 2715–2737. doi: 10.2174/092986712800609788

Schneider, R. O., Fogaca, N., S., Kmetzsch, L., Schrank, A., Vainstein, M. H., and Staats, C. C. (2012). Zap1 regulates zinc homeostasis and modulates virulence in *Cryptococcus gattii*. *PLoS ONE* 7:e43773. doi: 10.1371/journal.pone.0043773

Slavic, K., Krishna, S., Derbyshire, E. T., and Staines, H. M. (2011). Plasmodial sugar transporters as anti-malarial drug targets and comparisons with other protozoa. *Malar. J.* 10, 165. doi: 10.1186/1475-2875-10-165

Song, Y., Leonard, S. W., Traber, M. G., and Ho, E. (2009). Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses, and DNA repair in rats. *J. Nutr.* 139, 1626–1631. doi: 10.3945/jn.109.106369

Subramanian Vignesh, K., Landero Figueroa, J. A., Porollo, A., Caruso, J. A., and Deepe, G. S. Jr. (2013). Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival. *Immunity* 39, 697–710. doi: 10.1016/j.immuni.2013.09.006

Tekaia, F., and Latge, J. P. (2005). *Aspergillus fumigatus*: saprophyte or pathogen? *Curr. Opin. Microbiol.* 8, 385–392. doi: 10.1016/j.mib.2005.06.017

Torti, S. V., Torti, F. M., Whitman, S. P., Brechbiel, M. W., Park, G., and Planalp, R. P. (1998). Tumor cell cytotoxicity of a novel metal chelator. *Blood* 92, 1384–1389.

Urban, C. F., Ermert, D., Schmid, M., Abu-Abed, U., Goosmann, C., Nacken, W., et al. (2009). Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathog.* 5:e1000639. doi: 10.1371/journal.ppat.1000639

Vicentefranqueira, R., Moreno, M. A., Leal, F., and Calera, J. A. (2005). The zrfA and zrfB genes of *Aspergillus fumigatus* encode the zinc transporter proteins of a zinc uptake system induced in an acid, zinc-depleted environment. *Eukaryot. Cell* 4, 837–848. doi: 10.1128/EC.4.5.837-848.2005

Westerheide, S. D., Kawahara, T. L., Orton, K., and Morimoto, R. I. (2006). Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. *J. Biol. Chem.* 281, 9616–9622. doi: 10.1074/jbc.M512044200

Zarember, K. A., Cruz, A. R., Huang, C. Y., and Gallin, J. I. (2009). Antifungal activities of natural and synthetic iron chelators alone and in combination with azole and polyene antibiotics against *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 53, 2654–2656. doi: 10.1128/AAC.01547-08

Zuo, J., Schmitt, S. M., Zhang, Z., Prakash, J., Fan, Y., Bi, C., et al. (2012). Novel polypyridyl chelators deplete cellular zinc and destabilize the X-linked inhibitor of apoptosis protein (XIAP) prior to induction of apoptosis in human prostate and breast cancer cells. *J. Cell Biochem.* 113, 2567–2575. doi: 10.1002/jcb.24132

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 January 2015; accepted: 11 February 2015; published online: 27 February 2015.

Citation: Vicentefranqueira R, Amich J, Laskaris P, Ibrahim-Granet O, Latgé JP, Toledo H, Leal F and Calera JA (2015) Targeting zinc homeostasis to combat Aspergillus fumigatus infections. Front. Microbiol. ***6****:160. doi: 10.3389/fmicb.2015.00160*

This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology.

Copyright © 2015 Vicentefranqueira, Amich, Laskaris, Ibrahim-Granet, Latgé, Toledo, Leal and Calera. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 April 2015
doi: 10.3389/fmicb.2015.00252

AmcA—a putative mitochondrial ornithine transporter supporting fungal siderophore biosynthesis

*Lukas Schafferer[1], Nicola Beckmann[1], Ulrike Binder[2], Gerald Brosch[1] and Hubertus Haas[1]**

[1] Division of Molecular Biology/Biocenter, Medical University Innsbruck, Innsbruck, Austria, [2] Division of Hygiene and Medical Microbiology, Medical University Innsbruck, Innsbruck, Austria

Iron is an essential nutrient required for a wide range of cellular processes. The opportunistic fungal pathogen *Aspergillus fumigatus* employs low-molecular mass iron-specific chelators, termed siderophores, for uptake, storage and intracellular iron distribution, which play a crucial role in the pathogenicity of this fungus. Siderophore biosynthesis (SB) depends on coordination with the supply of its precursor ornithine, produced mitochondrially from glutamate or cytosolically via hydrolysis of arginine. In this study, we demonstrate a role of the putative mitochondrial transporter AmcA (AFUA_8G02760) in SB of *A. fumigatus.* Consistent with a role in cellular ornithine handling, AmcA-deficiency resulted in decreased cellular ornithine and arginine contents as well as decreased siderophore production on medium containing glutamine as the sole nitrogen source. In support, arginine and ornithine as nitrogen sources did not impact SB due to cytosolic ornithine availability. As revealed by Northern blot analysis, transcript levels of siderophore biosynthetic genes were unresponsive to the cellular ornithine level. In contrast to siderophore production, AmcA deficiency did only mildly decrease the cellular polyamine content, demonstrating cellular prioritization of ornithine use. Nevertheless, AmcA-deficiency increased the susceptibility of *A. fumigatus* to the polyamine biosynthesis inhibitor eflornithine, most likely due to the decreased ornithine pool. AmcA-deficiency decreased the growth rate particularly on ornithine as the sole nitrogen source during iron starvation and sufficiency, indicating an additional role in the metabolism and fitness of *A. fumigatus*, possibly in mitochondrial ornithine import. In the *Galleria mellonella* infection model, AmcA-deficiency did not affect virulence of *A. fumigatus,* most likely due to the residual siderophore production and arginine availability in this host niche.

Keywords: ***Aspergillus fumigatus*, AmcA, iron, siderophores, ornithine, mitochondria, virulence**

OPEN ACCESS

Edited by:
William J. Steinbach, Duke University Medical Center, USA

Reviewed by:
Elaine M. Bignell, The University of Manchester, UK
Anand K. Ramasubramanian, University of Texas at San Antonio, USA

***Correspondence:**
Hubertus Haas, Division of Molecular Biology/Biocenter, Medical University innsbruck, Innrain 80–82, Innsbruck, Austria
hubertus.haas@i-med.ac.at

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *15 January 2015*
Accepted: *13 March 2015*
Published: *07 April 2015*

Citation:
Schafferer L, Beckmann N, Binder U, Brosch G and Haas H (2015) AmcA—a putative mitochondrial ornithine transporter supporting fungal siderophore biosynthesis. Front. Microbiol. 6:252. doi: 10.3389/fmicb.2015.00252

Introduction

Iron is an essential nutrient for virtually every organism known to mankind. Its ability to adopt one of two ionic forms, reduced ferrous (Fe^{2+}) or oxidized ferric (Fe^{3+}) iron, makes it the major redox metal in cells. Although abundant in the earth's crust, its bioavailability is very low. The oxidized form, or more accurately oxyhydroxide colloid particles, is found in aerobic environments and shows a solubility below 10^{-9} M at neutral pH, which is insufficient to sustain vital

processes (Ratledge and Dover, 2000). Apart from its crucial role in metabolism, including respiration, oxidative stress detoxification as well as synthesis of amino acids, lipids and desoxyribonucleic acid, iron is able to generate toxic reactive species if accumulated excessively (Halliwell and Gutteridge, 1984). As a result, organisms have developed fine-tuned regulatory mechanisms regarding uptake, storage and consumption of iron.

The ascomycete *Aspergillus fumigatus* exhibits a typical saprophytic lifestyle and is usually found in soil and decaying matter. Nevertheless, it has become the most common airborne, pathogenic fungus causing life-threatening disease in immunocompromised patients. While it lacks specific uptake systems for host iron, it employs two high-affinity iron uptake systems; reductive iron assimilation (RIA) and siderophore-mediated iron uptake. Previous studies have demonstrated a crucial role of siderophores in virulence of *A. fumigatus*, as the elimination of both extra- and intracellular siderophores resulted in absolute avirulence in a mouse model of pulmonary aspergillosis (Schrettl et al., 2004).

Siderophores are small, high-affinity iron chelating compounds found in bacteria, fungi and plants. These molecules form a complex with Fe^{3+} to overcome the aforementioned bioavailability problem or to extract iron from host proteins. In the case of *A. fumigatus*, four different hydroxamate siderophores are produced: extracellular fusarinine C (FsC) and triacetylfusarinine C (TAFC) to mobilize extracellular iron, as well as intracellular ferricrocin (FC) for hyphal storage and distribution of iron, and hydroxyferricrocin (HFC) for conidial iron storage (Schrettl et al., 2007; Wallner et al., 2009).

FsC is the prototype of fusarinines and is made up of three N^5-*cis*-anhydromevalonyl-N^5-hydroxyornithine residues, which are linked cyclically by ester bonds (Haas et al., 2008). TAFC is derived from FsC by its tri-N^2-acetylation. FC is a cyclic hexapeptide of three N^5-acyl-N^5-hydroxyornthines and three amino acids (glycine, serine, or alanine), and HFC is the hydroxylated FC (Haas et al., 2008). Biosynthesis of all four siderophore-types starts with the N^5-hydroxylation of ornithine, which is catalyzed by the ornithine-N^5-monooxygenase SidA (Schrettl et al., 2004). This initial step is followed by N^5-acylation of N^5-hydroxyornithine to form the hydroxamate group (Haas et al., 2008). At this point, the biosynthesis pathway splits into two different pathways, depending on the choice of the acyl group (Schrettl et al., 2007). Biosynthesis of FC and HFC requires the addition of acetyl, mediated by the transacylase SidL and an uncharacterized enzyme (Blatzer et al., 2011). For formation of fusarinines, the addition of anhydromevalonyl catalyzed by the transacylase SidF is needed (Schrettl et al., 2007). In the next step, the hydroxamate and additional amino acid residues are covalently linked via ester or peptide bonds, which are achieved by nonribosomal peptide synthetases, SidC and SidD, resulting in FC and FsC (Schrettl et al., 2007).

The major precursor for all *A. fumigatus* siderophores is the non-proteinogenic amino acid ornithine, which is additionally involved in arginine metabolism, the urea cycle and polyamine biosynthesis. Ornithine can be produced in mitochondria or the cytosol (Schrettl et al., 2010; Beckmann et al., 2013). Within mitochondria, ornithine is synthesized from glutamate involving six enzymes. Subsequently, it is transported into the cytoplasm or converted to citrulline by the ornithine transcarbamoyl transferase ArgB (Jadoun et al., 2004), which is shuttled into the cytoplasm. In the cytoplasm, citrulline is converted via three enzymatic steps to arginine, which can be hydrolyzed to ornithine. A scheme of the enzymatic links between arginine/ornithine, polyamine and siderophore metabolism is depicted in **Figure 7** (Schrettl et al., 2010; Haas, 2012; Beckmann et al., 2013). In order to characterize the ornithine supply for siderophore biosynthesis (SB) in *A. fumigatus*, we aimed to identify the mitochondrial ornithine exporter.

Saccharomyces cerevisiae Arg11, a member of the mitochondrial carrier protein family, was suggested to play a role in arginine biosynthesis, either by importing glutamate into the mitochondrion or by exporting ornithine from the organelle to the cytosol (Crabeel et al., 1996). Further studies indicated that its main role is the transport of ornithine across the membrane into the cytosol (Palmieri et al., 1997). The *Neurospora crassa* ortholog Arg13 was similarly found to be involved in arginine metabolism, transporting ornithine from the cytosol into mitochondria or the other way around (Liu and Dunlap, 1996). Deficiency in the human ortholog ORNT1 is associated with hyperornithinaemia-hyperammonaemia-homocitrullinuria syndrome (HHH syndrome), an autosomal recessive disease with persistent hyperornithinaemia and episodic hyperammonaemia indicating that ORNT1 is crucial for transport of ornithine from the cytosol into the mitochondria, enabling proper urea cycle function as well as degradation of ornithine (Camacho et al., 1999).

The *A. fumigatus* homolog of *S. cerevisiae* Arg11 and *N. crassa* Arg13 was found to be transcriptionally upregulated under iron starvation (with glutamine as nitrogen source) in *Aspergillus nidulans* as well as *A. fumigatus* and subsequently termed AmcA (Oberegger et al., 2001; Schrettl et al., 2008, 2010). These studies indicated a role of the two major iron regulatory transcription factors in control of *amcA* expression: iron-repression mediated by the GATA-transcription factor SreA and activation during iron starvation by the bZip transcription factor HapX are involved in the transcriptional control of this mitochondrial ornithine transporter in *A. fumigatus*.

We previously demonstrated that the supply with the precursor ornithine plays an important role in siderophore production (Schrettl et al., 2010; Beckmann et al., 2013). Furthermore, studies employing arginine auxotrophic mutants indicated that SB is mainly fueled by mitochondrial rather than cytosolic ornithine production, at least with glutamine as nitrogen source (Beckmann et al., 2013). Here, we characterized the role of AmcA in general viability of *A. fumigatus* as well as its function in siderophore production on different nitrogen sources.

Materials and Methods

Fungal Strains and Growth Conditions

A. fumigatus strains were grown at 37°C in *Aspergillus* minimal medium containing 1% glucose as carbon source and 20 mM glutamine as nitrogen source (Pontecorvo et al., 1953). The use of other nitrogen sources is indicated in the text. Iron-replete media

contained 30 μM $FeSO_4$. For iron depleted conditions, iron was omitted. Bathophenanthroline disulfonate (BPS) was added to a concentration of 200 μM. For growth assays, 10^4 and 10^8 conidia were used for point-inoculation on plates or inoculation of 100 ml liquid media, respectively. Fungal strains used in this study are listed in Table S1.

Analysis of Siderophores, Free Amino Acids and Polyamines

Analysis of the free amino acid content was obtained by ethanol extraction and subsequent reversed-phase HPLC as described previously (Berger et al., 2008). Intracellular siderophores were analyzed from cell extracts as described previously (Schrettl et al., 2010). Extracellular siderophores were analyzed via reversed-phase HPLC. Chromatographic analyses were performed on a Dionex UltiMate 3000 HPLC system (Thermo Scientific, Waltham, Massachusetts, USA) with a diode array detector and a Nucleosil 100-5 C_{18} reversed phase column (250 mm × 4.6 mm I.D.; particle size 5.0 μm; Macherey-Nagel, Düren, Germany). A gradient elution was used at a constant flow rate of 0.5 ml/min with a mobile phase of water and 0.1% TFA (solvent A) and 85% acetonitril and 0.1% TFA (solvent B) starting at 6% B. For elution of siderophores, the following conditions were applied: 6–15% B during 10 min, 16–60% B during 15 min, 60% B for 5 min, 100% B for 5 min, return to 6% B for re-equilibration. For quantification of polyamines, 50 mg freeze-dried mycelia were homogenized and incubated with 6% perchloric acid for 3 h. Polyamine derivatization was carried out a ccording to Wongyai et al. (1989) with slight modifications (Beckmann et al., 2013).

DNA, RNA Isolation and Northern Blot Analysis

For extraction of genomic DNA, mycelia were homogenized and DNA was isolated according to Sambrook et al. (1989). RNA was isolated using TRI Reagent (Sigma) and peqGOLD Phase Trap (peqlab) reaction tubes. For Northern blot analysis, 10 μg of total RNA were analyzed as described in Oberegger et al. (2001). Hybridization probes were amplified by PCR using the primers listed in Table S2.

Deletion of *amcA* (AFUA_8G02760) and Reconstitution of the Δ*amcA* Strain

For generating the Δ*amcA* mutant strain, the bipartite marker technique was used (Nielsen et al., 2006). *A. fumigatus* AfS77 (ATCC46645 Δ*Ku70*) was co-transformed with two DNA fragments, each containing overlapping but incomplete fragments of the pyrithiamine resistance-conferring *ptrA* gene, fused to 1.4 kb *amcA* 5′- and 1.6 kb *amcA* 3′-flanking sequences, respectively. The *amcA* 5′-flanking region (1354 bp) was PCR-amplified from genomic DNA using primers oamcA-1 and oamcA-2. For amplification of the *amcA* 3′-flanking region (1630 bp) primers oamcA-3 and oamcA-4 were employed. Subsequent to gel-purification, these fragments were digested with *Spe*I (5′-flanking region) and *Xho*I (3′-flanking region), respectively. The *ptrA* selection marker was released from plasmid pSK275 (gift from Sven Krappmann, Goettingen, Germany) by digestion with *Spe*I and *Xho*I, and ligated with the 5′- and 3′-flanking region. The transformation construct A (2640 bp, fusion of the *amcA* 5′-flanking region and the *ptrA* split marker) was amplified from the ligation product using primers oamcA-5 and optrA-2.1. For amplification of the transformation construct B (2667 bp, fusion of the *amcA* 3′-flanking region and the supplementary *ptrA* split marker) primers oamcA-6 and optrA-1 were employed. For transformation of *A. fumigatus* AfS77 both constructs A and B were simultaneously used. This strategy deleted the sequence -33 to 1049 bp relative to the translation start site in *amcA*.

For reconstitution of the Δ*amcA* strain with a functional *amcA* copy, a 3987 bp PCR fragment was generated with primers oamcA-1 and oamcA-6 and was subsequently subcloned into a *Stu*I digested pAN7.1 plasmid (containing the hygromycin B resistance-conferring *hph* gene), resulting in plasmid pAN7.1_amcAreverse. The resulting 10.7 kb plasmid was linearized with *Sfi*I and used to transform *A. fumigatus* Δ*amcA*.

Transformation of *A. fumigatus* was carried out as described in Tilburn et al. (1983). For selection of transformants, 0.2 mg ml^{-1} hygromycin B (Calbiochem) was used. To obtain homokaryotic transformants, colonies from single homokaryotic spores were picked. Screening of transformants and confirmation of single genomic integration was performed by PCR and confirmed by Southern blot analysis. Hybridization probes were amplified by PCR using the primers listed in Table S2. Primers used for generating both Δ*amcA* and Δ*amcA*^{c} are listed in Table S3.

Eflornithine Inhibition Assay

Effects of eflornithine (Sigma-Aldrich) on fungal growth were analyzed by agar diffusion assays, which were carried out as described previously by Beckmann et al. (2013), with slight modifications. As the mutant strain analyzed in this study was not arginine auxotrophic, arginine supplementation was omitted.

Galleria Mellonella Virulence Assays

Comparison of the virulence potential of *A. fumigatus* wt and Δ*amcA* mutant strain in the *G. mellonella* model was carried out according to Fallon et al. (2011), and as described previously by Beckmann et al. (2013).

Statistical Analyses

For statistical analyses, an unpaired *t*-test was used, comparing mutant data with data from wt and the complemented strain. Significance levels are as follows: *indicates a *p*-value lower than 0.05 while **represent a *p*-value below 0.01.

Results/Discussion

Inactivation of amcA Reduces Radial Growth on Glutamine and Particularly Ornithine but not Arginine as Nitrogen Source

Previously it was shown, that iron deficiency causes an active upregulation of ornithine biosynthesis as well as the cellular ornithine content (Schrettl et al., 2010). This common precursor for all siderophores of *A. fumigatus* is produced either via the

conversion of arginine within the cytosol, or in the mitochondria from glutamate (Davis, 1986). The study of arginine auxotrophic mutants impaired (Δ*argEF*) or not impaired (Δ*argB*) in mitochondrial ornithine production indicated a dominant role of the latter in SB of this opportunistic fungal pathogen (Beckmann et al., 2013). To gain more insight into ornithine supply for SB, we compared a mutant lacking the putative ornithine transporter AmcA (Δ*amcA*) with its respective wild type (wt) strain on a variety of different nitrogen sources. Δ*amcA* was generated during this study in the *A. fumigatus* AfS77 strain, a Δ*akuA*-derivative of *A. fumigatus* ATCC 46645, lacking non-homologous recombination (Krappmann et al., 2006). The reconstituted Δ*amcA* mutant, termed Δ*amcA*^{c}, which was generated by re-integration of a wt gene copy, showed a wt-like phenotype in all experiments confirming that the mutant effects are caused specifically by *amcA* deletion (**Figure 1**). Plate assays were performed on solid minimal medium, containing glutamine, arginine, ornithine or arginine/ornithine as nitrogen sources, respectively, combined with different iron availability. Growth differences between Δ*amcA* and the wt were compared after spot inoculation with 10^4 conidia. **Figure 1A** shows a slight reduction in radial growth of Δ*amcA* with glutamine as the sole nitrogen source independent of the iron supply as well as reduced conidiation under iron starvation conditions (BPS, bathophenanthroline disulfonate). BPS is a ferrous iron-specific chelator, which blocks RIA, thereby rendering siderophore mediated iron uptake the only high-affinity uptake system of *A. fumigatus* (Schrettl et al., 2004). Ornithine as nitrogen source led to strongly reduced radial growth independent of the iron supply (**Figure 1C**). With arginine, be it either in combination with ornithine or as sole nitrogen source, Δ*amcA* displayed wt-like radial growth and conidiation (**Figures 1B,D**).

These data indicate that AmcA is important for adaptation to iron starvation (sporulation defect on BPS) on glutamine but not ornithine or arginine as nitrogen source. Moreover, AmcA is required for optimal utilization of glutamine and particularly ornithine as nitrogen source. Taking into account the homology of AmcA with predicted mitochondrial ornithine

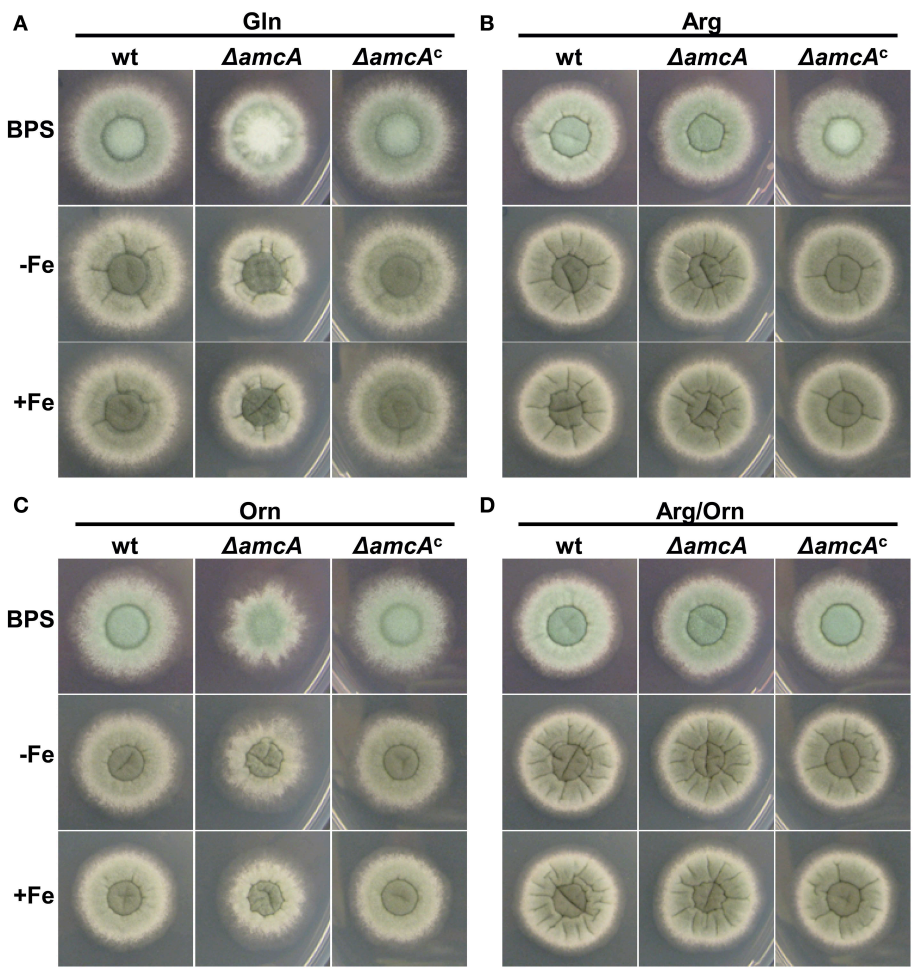


FIGURE 1 | AmcA-deficiency reduces radial growth on glutamine (Gln) or ornithine (Orn) but not arginine (Arg) or Arg/Orn. Fungal growth on solid medium was analyzed after 48 h on plates containing 20 mM Gln **(A)**, 20 mM Arg **(B)**, 20 mM Orn **(C)** or 10 mM Arg/10 mM Orn **(D)**. BPS plates contained 0.2 mM BPS to increase iron starvation. For -Fe plates, iron was omitted. Iron-replete plates (+Fe) contained 30 μM $FeSO_4$.

transporters (*S. cerevisiae* Arg11, *N. crassa* Arg13, *H. sapiens* ORNT1), the defects observed are most likely caused by defective ornithine trafficking between the mitochondria and the cytosol. The sporulation defect on glutamine during iron starvation (BPS) (**Figure 1A**) might be due to decreased ornithine supply for synthesis of the intracellular siderophore FC, which was previously shown to be crucial for intracellular iron distribution and in consequence for conidiation (Wallner et al., 2009). The latter would be consistent with a role in mitochondrial ornithine export for SB.

In Liquid Cultures, AmcA-Deficiency Decreases Growth on Ornithine and Production of Siderophores with Glutamine as Nitrogen Source

To further characterize the role of AmcA, biomass and siderophore production of wt, Δ*amcA* and Δ*amcA*^{c} were analyzed after growth in liquid cultures with glutamine, arginine, ornithine or arginine/ornithine as nitrogen sources during iron limitation and iron sufficiency (**Figure 2**). Biomass production displayed a clear correlation with the iron supply as the wt biomass production decreased to 27% during iron starvation conditions compared to iron sufficiency (see legend of **Figure 2**). In all these assays Δ*amcA*^{c} was wt-like. In contrast to the phenotype on solid medium, Δ*amcA* did not display a growth defect on glutamine, neither during iron starvation nor iron sufficiency (**Figures 2A,B**). Similar to growth on solid media, Δ*amcA* displayed wt-like growth on arginine and arginine/ornithine (**Figures 2A,B**). In agreement with the growth on solid media, Δ*amcA* showed markedly reduced growth with ornithine: 55% and 67% of the wt during iron starvation and sufficiency, respectively (**Figures 2A,B**), confirming that AmcA is required for optimal utilization of ornithine as nitrogen source, possibly for mitochondrial ornithine import. Interestingly, the wt showed increased growth on ornithine and arginine/ornithine

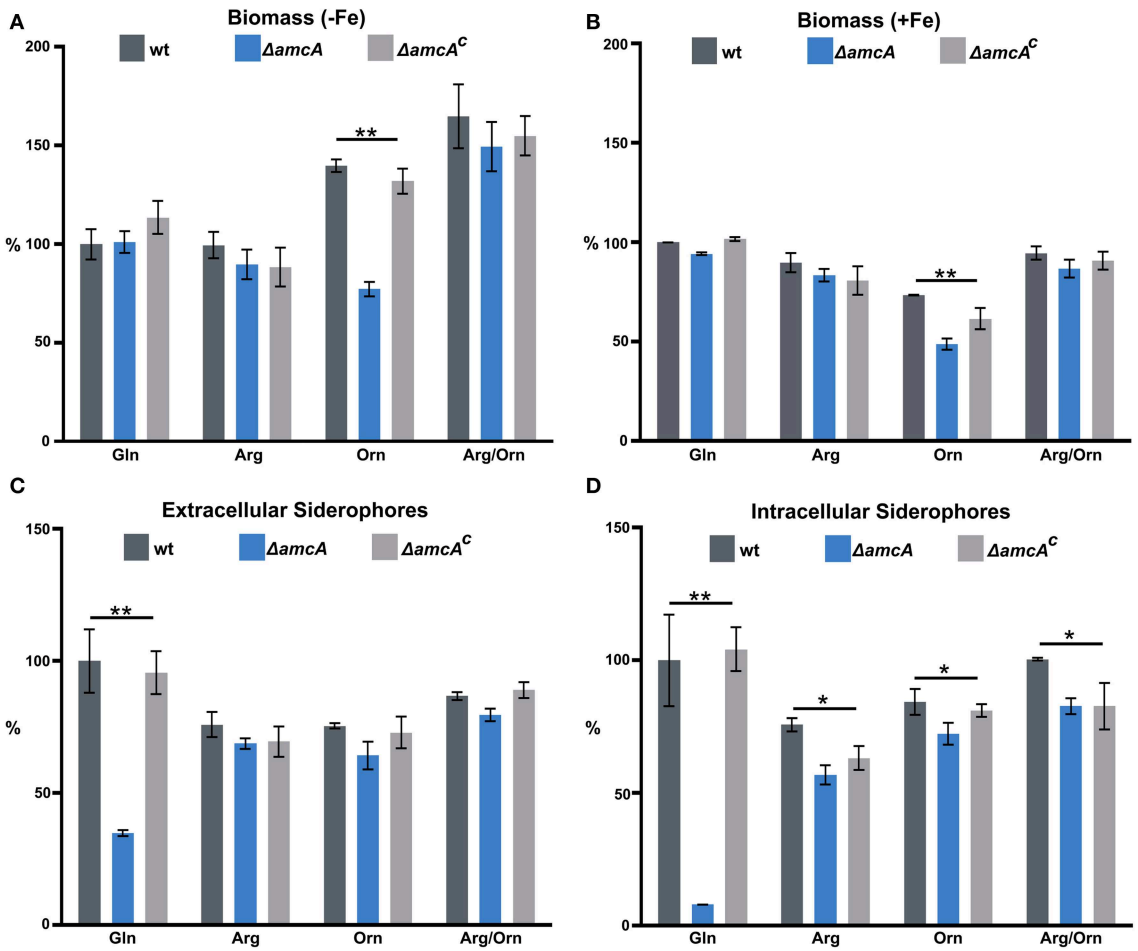


FIGURE 2 | In liquid cultures, AmcA-deficiency decreases growth on ornithine and production of siderophores on glutamine as nitrogen source. Fungal growth **(A,B)** and siderophore production **(C,D)** was quantified in liquid medium, containing 20 mM Gln, 20 mM Arg, 20 mM Orn or 10 mM Arg/10 mM Orn after 24 h during iron starvation (−Fe) and iron sufficiency (+Fe). Data represent the mean of three biological replicates ± standard deviation normalized to the biomass and to the wt on Gln. The wt biomass amounted to 0.691 g under +Fe and 0.188 g (27%) under −Fe. The differences in biomass production underline the difference in iron supply. (*≙ $P < 0.05$, **≙ $P < 0.01$).

during iron starvation compared to glutamine or arginine (**Figure 2A**). Inspection of the ornithine source proved that it was iron-contaminated, indicating that the increased biomass formation on this nitrogen source was a direct consequence of increased iron supply. Nevertheless, ornithine cultures still reflected iron starvation conditions as evident from the high siderophore production (**Figures 2C,D**) and Northern analysis of iron starvation-induced genes (**Figure 4**). In contrast, during iron sufficiency ornithine led to lower biomass production compared to the other nitrogen sources. These data demonstrate that iron was indeed growth limiting during the iron starvation conditions used, while nitrogen was limiting during the iron sufficient conditions used. Noteworthy, during iron starvation with glutamine as nitrogen source, AmcA-deficiency decreased production of extra- and intracellular siderophores to 35% and 8%, respectively, compared to wt (**Figures 2C,D**). On arginine, ornithine or arginine/ornithine, AmcA-deficiency decreased the production of extra- and intracellular siderophores only slightly to 80–95% of the wt. These data strongly indicate that AmcA is involved in ornithine supply for SB mainly on glutamine and to a lesser degree on the other nitrogen sources tested.

Consequently, the main function of AmcA on glutamine during iron starvation is most likely the export of mitochondrial ornithine into the cytosol. In line, the defective siderophore production of Δ*amcA* was largely rescued by supplementation with ornithine or arginine, which is hydrolyzed to ornithine by the arginase in the cytosol. Obviously, the reduced siderophore production of Δ*amcA* on glutamine is sufficient to support wt-like growth during iron starvation (**Figure 2A**). Either the residual siderophore production is sufficient for iron supply or the defect is compensated by reductive iron assimilation, the alternative high affinity iron uptake system (Schrettl et al., 2004). The reduced production of the intracellular siderophore FC is the most likely reason for the observed conidiation defect on BPS-solid medium with glutamine as nitrogen source (see above). Noteworthy, AmcA-deficiency led to decreased sporulation in the presence of BPS as well as decreased extra- and intracellular siderophore production with ammonium tartrate or sodium nitrate as nitrogen sources (**Figure S1**), as seen with glutamine.

Ornithine as Nitrogen Source Decreases TAFC and Increases FsC Production

Remarkably, the reversed-phase HPLC-guided analysis of siderophores revealed an influence of the nitrogen source on the composition of extracellular siderophores of *A. fumigatus* wt (**Figure 3**). As shown previously (Schrettl et al., 2007), *A. fumigatus* produced two extracellular siderophores, TAFC and FsC. On glutamine and arginine, TAFC exceeded FsC by about 3- and 2-fold, respectively. In contrast, on ornithine and arginine/ornithine, FsC surpassed TAFC levels about 7- and 2-fold. Iron supplementation to a final concentration of 1 μM of glutamine cultures did not influence the TafC/FsC ratio, ruling out the possibility that the slight iron contamination of ornithine (see above) is responsible for the decreased of TAFC/FsC ratio in ornithine cultures. Taken together, these data reveal a negative influence of ornithine on TAFC production.

Iron Starvation Upregulates *amcA* Expression Independently of the Nitrogen Source; Ornithine Downregulates Expression of the SB Gene *sidG*

To analyze possible effects of AmcA-deficiency on expression of genes involved in siderophore and arginine/ornithine metabolism, Northern blot analysis of selected genes was performed from iron-starved as well as iron replete cultures (**Figure 4**). Previously, expression of *amcA* (AFUA_8G02760) was found to be upregulated by iron starvation with glutamine as nitrogen source (Schrettl et al., 2010). Here we found that this transcription pattern also holds true for arginine, ornithine and arginine/ornithine as nitrogen sources (**Figure 4**). As the latter nitrogen sources render the necessity for mitochondrial ornithine export redundant, these data indicate additional functions of AmcA, such as mitochondrial ornithine import as discussed above. The lack of *amcA* transcript detection in Δ*amcA* confirmed the deletion of the gene. The following genes involved in adaptation to iron starvation (Schrettl et al., 2004, 2008, 2010; Raymond-Bouchard et al., 2012), *sidA* (AFUA_2G07680, an ornithine monooxygenase catalyzing the first committed enzymatic step in the SB pathway), *mirB* (AFUA_3G03640, a siderophore transporter), *hapX* (AFUA_5G03920, the iron regulatory bZip transcription factor) and *sidG* (AFUA_3G03650, the N^2-transacetylase) were upregulated during iron starvation and unaffected by AmcA-deficiency (**Figure 4**). The fact that AmcA-deficiency did not affect transcript levels of any of the analyzed SB genes indicates that the decreased SB of Δ*amcA* with glutamine as nitrogen source has other reasons. Both ornithine and arginine/ornithine as nitrogen source did not significantly affect expression of *mirB* or *hapX*, mildly upregulated *sidA*, but downregulated *sidG*. As *sidG* encodes the enzyme that is responsible for conversion of FsC into TAFC, these data explain the increase of FsC and decrease of TAFC in the presence of ornithine or arginine/ornithine (**Figure 3**). The reason for the negative effect of ornithine on *sidG* expression and consequently TAFC production remains elusive. As expected, the presence of arginine upregulated the arginase-encoding gene *agaA* (AFUA_3G11430) during both iron starvation and sufficiency, largely unaffected by AmcA-deficiency (**Figure 4**). These data are in line with arginine catabolism and cytosolic ornithine production under these growth conditions. Expression of the ornithine-biosynthetic gene *argEF* (AFUA_6G02910, acetylglutamate kinase/N-acetyl-γ-glutamyl-phosphate reductase) was upregulated during iron starvation compared to iron sufficiency, as previously reported (Schrettl et al., 2007), and unaffected by AmcA-deficiency. Expression of the arginine-biosynthetic gene *argB* (AFUA_4G07190, ornithine carbamoyltransferase) and the ornithine decarboxylase encoding *odcA* (AFUA_4G08010), which is responsible for the first step in the conversion of ornithine to polyamines, were largely unaffected by both AmcA-deficiency and nitrogen source (**Figure 4**) (Oberegger et al., 2001; Jin et al., 2002; Beckmann et al., 2013).

Taken together with the data obtained by Northern blot analysis, which indicated wt-like expression of selected SB genes in Δ*amcA* (**Figure 4**), the decrease of SB of Δ*amcA* with glutamine as nitrogen source is most likely caused by the decrease in the cellular ornithine pool. Moreover, AmcA-deficiency does

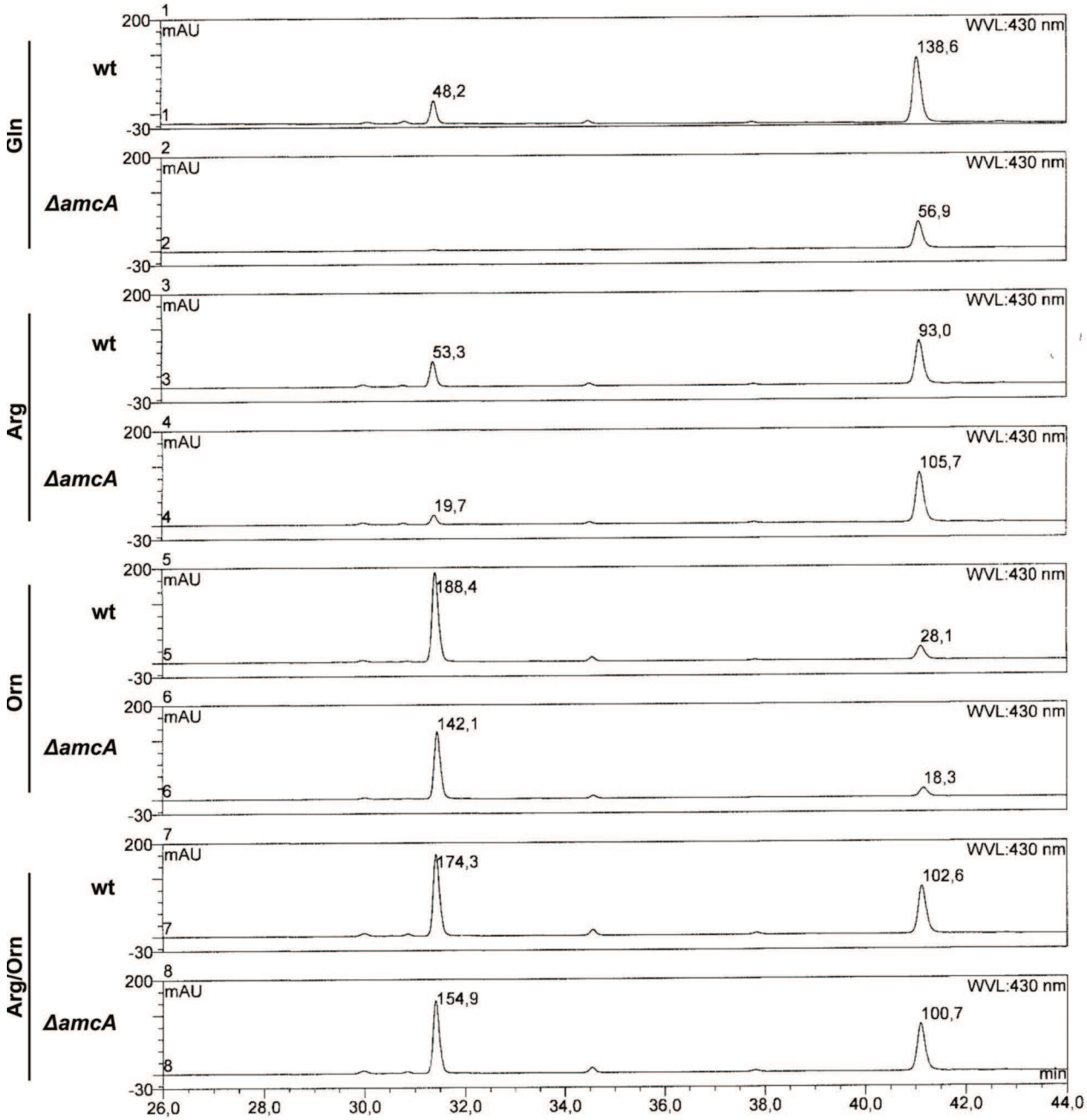


FIGURE 3 | Reversed-phase HPLC analysis of wt and ΔamcA grown with different nitrogen sources (20 mM Gln, 20 mM Arg, 20 mM Orn, 10 mM Arg/10 mM Orn). Ornithine as nitrogen source leads to a shift in siderophore composition from TAFC toward FsC. The peaks at 31.5 and 41 min represent TAFC and FsC, respectively. Absorbance of selected peaks is indicated.

not appear to affect arginine/ornithine biosynthesis at the transcriptional level.

AmcA-Deficiency Decreases the Cellular Pool of Arginine and Particularly Ornithine during Iron Starvation

Effects of AmcA-deficiency on amino acid composition were analyzed by comparison of the free amino acid pools of wt and Δ*amcA* in iron-starved and iron replete liquid cultures with 20 mM glutamine supplementation (**Table 1**). As previously shown for another *A. fumigatus* strain (Schrettl et al., 2010), iron starvation dramatically altered the composition of the free amino acid pool, including a 16-fold increase in both ornithine and arginine (**Table 1**). In comparison to the wt, during iron starvation AmcA-deficiency decreased the levels of arginine and ornithine by 48% and 84%, respectively, with the effect on the

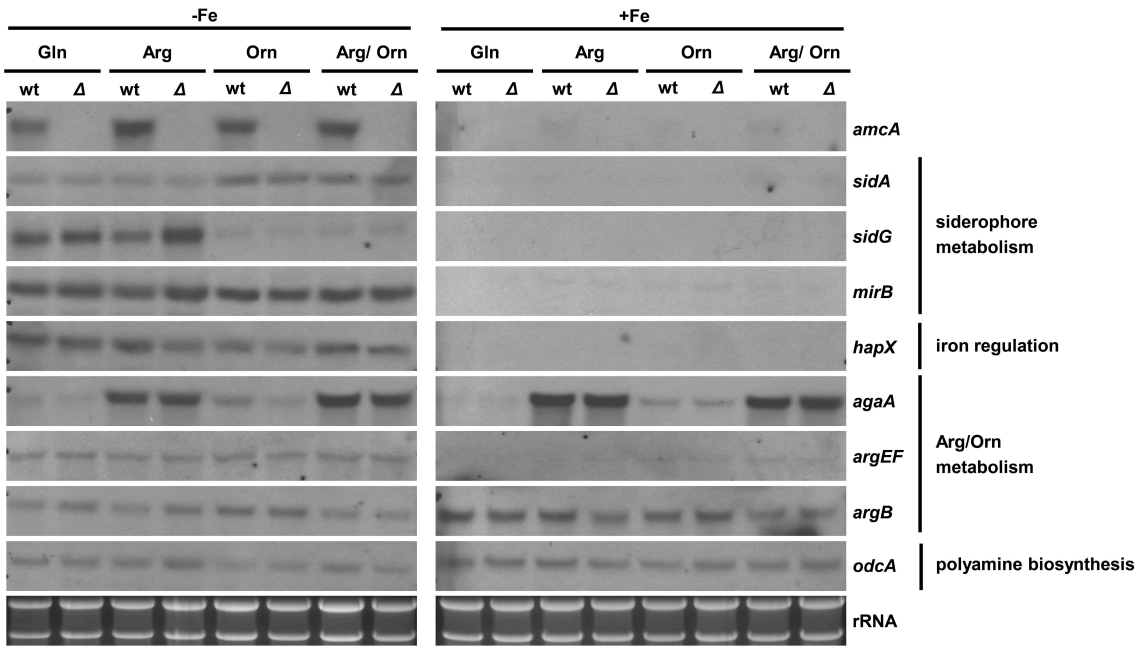


FIGURE 4 | Iron starvation upregulates *amcA* expression independent of the nitrogen source and AmcA-deficiency downregulates expression of the siderophore biosynthetic gene *sidG*. Northern analysis was performed with RNA from 18 h liquid cultures representing iron starvation (−Fe) as well as iron replete conditions (+Fe) with different nitrogen sources (20 mM Gln, 20 mM Arg, 20 mM Orn, 10 mM Arg/10 mM Orn).

TABLE 1 | Analysis of the amino acid composition of the wt and mutant strain under iron-replete and iron depleted conditions with glutamine as nitrogen source after 24 h of growth in % of total free amino acids.

aa	Wt			Δ*amcA*			Δ*amcA*/wt	
	+Fe	−Fe	−/+Fe	+Fe	−Fe	−/+Fe	+Fe	−Fe
Ala	15.75 ± 0.27	4.51 ± 0.13	***0.29***	20.87 ± 0.51	6.10 ± 0.22	***0.29***	1.33	1.35
Arg	1.05 ± 0.03	16.67 ± 0.16	***15.94***	0.86 ± 0.01	8.61 ± 0.22	***9.96***	0.83	**0.52**
Asn	0.72 ± 0.03	3.45 ± 0.11	***4.78***	0.80 ± 0.02	3.28 ± 0.10	***4.11***	1.11	0.95
Asp	16.80 ± 0.14	4.47 ± 0.13	***0.27***	7.49 ± 0.09	4.58 ± 0.19	**0.61**	**0.45**	1.03
Gln	9.17 ± 0.22	36.94 ± 0.33	***4.03***	5.11 ± 0.38	48.17 ± 0.45	***9.42***	**0.56**	1.30
Glu	44.58 ± 0.61	15.95 ± 0.42	**0.36**	53.60 ± 0.28	18.41 ± 0.73	**0.34**	1.20	1.15
Gly	0.98 ± 0.07	1.23 ± 0.07	1.26	1.00 ± 0.05	0.82 ± 0.03	0.82	1.02	**0.66**
His	0.57 ± 0.01	2.73 ± 0.07	***4.80***	0.60 ± 0.02	2.80 ± 0.00	***4.68***	1.05	1.03
Ile	0.51 ± 0.06	0.23 ± 0.02	**0.45**	0.43 ± 0.00	0.22 ± 0.01	**0.50**	0.83	0.93
Leu	0.59 ± 0.01	0.32 ± 0.01	**0.54**	0.42 ± 0.01	0.20 ± 0.00	**0.47**	0.72	**0.63**
Lys	1.40 ± 0.07	4.28 ± 0.04	***3.06***	1.83 ± 0.04	2.13 ± 0.06	1.17	1.31	**0.50**
Met	0.09 ± 0.02	0.11 ± 0.01	1.24	0.07 ± 0.03	0.06 ± 0.02	0.80	0.86	**0.56**
Orn	0.30 ± 0.01	5.11 ± 0.11	***16.78***	0.45 ± 0.04	0.83 ± 0.00	**1.86**	1.47	***0.16***
Phe	0.24 ± 0.02	0.13 ± 0.02	**0.55**	0.17 ± 0.01	0.10 ± 0.01	**0.57**	0.72	0.74
Ser	2.49 ± 0.05	1.52 ± 0.03	**0.61**	2.16 ± 0.04	1.37 ± 0.05	**0.63**	0.87	0.90
Thr	3.31 ± 0.03	1.35 ± 0.05	**0.41**	2.08 ± 0.00	1.52 ± 0.07	0.73	**0.63**	1.12
Trp	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00	0.00
Tyr	0.29 ± 0.01	0.39 ± 0.01	1.37	0.29 ± 0.03	0.31 ± 0.00	1.07	1.01	0.79
Val	1.15 ± 0.03	0.60 ± 0.03	0.52	1.76 ± 0.01	0.49 ± 0.01	***0.28***	**1.53**	0.81

*Individual amino acid pools are given in % of the total free amino acids. Amino acid pools up-regulated >1.5- and >3-fold are in **bold** and **bold/italics** respectively. Down-regulation of >1.5- and >3-fold is marked in **bold** and **bold/italics**. Given values represent the mean of two biological replicates ± standard deviation.*

TABLE 2 | Analysis of polyamine production in wt and Δ*amcA* under iron-replete and iron depleted conditions after 24 h cultivation with glutamine as nitrogen source.

Polyamines	wt		Δ*amcA*	
	+Fe	−Fe	+Fe	−Fe
Putrescine	20.94 ± 18.71	9.39 ± 10.75	7.31 ± 2.09	5.07 ± 0.85
Spermidine	294.39 ± 32.40	105.45 ± 16.40	231.42 ± 3.59	99.04 ± 21.73
Spermine	64.15 ± 1.46	63.39 ± 1.65	77.11 ± 16.42	51.26 ± 21.70
Total	**379.48**	**178.23**	**315.84**	**155.37**

The values are given in nmol/ml and represent the mean of two biological replicates ± standard deviation.

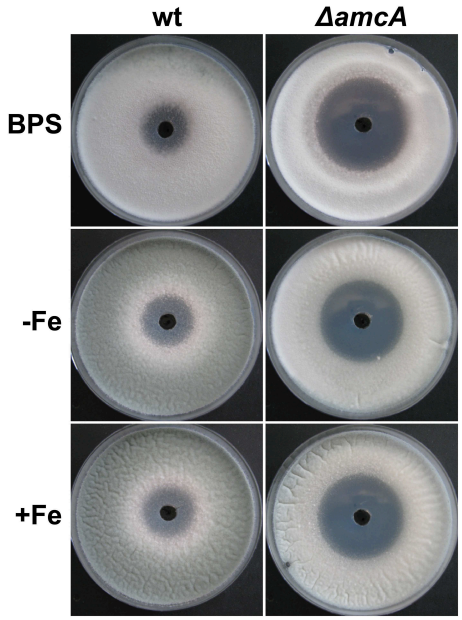


FIGURE 5 | Deficiency in AmcA increased susceptibility to eflornithine under different availability for iron (BPS, −Fe, +Fe) with glutamine as nitrogen source. 100 μl of a 0.3 M eflornithine solution were added to a hole pricked in the middle of the plate. Growth inhibition on solid medium was scored after 48 h.

cellular ornithine content being the most severe of all amino acids.

A significant decrease of arginine and particularly ornithine pools was previously also found for the *S. cerevisiae* mutant lacking the AmcA ortholog Arg11 (Crabeel et al., 1996). These data stress the role of AmcA as a mitochondrial ornithine transporter. The possible reasons for the decreased arginine pool include increased conversion of arginine to ornithine via arginase, although not visible at the transcript level of arginase-encoding *agaA* in Northern blot analysis (**Figure 4**). Most likely, the decreased cellular ornithine and arginine levels are caused by feed-back inhibition of mitochondrial arginine/ornithine biosynthetic enzymes due to the increased mitochondrial ornithine content resulting from blocked ornithine export. The change in content of the other amino acids underlines the interconnection of metabolic pathways.

AmcA-Deficiency Mildly Decreases Polyamine Levels

Polyamines (putrescine, spermine and spermidine) are organic cations required for normal growth as well as cell proliferation and differentiation of all organisms (McCann et al., 1987; Hu and Pegg, 1997; Pegg, 2009). After having observed decreased ornithine, in addition to decreased siderophore levels under glutamine supplementation, we considered it worthwhile to have a closer look at polyamine levels in the Δ*amcA* mutant, as ornithine is the precursor for biosynthesis of not only siderophores, but also polyamines. In the first and rate-limiting step, ornithine is converted via the ornithine decarboxylase to putrescine, which is then further converted to spermidine, the major polyamine in *Aspergilli*, and later to spermine (Jin et al., 2002). The analysis confirmed spermidine as the major polyamine of *A. fumigatus* under iron-replete as well as iron depleted conditions (**Table 2**). During iron starvation, the total polyamine content of *A. fumigatus* wt decreased to 47% compared to iron-replete conditions (**Table 2**). AmcA-deficiency mildly decreased the total polyamine content compared to wt, to 83% and 87% during iron sufficiency and iron starvation, respectively (**Table 2**).

These data indicate cellular prioritization of ornithine flux into polyamine rather than SB as the experiments described above revealed a significant decrease of the cellular ornithine pool and siderophore production in Δ*amcA* (**Table 1** and **Figure 2**). These observations underline the importance of fine-tuned cellular polyamine levels within an organism. Of course iron supply is as essential as polyamine biosynthesis. However, *A. fumigatus* harbors an ornithine-independent, high-affinity iron uptake system, namely RIA (Schrettl et al., 2004), while there is no alternative for polyamine biosynthesis. This might explain the prioritization, which was also indicated in the study investigating the link between arginine/ornithine metabolism and SB using arginine auxotrophic mutants (Beckmann et al., 2013).

AmcA-Deficiency Leads to Increased Sensitivity against the Ornithine Decarboxylase (OdcA) Inhibitor Eflornithine

To further analyze the link between AmcA-deficiency, the ornithine pool and polyamine homeostasis, we analyzed the effect of AmcA-deficiency on susceptibility to eflornithine (α-difluormethylornithine) using plate-diffusion assays (**Figure 5**). Eflornithine is the most effective inhibitor of the ornithine

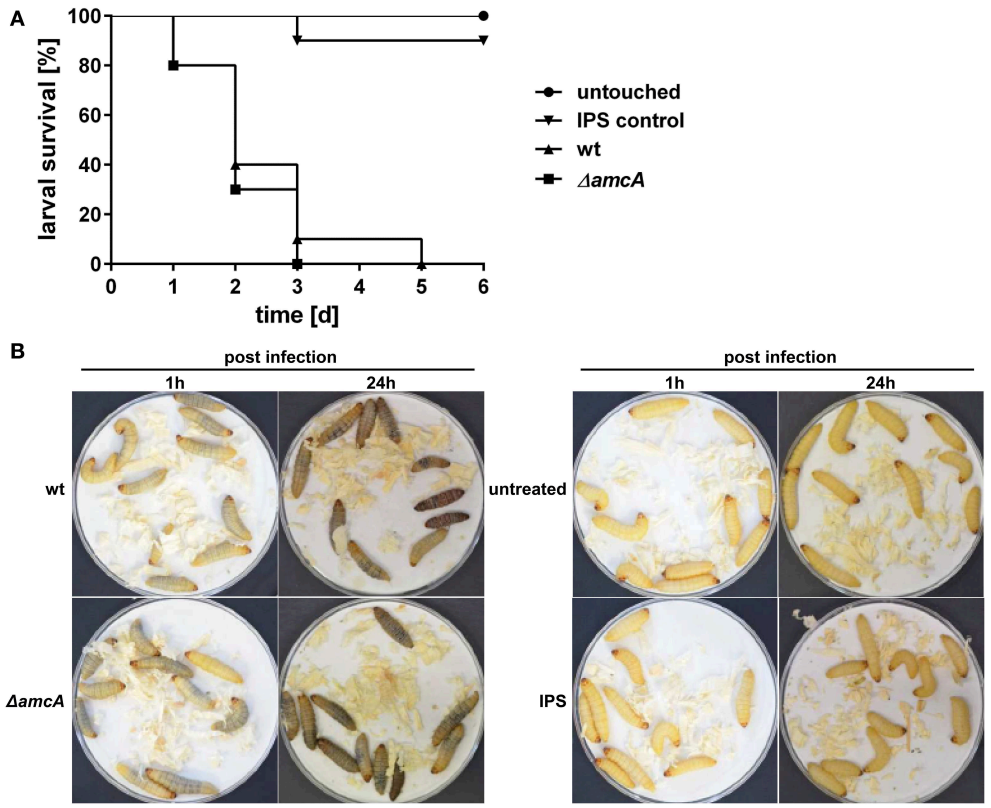


FIGURE 6 | Deficiency in AmcA does not attenuate virulence of *A. fumigatus* in the *Galleria mellonella* infection model. (A) No significant difference in virulence between Δ*amcA* and wt could be detected ($P = 0.5253$). Insect physiological saline (IPS) was used as an injection control. 90% of all larvae in this group remained viable for the entire experiment. **(B)** Exemplary infection assay with *Galleria mellonella larvae*. Δ*amcA* and wt (AfS77) exhibit same rate of melanisation 1 h and 24 h post infection, indicating progression of infection, and resulting in 100% mortality at day 3 and 5, respectively **(A)**.

decarboxylase and is used in treatment against African trypanosomiasis and hirsutism (Pegg, 1986; Ramot et al., 2010; Kennedy, 2013). Confirming previous data (Beckmann et al., 2013), *A. fumigatus* is susceptible to eflornithine under iron sufficiency and iron starvation (**Figure 5**). However, AmcA-deficiency significantly increased susceptibility to eflornithine (inhibition zone of 40 mm of Δ*amcA* compared to 23 mm of wt) (**Figure 5**).

These data indicate that inhibition of the ornithine decarboxylase is more detrimental to Δ*amcA* than the wt, which is most likely a consequence of the decreased ornithine content (**Table 1**).

Deficiency in AmcA does not Impede Virulence of *A. fumigatus* in an Insect Host Model

In order to assess the role of AmcA in the virulence of *A. fumigatus*, we compared the Δ*amcA* mutant with the wt strain in the *Galleria mellonella* infection model (Fallon et al., 2011). AmcA does not seem to play a major role in the pathogenicity of *A. fumigatus*, as AmcA-deficiency resulted in no significant differences in survival rates ($P = 0.5253$) over a period of 6 days (**Figures 6A,B**).

Previously, defective SB was shown to result in attenuated virulence in different models, including pulmonary and keratitis mouse models as well as *G. mellonella* (Schrettl et al., 2004, 2007; Slater et al., 2011; Yasmin et al., 2012; Beckmann et al., 2013; Leal et al., 2013). The dramatic decrease in siderophore production of the Δ*amcA* mutant with glutamine as nitrogen source might have led to speculate about an attenuated virulence of this strain. However, previous studies have demonstrated wt-like virulence of an *A. fumigatus argB* mutant in *G. mellonella* (Beckmann et al., 2013), which indicates arginine availability in the host niche of this virulence model. The latter might explain wt-like virulence of Δ*amcA* as arginine restored SB in Δ*amcA* (**Figure 2**). Alternatively, or combined with the arginine availability, the residual siderophore production of Δ*amcA* could be the reason for unaltered virulence of *A. fumigatus* Δ*amcA*.

Conclusion

This study identified a novel cellular component supporting SB of *A. fumigatus*. Despite the lack of direct prove, several lines of evidence indicate a function of AmcA in the supply of the precursor ornithine via mitochondrial ornithine export: (i) the

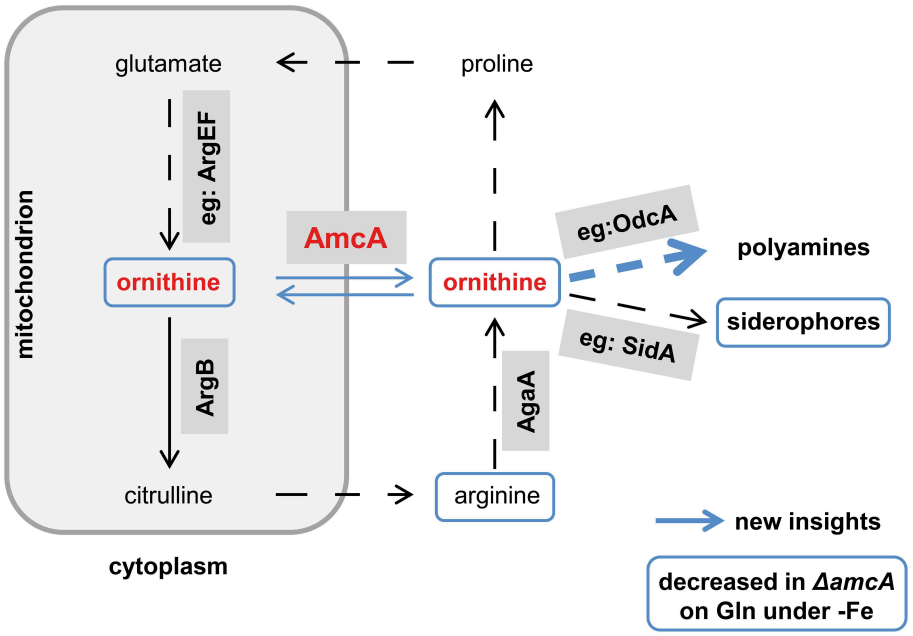


FIGURE 7 | Postulated working model for AmcA function. This schematic view of the link between AmcA, ornithine and siderophore production was adopted from Beckmann et al. (2013). Novel findings of this study are marked in blue. Whether the decreased cellular ornithine level shown in **Table 1** is caused mitochondrially or cytosolically remains to be elucidated. Prioritization of polyamine synthesis over siderophore production is indicated by the bulky blue arrow.

AmcA protein is a member of the mitochondrial carrier protein family with homologs involved in ornithine exchange between the mitochondrial matrix and the cytosol, (ii) AmcA-deficiency in *A. fumigatus* resulted in a dramatically decreased cellular ornithine content and SB during iron starvation with glutamine as nitrogen source but not ornithine or arginine, which stresses cytosolic ornithine production, (iii) AmcA-deficiency led to mildly decreased cellular polyamine content and significantly increased susceptibility to the polyamine biosynthesis inhibitor eflornithine. The novel findings and the postulated function of AmcA are summarized in **Figure 7**.

In line with its role in precursor supply for siderophore biosynthesis, *amcA* was transcriptionally upregulated during iron starvation. The fact that this upregulation was independent of the nitrogen source (e.g., with arginine and ornithine) indicates additional roles of AmcA such as mitochondrial ornithine import. In line, AmcA-deficiency impaired growth on ornithine as nitrogen source.

In the *G. mellonella* model, AmcA-deficiency did not affect virulence of *A. fumigatus*, most likely due to the residual siderophore production and/or arginine availability in this host niche.

Acknowledgments

This work was supported in part by the Austrian Science Foundation FWF P21643−B11 and DACH program FWF I1346−B22.

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00252/abstract

Figure S1 | (A) AmcA-deficiency decreases radial growth independent of the iron supply and sporulation in the presence of BPS on 20 mM glutamine (Gln), 20 mM ammonium tartrate (ammonium), and 70 mM sodium nitrate (nitrate) as nitrogen sources. Fungal growth on solid medium was analyzed after 48 h on plates. BPS plates contained 0.2 mM BPS to increase iron starvation. For −Fe plates, iron was omitted. Iron-replete plates (+Fe) contained 30 μM $FeSO_4$. **(B,C)** Fungal growth was quantified in liquid culture, containing 20 mM glutamine (Gln), 20 mM ammonium tartrate (ammonium) or 70 mM sodium nitrate (nitrate) after 24 h during iron starvation (−Fe) and iron sufficiency (+Fe), respectively. **(D,E)** AmcA-deficiency decreases production of extra- and intra-cellular siderophores on glutamine, ammonium tartrate and sodium nitrate as nitrogen sources. Data represent the mean of three biological replicates ± standard deviation normalized to the biomass and to the wt on Gln. ($* \hat{=} P < 0.05$, $** \hat{=} P < 0.01$).

References

Beckmann, N., Schafferer, L., Schrettl, M., Binder, U., Talasz, H., Lindner, H., et al. (2013). Characterization of the link between ornithine, arginine, polyamine and siderophore metabolism in *Aspergillus fumigatus*. *PLoS ONE* 8:e67426. doi: 10.1371/journal.pone.0067426

Berger, H., Basheer, A., Bock, S., Reyes-Dominguez, Y., Dalik, T., Altmann, F., et al. (2008). Dissecting individual steps of nitrogen transcription factor cooperation in the *Aspergillus nidulans* nitrate cluster. *Mol. Microbiol.* 69, 1385–1398. doi: 10.1111/j.1365-2958.2008.06359.x

Blatzer, M., Schrettl, M., Sarg, B., Lindner, H. H., Pfaller, K., and Haas, H. (2011). SidL, an *Aspergillus fumigatus* transacetylase involved in biosynthesis of the

siderophores ferricrocin and hydroxyferricrocin. *Appl. Environ. Microbiol.* 77, 4959–4966. doi: 10.1128/AEM.00182-11

Camacho, J. A., Obie, C., Biery, B., Goodman, B. K., Hu, C., Almashanu, S., et al. (1999). Hyperornithinaemia-hyperammonaemia-homocitrullinuria syndrome is caused by mutations in a gene encoding a mitochondrial ornithine transporter. *Nat. Genet.* 22, 151–158. doi: 10.1038/9658

Crabeel, M., Soetens, O., De Rijcke, M., Pratiwi, R., and Pankiewicz, R. (1996). The ARG11 gene of *Saccharomyces cerevisiae* encodes a mitochondrial integral membrane protein required for arginine biosynthesis. *J. Biol. Chem.* 271, 25011–25018. doi: 10.1074/jbc.271.40.25011

Davis, R. H. (1986). Compartmental and regulatory mechanisms in the arginine pathways of *Neurospora crassa* and *Saccharomyces cerevisiae*. *Microbiol. Rev.* 50, 280–313.

Fallon, J. P., Troy, N., and Kavanagh, K. (2011). Pre-exposure of *Galleria mellonella* larvae to different doses of *Aspergillus fumigatus* conidia causes differential activation of cellular and humoral immune responses. *Virulence* 2, 413–421. doi: 10.4161/viru.2.5.17811

Haas, H. (2012). Iron - a key nexus in the virulence of *Aspergillus fumigatus*. *Front. Microbiol.* 6:28. doi: 10.3389/fmicb.2012.00028

Haas, H., Eisendle, M., and Turgeon, B. G. (2008). Siderophores in fungal physiology and virulence. *Annu. Rev. Phytopathol.* 46, 149–187. doi: 10.1146/annurev.phyto.45.062806.094338

Halliwell, B., and Gutteridge, J. M. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219, 1–14.

Hu, R. H., and Pegg, A. E. (1997). Rapid induction of apoptosis by deregulated uptake of polyamine analogues. *Biochem. J.* 328, 307–316.

Jadoun, J., Shadkchan, Y., and Osherov, N. (2004). Disruption of the *Aspergillus fumigatus* argB gene using a novel *in vitro* transposon-based mutagenesis approach. *Curr. Genet.* 45, 235–241. doi: 10.1007/s00294-003-0480-6

Jin, Y., Bok, J. W., Guzman-de-Pena, D., and Keller, N. P. (2002). Requirement of spermidine for developmental transitions in *Aspergillus nidulans*. *Mol. Microbiol.* 46, 801–812. doi: 10.1046/j.1365-2958.2002.03201.x

Kennedy, P. G. (2013). Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). *Lancet Neurol.* 12, 186–194. doi: 10.1016/S1474-4422(12)70296-X

Krappmann, S., Sasse, C., and Braus, G. H. (2006). Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end- joining-deficient genetic background. *Eukaryot. Cell* 5:212–215. doi: 10.1128/EC.5.1.212-215.2006

Leal, S. M. Jr., Roy, S., Vareechon, C., Carrion, S. D., Clark, H., Lopez-Berges, M. S., et al. (2013). Targeting iron acquisition blocks infection with the fungal pathogens *Aspergillus fumigatus* and *Fusarium oxysporum*. *PLoS Pathog.* 9:e1003436. doi: 10.1371/journal.ppat.1003436

Liu, Q., and Dunlap, J. C. (1996). Isolation and analysis of the arg-13 gene of *Neurospora crassa*. *Genetics* 143, 1163–1174.

McCann, P. P., Pegg, A. E., Sjoerdsma, A. (eds.). (1987). *Inhibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies*. New York, NY: Academic Press, Inc.

Nielsen, M. L., Albertsen, L., Lettier, G., Nielsen, J. B., and Mortensen, U. H. (2006). Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. *Fungal Genet. Biol.* 43, 54–64. doi: 10.1016/j.fgb.2005.09.005

Oberegger, H., Schoeser, M., Zadra, I., Abt, B., and Haas, H. (2001). SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*. *Mol. Microbiol.* 41, 1077–1089. doi: 10.1046/j.1365-2958.2001.02586.x

Palmieri, L., De Marco, V., Iacobazzi, V., Palmieri, F., Runswick, M. J., and Walker, J. E. (1997). Identification of the yeast *ARG-11* gene as a mitochondrial ornithine carrier involved in arginine biosynthesis. *FEBS Lett.* 410, 447–451. doi: 10.1016/S0014-5793(97)00630-3

Pegg, A. E. (1986). Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* 234, 249–262.

Pegg, A. E. (2009). Mammalian polyamine metabolism and function. *IUBMB Life* 61, 880–894. doi: 10.1002/iub.230

Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D., and Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv. Genet.* 5, 141–238. doi: 10.1016/S0065-2660(08)60408-3

Ramot, Y., Pietila, M., Giuliani, G., Rinaldi, F., Alhonen, L., and Paus, R. (2010). Polyamines and hair: a couple in search of perfection. *Exp. Dermatol.* 19, 784–790. doi: 10.1111/j.1600-0625.2010.01111.x

Ratledge, C., and Dover, L. G. (2000). Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* 54, 881–941. doi: 10.1146/annurev.micro.54.1.881

Raymond-Bouchard, I., Carroll, C. S., Nesbitt, J. R., Henry, K. A., Pinto, L. J., Moinzadeh, M., et al. (2012). Structural requirements for the activity of the MirB Ferrisiderophore transporter of *Aspergillus fumigatus*. *Eukaryot. cell* 11, 1333–1344. doi: 10.1128/EC.00159-12

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. New York, NY: Cold Spring Harbor Laboratory Press.

Schrettl, M., Beckmann, N., Varga, J., Heinekamp, T., Jacobsen, I. D., Jöchl, C., et al. (2010). HapX-mediated adaption to iron starvation is crucial for virulence of *Aspergillus fumigatus*. *PLoS Pathog.* 6:e1001124. doi: 10.1371/journal.ppat.1001124

Schrettl, M., Bignell, E., Kragl, C., Sabiha, Y., and Loss, O., Eisendle, M., et al. (2007). Distinct Roles for Intra- and Extracellular Siderophores during *Aspergillus fumigatus* Infection. *PLoS Pathog.* 3:e128. doi: 10.1371/journal.ppat.0030128

Schrettl, M., Bignell, E., Kragl, C., Joechl, C., Rogers, T., Arst, H. N. Jr., et al. (2004). Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. *J. Exp. Med.* 200, 1213–1219. doi: 10.1084/jem.20041242

Schrettl, M., Kim, H. S., Eisendle, M., Kragl, C., Nierman, W. C., Heinekamp, T., et al. (2008). SreA mediated iron regulation in *Aspergillus fumigatus*. *Mol. Microbiol.* 70, 27–43. doi: 10.1111/j.1365-2958.2008.06376.x

Slater, J. L., Gregson, L., Denning, D. W., and Warn, P. A. (2011). Pathogenicity of *Aspergillus fumigatus* mutants assessed in *Galleria mellonella* matches that in mice. *Med. Mycol.* 49, 107–113. doi: 10.3109/13693786.2010.523852

Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A., and Davies, R. W. (1983). Transformation by integration in *Aspergillus nidulans*. *Gene* 26, 205–221. doi: 10.1016/0378-1119(83)90191-9

Wallner, A., Blatzer, M., Schrettl, M., Sarg, B., Lindner, H., and Haas, H. (2009). Ferricrocin, a siderophore involved in intra- and transcellular iron distribution in *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 75, 4194–4196. doi: 10.1128/AEM.00479-09

Wongyai, S. O., Oefner, P. J., and Bonn, G. K. (1989). High resolution analysis of polyamines and their acetyl derivatives using RP-HPLC. *J. Liq. Chromatogr.* 12, 2249–2261.

Yasmin, S., Alaczar-Fuoli, L., Gründlinger, M., Puempel, T., Cairns, T., Blatzer, M., et al. (2012). Mevalonate governs interdependency of ergosterol and siderophore biosynthesis in the fungal pathogen *Aspergillus fumigatus*. *Proc. Natl. Acad. Sci. U.S.A.* 109, E497–E504. doi: 10.1073/pnas.1106399108

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Schafferer, Beckmann, Binder, Brosch and Haas. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

PERSPECTIVE
published: 21 April 2015
doi: 10.3389/fmicb.2015.00344

Co-recognition of β-glucan and chitin and programming of adaptive immunity to *Aspergillus fumigatus*

*Nansalmaa Amarsaikhan and Steven P. Templeton**

Department of Microbiology and Immunology, Indiana University School of Medicine – Terre Haute, Terre Haute, IN, USA

The prevalence of fungal infections has increased concurrently with increases in immune suppressive therapies and susceptible individuals. Opportunistic fungal pathogens such as *Aspergillus fumigatus* may exhibit invasive growth and dissemination resulting in a high mortality rate. Herein, we discuss how immune sensing of germination directs innate immune responses and programs adaptive responses that could promote or impair immune protection during periods of heightened susceptibility. In infected individuals, Th1 responses are the most protective, while Th2 responses lead to poor disease outcomes. In particular, the roles of β-glucan and chitin co-recognition in shaping Th1- and Th2-type immunity to fungal infection are explored. We discuss how fungal responses to environmental stresses could result in decreased immune protection from infection, particularly in response to anti-fungal drugs that target β-glucan synthesis. Furthermore, we consider how experimental modulation of host-pathogen interactions might elucidate the mechanisms of protective and detrimental immunity and the potential of current and future studies to promote the development of improved treatments for patients that respond poorly to existing therapies.

Keywords: *Aspergillus fumigatus*, fungal infection, aspergillosis, innate recognition, adaptive immunity, β-glucan, chitin, cell wall modulation

OPEN ACCESS

Edited by:
Frederic Lamoth, Duke University, USA

Reviewed by:
Peter R. Williamson, National Institutes of Health, USA
Anne Beauvais, Institut Pasteur, France

***Correspondence:**
Steven P. Templeton, Department of Microbiology and Immunology, Indiana University School of Medicine – Terre Haute, 620 Chestnut Street HH135, Terre Haute, IN 47809, USA
sptemple@iupui.edu

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: *13 February 2015*
Accepted: *07 April 2015*
Published: *21 April 2015*

Citation:
Amarsaikhan N and Templeton SP (2015) Co-recognition of β-glucan and chitin and programming of adaptive immunity to Aspergillus fumigatus. Front. Microbiol. 6:344. doi: 10.3389/fmicb.2015.00344

Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen abundant in indoor and outdoor environments, causing fungal infection in immune suppressed individuals and exacerbating chronic pulmonary conditions (Hohl and Feldmesser, 2007; Kwon-Chung and Sugui, 2013). The small size of *A. fumigatus* conidia promotes aerosol formation and thus frequent contact with airways of potential hosts. Small to moderate amounts of conidia are often removed by alveolar macrophages without a significant inflammatory response. When larger numbers of conidia are inhaled, more conidia are allowed to germinate, stimulating in an inflammatory response. Swelling of *A. fumigatus* conidia follows the degradation of the outermost hydrophobic rodlet layer, thus exposing the inner cell wall layer composed of a complex network of immune-stimulating polysaccharides (Thau et al., 1994; Latgé, 1999; Paris et al., 2003; Aimanianda et al., 2009). Since these cell wall components are not found in mammalian hosts, specific host recognition receptors have evolved as a mechanism to signal appropriate early inflammation and the subsequent development of protective innate and adaptive immune responses.

Recent studies have indicated a variety of responses to particulate forms of fungal pathogen-associated molecular patterns (PAMPs). However, in natural *A. fumigatus* exposure and infection, fungal PAMPs are recognized in concert on the germinating conidial surface, and it is thus likely

that this combined recognition results in programming of immune profiles not observed in studies of purified, particulate cell wall components. Of the PAMPs contained in the cell wall of *A. fumigatus*, many studies have focused on the covalently linked fibrillary core polysaccharides β1-3-glucan (β-glucan) and chitin; both known to be immune stimulatory in purified, particulate form (Lenardon et al., 2010; Drummond and Brown, 2011). Furthermore, the amorphous cell wall components α1-3-linked glucan (α-glucan), galactomannan, and galactosaminogalactan (GAG) also act to modify immune responses to infection (Bozza et al., 2009; Gravelat et al., 2013; Latge and Beauvais, 2014), though their direct contributions to the development of protective or detrimental immunity are less clear. Recent studies reported that the expression of fungal PAMPs varies considerably depending on available nutrients, temperature, oxygen levels, and the presence of anti-fungal drugs (Verwer et al., 2012; Shepardson et al., 2013; Beauvais et al., 2014). When combined, these and other potential cellular and metabolic stressors may ultimately result in distinct patterns of recognition and cell signaling with the potential to program equally distinct profiles of adaptive immunity. However, our current understanding of these pathways of recognition and their influence on adaptive immunity is basic and preliminary, and more detailed studies of combined recognition of fungal PAMPs during germination are needed. Herein, we examine the role of individual and aggregate pattern recognition in the programming of immunity to *A. fumigatus*, focusing on the immune responses to β-glucan and chitin. We also consider the evidence that fungal cell wall modulation due to environmental stresses like antifungal drug exposure could either enhance or diminish immune protection from infection.

Early Recognition of Fungal Germination

β-Glucan/dectin-1

The *A. fumigatus* cell wall consists of covalently bound β-glucan, chitin, galactomannan and α-glucan that are absent in mammals, and thus present prime targets for pattern recognition receptors (PRRs) on host cells (Chai et al., 2011). β-glucan is recognized by the C-type lectin receptor dectin-1 and has been studied extensively with infection models of *A. fumigatus* and other pathogenic fungi (Drummond and Brown, 2011). Downstream signaling of dectin-1 activation promotes cellular antifungal responses including phagocytosis, ROS production, and inflammatory cytokine production. Mutations in human dectin-1 rendered individuals more susceptible to invasive aspergillosis, and infected dectin-1-deficient mice displayed increased pathology with decreased neutrophil recruitment and impaired cytokine production (Steele et al., 2005; Gersuk et al., 2006; Cunha et al., 2010; Gessner et al., 2012). The inflammatory response initiated by binding of dectin-1 receptor on resident cells is strengthened when combined with signaling through toll-like receptors (TLR) that are co-expressed within an immunological synapse (Goodridge et al., 2011; Inoue and Shinohara, 2014), resulting in synergistically increased cytokine production and activation of inflammatory signaling pathways (Mambula et al., 2002; Hohl et al., 2005; Gersuk et al., 2006; Dennehy et al., 2008). Thus, dectin-1 and associated PRR recognition provide immune signals essential for protective immunity to *A. fumigatus* infection.

Conidial surface β-glucan is initially recognized by epithelial cells, macrophages and dendritic cells (Drummond and Brown, 2011; Osherov, 2012). Epithelial cells act as the first barrier and immunologically active surface in host tissues, serving as non-professional phagocytes where engulfed conidia persist in the pulmonary epithelial space (Heinekamp et al., 2015). Airway epithelial cells activated a panel of antimicrobial genes in a β-glucan-mediated response to *A. fumigatus* (Evans et al., 2010; Sun et al., 2012), and secreted TNF-α, IL-8 (CXCL-8) and GM-CSF (Sun et al., 2012), indicating an important role for these cells in neutrophil recruitment that is essential for protection from invasive infection (Bonnett et al., 2006; Mircescu et al., 2009). Similar to epithelial cells, alveolar macrophages from dectin-1 knockout mice lacked the ability to produce IL-1α/β, TNF-α, CCL3/4 (MIP-1α/β), and CXCL1 (KC) in response to *A. fumigatus* (Werner et al., 2011). A dectin-1/CARD9 pathway promoted early neutrophil influx, although initial recruitment may be mediated by a hypoxia inducible factor-α/IL1R1/MyD88 pathway (Shepardson et al., 2014; Caffrey et al., 2015; Jhingran et al., 2015). In neutrophils, β-glucan recognition by dectin-1 promoted production of reactive oxygen species (Kennedy et al., 2007). Neutrophils produced dectin-1-mediated IL-17A in the presence myeloid cells in response to *A. fumigatus* (Werner et al., 2011) that likely serves as a feedback signal for increased neutrophil recruitment via stimulation of epithelial cells to produce TNF-α, IL-8, and G-CSF (Iwakura et al., 2011). In addition to neutrophils, NK cells are recruited early after *A. fumigatus* infection by a CCL2-dependent mechanism, and provide protection through IFN-γ secretion and subsequent activation of macrophages (Morrison et al., 2003; Park et al., 2009) and also potentially through enhanced neutrophil killing (Roilides et al., 1993). Inflammatory monocytes also provide protection from invasive infection, similarly in part by enhancing neutrophil conidiacidal activity (Espinosa et al., 2014). In DCs, TLR and dectin-1 signaling mediated β-glucan-induced secretion of TNF-α and IL-12 (Gantner et al., 2003; Mezger et al., 2008). In response to *A. fumigatus* conidia, Dectin-1 also promoted early lung protection and fungal allergy via secretion of IL-22, a cytokine important in activation of antimicrobial effectors at mucosal surfaces (Gessner et al., 2012; Lilly et al., 2012). Thus, dectin-1 recognition of β-glucan exposure in *A. fumigatus* results in the activation of an array of inflammatory cytokines and chemokines that promote early protection from infection.

Chitin

Chitin is a fungal cell wall polysaccharide that is abundant in parasites, insects, and crustaceans (Da Silva et al., 2010; Lenardon et al., 2010; Muzzarelli, 2010). Chitin microfibrils, covalently linked with β-glucan, impart a strong rigidity to the cell wall of fungal hyphae. The results of several studies examining immune responses to purified chitin indicate that particle size, concentration, and degree of acetylation are important determinants of cytokine profiles and inflammatory cell recruitment (Shibata et al., 1997; Da Silva et al., 2009; Wagener et al., 2014). Low concentrations of chitin particles between 1 and 10 μm

induced macrophage IL-10 secretion, while increased concentrations resulted in increased TNF secretion. In contrast, larger chitin particles (50–100 μm) promoted lung eosinophilia and alternative macrophage activation (Reese et al., 2007; Kogiso et al., 2011; Roy et al., 2012). Chitin exposure increased expression of lung epithelial CCL2, IL-25, IL-33, and TLSP that mediated recruitment of eosinophils and promoted M2 (alternatively activated) macrophage activation (Islam and Luster, 2012; Roy et al., 2012; Van Dyken et al., 2014). Furthermore, chitin-induced IL-25, IL-33, and TLSP induced type 2 innate lymphoid cells (ILC2) to secrete IL-5 and IL-13, cytokines essential for eosinophil recruitment and M2 macrophage activation (Van Dyken et al., 2014). In addition to purified particles, inhaled fungal chitin from house dust, hyphal extracts, and conidia also promoted lung eosinophil recruitment in mice that was decreased in the presence of constitutively expressed acidic mammalian chitinase (AMCase; Van Dyken et al., 2011; O'Dea et al., 2014). These studies demonstrate that innate recognition of purified or fungal chitin induces recruitment of eosinophils and promotes M2 macrophage activation.

In contrast to β-glucan, a distinct chitin recognition receptor has not been fully characterized. To date, the only chitin-specific receptor identified is FIBCD1, a type II transmembrane protein apically expressed in gut tissues (Schlosser et al., 2009). However, several PRRs specific for other microbial PAMPs are associated with chitin-mediated responses. Chitin-induced macrophage secretion of IL-17A and TNF-α were dependent on the TLR-2/MyD88 pathway and dectin-1/TLR2 expression, respectively (Da Silva et al., 2008, 2009). IL-10 secretion in response to smaller chitin particles was dependent on mannose receptor, NOD2 and TLR9 (Wagener et al., 2014). In addition, the cytosolic C-type lectin RegIIIγ also binds chitin (Cash et al., 2006). Notably, the well-described ligand shared by TLR2, NOD2, and RegIIIγ is peptidoglycan, an essential cell wall component of gram-positive bacteria that, like chitin, consists of a carbohydrate backbone containing *N*-acetylglucosamine residues (GLcNAc). Furthermore, RegIIIγ and other C-type lectins that bind GlcNAc containing polysaccharides also bind mannan (Drickamer, 1992; Cash et al., 2006). It is possible that this structural similarity enables mannose receptor-mediated responses to chitin particles. The innate immune signals involved in chitin recognition are nonetheless complex, and future studies are needed to determine the importance of each of these recognition molecules in innate immune responses to chitin-containing pathogens.

Innate Immune Effectors Program Adaptive Immunity

Although alveolar macrophages and neutrophils are critical for killing dormant or germinating conidia and hyphae, monocytes, NK cells, NKT cells, plasmacytoid DCs, and eosinophils may also provide early protection from infection (Morrison et al., 2003; Mircescu et al., 2009; Cohen et al., 2011; Ramirez-Ortiz et al., 2011; Espinosa et al., 2014; Lilly et al., 2014). Furthermore, cytokines produced by these cells are involved in the programming of protective or non-protective adaptive immune responses. In particular, CD4 (T-helper) and CD8 (cytotoxic) T cells provide significant protection from *A. fumigatus* infection and are therefore considered important targets for vaccination studies (Cenci et al., 2000; Perruccio et al., 2005; Chai et al., 2010; Romani, 2011; Carvalho et al., 2012). However, Th1 responses are the most protective, while Th2 responses result in poor disease outcomes. The level of protection conferred by Th17 cells and IL-17 is not clear, as conflicting studies reported impaired or enhanced early protection after antibody depletion of IL-17A (Zelante et al., 2007; Werner et al., 2009). In a model of fungal keratitis, IL-17A was protective, although the cellular sources of IL-17A attributed to this protection included neutrophils in addition to Th17 cells (Taylor et al., 2014). In addition to neutrophils, γδ T cells may also be an important source of IL-17A, particularly in the lung, although their role in protection is unclear and may be subset-dependent (Roark et al., 2008; Romani et al., 2008). NK and invariant NKT cells may be early sources of IFN-γ during infection (Bouzani et al., 2011; Cohen et al., 2011), while basophils or NKT cells may provide innate production of IL-4 in the development of allergy/Th2 responses (Taniguchi et al., 2003; Liang et al., 2012). Therefore, in addition to proinflammatory cytokines produced by innate cells, early production of T helper cytokines provides an early window into the subsequent development of protective or detrimental adaptive responses.

Perhaps the most consequential cell in initiating adaptive immunity to fungal infection is the DC (Romani, 2011; Wuthrich et al., 2012). Initiation of a protective adaptive immune response against *A. fumigatus* is partly dependent on the actions of DCs stimulated through activation of fungal PRRs. Monocytes recruited into the lung shortly after *A. fumigatus* infection differentiated into DCs that were critical for induction of Th1 responses that are increased in the absence of dectin-1 (Hohl et al., 2009; Rivera et al., 2011). Rather than promote Th1 responses, dectin-1 recognition induced Th17 responses to *A. fumigatus* (Werner et al., 2009; Rivera et al., 2011). Accordingly, direct stimulation of DCs with purified β-glucan stimulated TNFα, yet inhibited TLR-mediated induction of IL-12 (Huang et al., 2009). DC priming of Th2 responses was promoted by the epithelial cytokines TSLP and IL-33 that were also induced in epithelial cells by chitin stimulation (Paul and Zhu, 2010; Van Dyken et al., 2014). Chitin particles also induced generation of C3a in the lungs of mice that is required for DC stimulation of Th2 responses to *Aspergillus fumigatus* hyphal extracts (Roy et al., 2013). DCs thus respond to different fungal PAMPs with distinct cytokine profiles and differentially prime Th responses.

Co-recognition of β-glucan and Chitin and Programming of Adaptive Immunity

Although many studies have focused on responses to purified fungal PAMPs, actual responses to viable *A. fumigatus* are programmed as a result of co-recognition of multiple PAMPs by multiple PRRs after these ligands are revealed on the surface of germinating conidia. Furthermore, since soluble forms of these ligands are often inhibitory, it has been hypothesized that long fibrillar polysaccharide fungal PAMPs are able to bind to multiple PRRs, thus increasing activation signals in PRR-expressing cells (Latge, 2010). Recognition by multiple PRRs would also be facilitated by clustering formations within the immunological

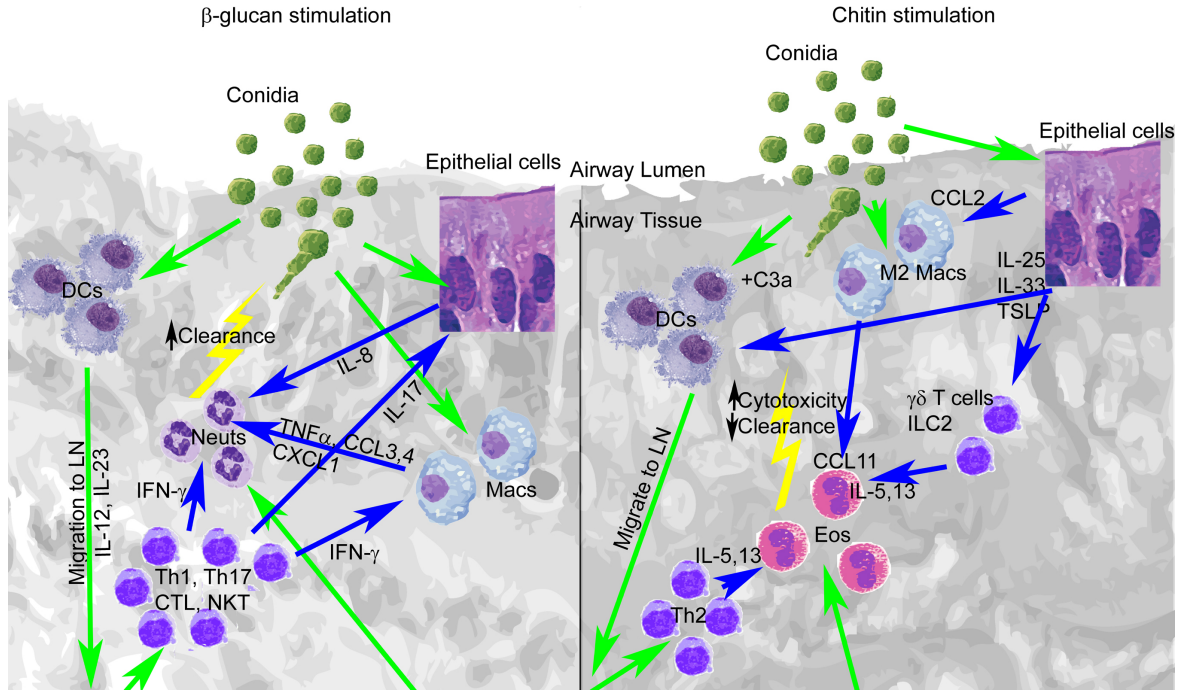


FIGURE 1 | Early lung recognition of β-glucan and chitin programs distinct profiles of cytokine secretion, leukocyte recruitment, and adaptive immunity. Left, β-glucan stimulation. Right, chitin stimulation. Recognition of germinating conidia or migration of cells is displayed with green arrows, while cytokine stimulation is shown with blue arrows.

synapse (Goodridge et al., 2011; Inoue and Shinohara, 2014). Results of studies that examined the effects of co-recognition of multiple PAMPs with mixtures of particles or in response to intact conidia provide a contrast to studies focused solely on responses to purified particles. For example, covalently-linked chitin-β-glucan particles induced neutrophil and eosinophil recruitment as well increased chitinase activity, TNF-α and TSLP production in mouse lungs (Dubey et al., 2014). Furthermore, multiple aspirations of viable *A. fumigatus* conidia activated Th1, Th2, and Th17 responses, and the relative expansion of these subsets may depend on the dose, frequency of aspirations, and strain characteristics of the conidia used (Fei et al., 2011; Murdock et al., 2011; O'Dea et al., 2014). Our laboratory identified an isolate of *A. fumigatus* (Af5517) that expressed increased levels of chitin and induced Th2-skewed immunity in the lungs of mice after repeated conidial aspiration (O'Dea et al., 2014). However, an isolate that we identified as relatively low chitin-expressing (Af13073) induced allergic sensitization when the frequency of aspiration was increased and the interval between aspirations was decreased (Fei et al., 2011; Lilly et al., 2012; Amarsaikhan et al., 2014). Interestingly, dectin-1−/− mice displayed increased eosinophil recruitment in response to single or multiple aspirations of *Aspergillus* conidia (Werner et al., 2011; Mintz-Cole et al., 2012). Although not discussed in either report, it is possible that co-recognition of β-glucan by dectin-1 may inhibit signals generated by chitin recognition and early programming of Th2 responses. However, this effect may be overcome by allergic sensitization, as dectin-1 deficient mice did not exhibit increased lung eosinophilia in a model of fungal asthma (Lilly et al., 2012). This is not surprising, considering other differences between models of exposure and sensitization. For example, chitinase expression promotes allergic inflammation in models of allergic sensitization, while in the absence of sensitization chitinase expression decreases eosinophil recruitment in response to chitin particles, fungal extracts, or conidia (Zhu et al., 2004; Reese et al., 2007; Van Dyken et al., 2011; O'Dea et al., 2014). Taken together, these results suggest that in the absence of sensitization, co-recognition of chitin and β-glucan may provide antagonistic signals that result in differential programming of adaptive immunity to *A. fumigatus*.

Fungal Stress, Cell Wall Modulation, and Consequences for Treatment of Infection

The clinical relevance of cell wall modulation is an important area of current and future investigation. Several lines of evidence suggest that stresses encountered by pathogenic fungi during infection alter the metabolism and cell wall architecture, and thus modulate immune responses toward non-protective programs of adaptive immunity. In the case of *A. fumigatus* and other fungal infection, Th2 immune responses inhibit protective immunity (Cenci et al., 1998, 1999, 2000; Wuthrich et al., 2012). Eosinophils may be partly responsible for this impairment, as Th2-responding mice that lacked eosinophils increased fungal clearance, although the mechanism for this inhibition remains unknown (O'Dea et al., 2014). Numerous reports have demonstrated alteration of fungal cell wall architecture in response to changes in growth conditions or environmental stresses encountered during infection, in both

A. fumigatus and *Candida albicans* (Ene et al., 2012; Shepardson et al., 2013; Beauvais et al., 2014). *A. fumigatus* growth under hypoxic conditions resulted in increased cell wall β-glucan and chitin that stimulated increased macrophage and neutrophil activation (Shepardson et al., 2013). More importantly, classes of antifungal drugs that directly target the synthesis of cell wall chitin and β-glucan modulate cell wall architecture over the course of infection, and these changes may concomitantly affect host pattern recognition and pathogen clearance. For example, echinocandins directly target the synthesis of β-glucan, while nikkomycins target chitin synthesis (Ostrosky-Zeichner et al., 2010). Moreover, growth of *A. fumigatus* in the presence of the echinocandin caspofungin resulted in increased cell wall chitin, while growth on nikkomycin Z increased β-glucan (Verwer et al., 2012). In a mouse model of *C. albicans* infection, increased cell wall chitin induced by caspofungin treatment mediated echinocandin resistance (Lee et al., 2012). Thus, cell wall modulation in response to the stresses of infection may influence the development of protective immunity and the efficacy of antifungal drug treatment.

Summary/Conclusion

Among the cell wall components of *A. fumigatus*, chitin and β-glucan may stimulate protective or detrimental immune responses, depending on their level of expression and recognition. Other cell wall components such as α-glucan, galactomannan, and GAG may also promote or inhibit the development of protective immunity, although their roles are less understood, and thus require further examination. Early cellular and cytokine signals induced by innate recognition of covalently linked β-glucan and chitin initiate Th1/Th17 or Th2 responses that may alter the balance between protective immunity and damaging inflammation (**Figure 1**). This co-recognition may be altered by pathogen mutation or in response to environmental stresses encountered during infection, particularly by exposure to antifungal drugs that directly target β-glucan or chitin synthesis. However, the consequences of changes in this recognition to protection from infection are not well understood. Future studies will be required to more completely define the development of protective immunity at the level of host-pathogen interaction, with the goal of introducing and validating new therapies that promote protection and/or target detrimental inflammatory processes that arise within the spectrum of *A. fumigatus*-associated disease.

Acknowledgment

NA is supported by an American Association of Immunologists Careers in Immunology Fellowship.

References

Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S. R., et al. (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 460, 1117–1121. doi: 10.1038/nature08264

Amarsaikhan, N., O'dea, E. M., Tsoggerel, A., Owegi, H., Gillenwater, J., and Templeton, S. P. (2014). Isolate-dependent growth, virulence, and cell wall composition in the human pathogen *Aspergillus fumigatus*. *PLoS ONE* 9:e100430. doi: 10.1371/journal.pone.0100430

Beauvais, A., Fontaine, T., Aimanianda, V., and Latge, J. P. (2014). *Aspergillus* cell wall and biofilm. *Mycopathologia* 178, 371–377. doi: 10.1007/s11046-014-9766-0

Bonnett, C. R., Cornish, E. J., Harmsen, A. G., and Burritt, J. B. (2006). Early neutrophil recruitment and aggregation in the murine lung inhibit germination of *Aspergillus fumigatus* Conidia. *Infect. Immun.* 74, 6528–6539. doi: 10.1128/IAI.00909-06

Bouzani, M., Ok, M., Mccormick, A., Ebel, F., Kurzai, O., Morton, C. O., et al. (2011). Human NK cells display important antifungal activity against *Aspergillus fumigatus*, which is directly mediated by IFN-gamma release. *J. Immunol.* 187, 1369–1376. doi: 10.4049/jimmunol.1003593

Bozza, S., Clavaud, C., Giovannini, G., Fontaine, T., Beauvais, A., Sarfati, J., et al. (2009). Immune sensing of *Aspergillus fumigatus* proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination. *J. Immunol.* 183, 2407–2414. doi: 10.4049/jimmunol.0900961

Caffrey, A. K., Lehmann, M. M., Zickovich, J. M., Espinosa, V., Shepardson, K. M., Watschke, C. P., et al. (2015). IL-1α signaling is critical for leukocyte recruitment after pulmonary *Aspergillus fumigatus* challenge. *PLoS Pathog.* 11:e1004625. doi: 10.1371/journal.ppat.1004625

Carvalho, A., De Luca, A., Bozza, S., Cunha, C., D'angelo, C., Moretti, S., et al. (2012). TLR3 essentially promotes protective class I-restricted memory CD8 T-cell responses to *Aspergillus fumigatus* in hematopoietic transplanted patients. *Blood* 119, 967–977. doi: 10.1182/blood-2011-06-362582

Cash, H. L., Whitham, C. V., Behrendt, C. L., and Hooper, L. V. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313, 1126–1130. doi: 10.1126/science.1127119

Cenci, E., Mencacci, A., Bacci, A., Bistoni, F., Kurup, V. P., and Romani, L. (2000). T cell vaccination in mice with invasive pulmonary aspergillosis. *J. Immunol.* 165, 381–388. doi: 10.4049/jimmunol.165.1.381

Cenci, E., Mencacci, A., Del Sero, G., Bacci, A., Montagnoli, C., D'ostiani, C. F., et al. (1999). Interleukin-4 causes susceptibility to invasive pulmonary aspergillosis through suppression of protective type I responses. *J. Infect. Dis.* 180, 1957–1968. doi: 10.1086/315142

Cenci, E., Mencacci, A., Fe D'ostiani, C., Del Sero, G., Mosci, P., Montagnoli, C., et al. (1998). Cytokine- and T helper-dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. *J. Infect. Dis.* 178, 1750–1760. doi: 10.1086/314493

Chai, L. Y., Van De Veerdonk, F., Marijnissen, R. J., Cheng, S. C., Khoo, A. L., Hectors, M., et al. (2010). Anti-*Aspergillus* human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity. *Immunology* 130, 46–54. doi: 10.1111/j.1365-2567.2009.03211.x

Chai, L. Y. A., Vonk, A. G., Kullberg, B. J., Verweij, P. E., Verschueren, I., Van Der Meer, J. W. M., et al. (2011). *Aspergillus fumigatus* cell wall components differentially modulate host TLR2 and TLR4 responses. *Microbes Infect.* 13, 151–159. doi: 10.1016/j.micinf.2010.10.005

Cohen, N. R., Tatituri, R. V., Rivera, A., Watts, G. F., Kim, E. Y., Chiba, A., et al. (2011). Innate recognition of cell wall β-glucans drives invariant natural killer T cell responses against fungi. *Cell Host Microbe* 10, 437–450. doi: 10.1016/j.chom.2011.09.011

Cunha, C., Di Ianni, M., Bozza, S., Giovannini, G., Zagarella, S., Zelante, T., et al. (2010). Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. *Blood* 116, 5394–5402. doi: 10.1182/blood-2010-04-279307

Da Silva, C. A., Chalouni, C., Williams, A., Hartl, D., Lee, C. G., and Elias, J. A. (2009). Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J. Immunol.* 182, 3573–3582. doi: 10.4049/jimmunol.0802113

Da Silva, C. A., Hartl, D., Liu, W., Lee, C. G., and Elias, J. A. (2008). TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation. *J. Immunol.* 181, 4279–4286. doi: 10.4049/jimmunol.181.6.4279

Da Silva, C. A., Pochard, P., Lee, C. G., and Elias, J. A. (2010). Chitin particles are multifaceted immune adjuvants. *Am. J. Respir. Crit. Care Med.* 182, 1482–1491. doi: 10.1164/rccm.200912-1877OC

Dennehy, K. M., Ferwerda, G., Faro-Trindade, I., Pyz, E., Willment, J. A., Taylor, P. R., et al. (2008). Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur. J. Immunol.* 38, 500–506. doi: 10.1002/eji.200737741

Drickamer, K. (1992). Engineering galactose-binding activity into a C-type mannose-binding protein. *Nature* 360, 183–186. doi: 10.1038/360183a0

Drummond, R. A., and Brown, G. D. (2011). The role of Dectin-1 in the host defence against fungal infections. *Curr. Opin. Microbiol.* 14, 392–399. doi: 10.1016/j.mib.2011.07.001

Dubey, L. K., Moeller, J. B., Schlosser, A., Sorensen, G. L., and Holmskov, U. (2014). Induction of innate immunity by *Aspergillus fumigatus* cell wall polysaccharides is enhanced by the composite presentation of chitin and β-glucan. *Immunobiology* 219, 179–188. doi: 10.1016/j.imbio.2013.10.003

Ene, I. V., Adya, A. K., Wehmeier, S., Brand, A. C., Maccallum, D. M., Gow, N. A., et al. (2012). Host carbon sources modulate cell wall architecture, drug resistance and virulence in a fungal pathogen. *Cell. Microbiol.* 14, 1319–1335. doi: 10.1111/j.1462-5822.2012.01813.x

Espinosa, V., Jhingran, A., Dutta, O., Kasahara, S., Donnelly, R., Du, P., et al. (2014). Inflammatory monocytes orchestrate innate antifungal immunity in the lung. *PLoS Pathog.* 10:e1003940. doi: 10.1371/journal.ppat.1003940

Evans, S. E., Scott, B. L., Clement, C. G., Larson, D. T., Kontoyiannis, D., Lewis, R. E., et al. (2010). Stimulated innate resistance of lung epithelium protects mice broadly against bacteria and fungi. *Am. J. Respir. Cell Mol. Biol.* 42, 40–50. doi: 10.1165/rcmb.2008-0260OC

Fei, M., Bhatia, S., Oriss, T. B., Yarlagadda, M., Khare, A., Akira, S., et al. (2011). TNF-α from inflammatory dendritic cells (DCs) regulates lung IL-17A/IL-5 levels and neutrophilia versus eosinophilia during persistent fungal infection. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5360–5365. doi: 10.1073/pnas.1015476108

Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003). Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197, 1107–1117. doi: 10.1084/jem.20021787

Gersuk, G. M., Underhill, D. M., Zhu, L., and Marr, K. A. (2006). Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. *J. Immunol.* 176, 3717–3724. doi: 10.4049/jimmunol.176.6.3717

Gessner, M. A., Werner, J. L., Lilly, L. M., Nelson, M. P., Metz, A. E., Dunaway, C. W., et al. (2012). Dectin-1-dependent interleukin-22 contributes to early innate lung defense against *Aspergillus fumigatus*. *Infect. Immun.* 80, 410–417. doi: 10.1128/IAI.05939-11

Goodridge, H. S., Reyes, C. N., Becker, C. A., Katsumoto, T. R., Ma, J., Wolf, A. J., et al. (2011). Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. *Nature* 472, 471–475. doi: 10.1038/nature10071

Gravelat, F. N., Beauvais, A., Liu, H., Lee, M. J., Snarr, B. D., Chen, D., et al. (2013). *Aspergillus* galactosaminogalactan mediates adherence to host constituents and conceals hyphal β-glucan from the immune system. *PLoS Pathog.* 9:e1003575. doi: 10.1371/journal.ppat.1003575

Heinekamp, T., Schmidt, H., Lapp, K., Pahtz, V., Shopova, I., Koster-Eiserfunke, N., et al. (2015). Interference of *Aspergillus fumigatus* with the immune response. *Semin. Immunopathol.* 37, 141–152. doi: 10.1007/s00281-014-0465-1

Hohl, T. M., and Feldmesser, M. (2007). *Aspergillus fumigatus*: principles of pathogenesis and host defense. *Eukaryot. Cell* 6, 1953–1963. doi: 10.1128/EC.00274-07

Hohl, T. M., Rivera, A., Lipuma, L., Gallegos, A., Shi, C., Mack, M., et al. (2009). Inflammatory monocytes facilitate adaptive CD4 T cell responses during respiratory fungal infection. *Cell Host Microbe* 6, 470–481. doi: 10.1016/j.chom.2009.10.007

Hohl, T. M., Van Epps, H. L., Rivera, A., Morgan, L. A., Chen, P. L., Feldmesser, M., et al. (2005). *Aspergillus fumigatus* triggers inflammatory responses by stage-specific β-glucan display. *PLoS Pathog.* 1:e30. doi: 10.1371/journal.ppat.0010030

Huang, H., Ostroff, G. R., Lee, C. K., Wang, J. P., Specht, C. A., and Levitz, S. M. (2009). Distinct patterns of dendritic cell cytokine release stimulated by fungal β-glucans and toll-like receptor agonists. *Infect. Immun.* 77, 1774–1781. doi: 10.1128/IAI.00086-09

Inoue, M., and Shinohara, M. L. (2014). Clustering of pattern recognition receptors for fungal detection. *PLoS Pathog.* 10:e1003873. doi: 10.1371/journal.ppat.1003873

Islam, S. A., and Luster, A. D. (2012). T cell homing to epithelial barriers in allergic disease. *Nat. Med.* 18, 705–715. doi: 10.1038/nm.2760

Iwakura, Y., Ishigame, H., Saijo, S., and Nakae, S. (2011). Functional specialization of interleukin-17 family members. *Immunity* 34, 149–162. doi: 10.1016/j.immuni.2011.02.012

Jhingran, A., Kasahara, S., Shepardson, K. M., Junecko, B. A., Heung, L. J., Kumasaka, D. K., et al. (2015). Compartment-specific and sequential role of MyD88 and CARD9 in chemokine induction and innate defense during respiratory fungal infection. *PLoS Pathog.* 11:e1004589. doi: 10.1371/journal.ppat.1004589

Kennedy, A. D., Willment, J. A., Dorward, D. W., Williams, D. L., Brown, G. D., and Deleo, F. R. (2007). Dectin-1 promotes fungicidal activity of human neutrophils. *Eur. J. Immunol.* 37, 467–478. doi: 10.1002/eji.200636653

Kogiso, M., Nishiyama, A., Shinohara, T., Nakamura, M., Mizoguchi, E., Misawa, Y., et al. (2011). Chitin particles induce size-dependent but carbohydrate-independent innate eosinophilia. *J. Leukoc. Biol.* 90, 167–176. doi: 10.1189/jlb.1110624

Kwon-Chung, K. J., and Sugui, J. A. (2013). *Aspergillus fumigatus*—what makes the species a ubiquitous human fungal pathogen? *PLoS Pathog.* 9:e1003743. doi: 10.1371/journal.ppat.1003743

Latgé, J. P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12, 310–350.

Latge, J. P. (2010). Tasting the fungal cell wall. *Cell. Microbiol.* 12, 863–872. doi: 10.1111/j.1462-5822.2010.01474.x

Latge, J. P., and Beauvais, A. (2014). Functional duality of the cell wall. *Curr. Opin. Microbiol.* 20, 111–117. doi: 10.1016/j.mib.2014.05.009

Lee, K. K., Maccallum, D. M., Jacobsen, M. D., Walker, L. A., Odds, F. C., Gow, N. A., et al. (2012). Elevated cell wall chitin in Candida albicans confers echinocandin resistance *in vivo*. *Antimicrob. Agents Chemother.* 56, 208–217. doi: 10.1128/AAC.00683-11

Lenardon, M. D., Munro, C. A., and Gow, N. A. (2010). Chitin synthesis and fungal pathogenesis. *Curr. Opin. Microbiol.* 13, 416–423. doi: 10.1016/j.mib.2010.05.002

Liang, H. E., Reinhardt, R. L., Bando, J. K., Sullivan, B. M., Ho, I. C., and Locksley, R. M. (2012). Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat. Immunol.* 13, 58–66. doi: 10.1038/ni.2182

Lilly, L. M., Gessner, M. A., Dunaway, C. W., Metz, A. E., Schwiebert, L., Weaver, C. T., et al. (2012). The β-glucan receptor Dectin-1 promotes lung immunopathology during fungal allergy via IL-22. *J. Immunol.* 189, 3653–3660. doi: 10.4049/jimmunol.1201797

Lilly, L. M., Scopel, M., Nelson, M. P., Burg, A. R., Dunaway, C. W., and Steele, C. (2014). Eosinophil deficiency compromises lung defense against *Aspergillus fumigatus*. *Infect. Immun.* 82, 1315–1325. doi: 10.1128/IAI.01172-13

Mambula, S. S., Sau, K., Henneke, P., Golenbock, D. T., and Levitz, S. M. (2002). Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. *J. Biol. Chem.* 277, 39320–39326. doi: 10.1074/jbc.M201683200

Mezger, M., Kneitz, S., Wozniok, I., Kurzai, O., Einsele, H., and Loeffler, J. (2008). Proinflammatory response of immature human dendritic cells is mediated by dectin-1 after exposure to *Aspergillus fumigatus* germ tubes. *J. Infect. Dis.* 197, 924–931. doi: 10.1086/528694

Mintz-Cole, R. A., Gibson, A. M., Bass, S. A., Budelsky, A. L., Reponen, T., and Hershey, G. K. (2012). Dectin-1 and IL-17A suppress murine asthma induced by *Aspergillus versicolor* but not *Cladosporium cladosporioides* due to differences in β-glucan surface exposure. *J. Immunol.* 189, 3609–3617. doi: 10.4049/jimmunol.1200589

Mircescu, M. M., Lipuma, L., Van Rooijen, N., Pamer, E. G., and Hohl, T. M. (2009). Essential role for neutrophils but not alveolar macrophages at early time points following *Aspergillus fumigatus* infection. *J. Infect. Dis.* 200, 647–656. doi: 10.1086/600380

Morrison, B. E., Park, S. J., Mooney, J. M., and Mehrad, B. (2003). Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. *J. Clin. Invest.* 112, 1862–1870. doi: 10.1172/JCI18125

Murdock, B. J., Shreiner, A. B., Mcdonald, R. A., Osterholzer, J. J., White, E. S., Toews, G. B., et al. (2011). Coevolution of TH1, TH2, and TH17 responses during repeated pulmonary exposure to *Aspergillus fumigatus* conidia. *Infect. Immun.* 79, 125–135. doi: 10.1128/IAI.00508-10

Muzzarelli, R. A. A. (2010). Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Marine Drugs* 8, 292–312. doi: 10.3390/md8020292

O'Dea, E. M., Amarsaikhan, N., Li, H., Downey, J., Steele, E., Van Dyken, S. J., et al. (2014). Eosinophils are recruited in response to chitin exposure and enhance Th2-mediated immune pathology in *Aspergillus fumigatus* infection. Infect Immun. 82, 3199–3205. doi: 10.1128/IAI.01990-14

Osherov, N. (2012). Interaction of the pathogenic mold *Aspergillus fumigatus* with lung epithelial cells. *Front. Microbiol.* 3:346. doi: 10.3389/fmicb.2012.00346

Ostrosky-Zeichner, L., Casadevall, A., Galgiani, J. N., Odds, F. C., and Rex, J. H. (2010). An insight into the antifungal pipeline: selected new molecules and beyond. *Nat. Rev. Drug Discov.* 9, 719–727. doi: 10.1038/nrd3074

Paris, S., Debeaupuis, J. P., Crameri, R., Carey, M., Charles, F., Prevost, M. C., et al. (2003). Conidial hydrophobins of *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 69, 1581–1588. doi: 10.1128/AEM.69.3.1581-1588.2003

Park, S. J., Hughes, M. A., Burdick, M., Strieter, R. M., and Mehrad, B. (2009). Early NK cell-derived IFN-{gamma} is essential to host defense in neutropenic invasive aspergillosis. *J. Immunol.* 182, 4306–4312. doi: 10.4049/jimmunol.0803462

Paul, W. E., and Zhu, J. F. (2010). How are T(H)2-type immune responses initiated and amplified? *Nat. Rev. Immunol.* 10, 225–235. doi: 10.1038/nri2735

Perruccio, K., Tosti, A., Burchielli, E., Topini, F., Ruggeri, L., Carotti, A., et al. (2005). Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. *Blood* 106, 4397–4406. doi: 10.1182/blood-2005-05-1775

Ramirez-Ortiz, Z. G., Lee, C. K., Wang, J. P., Boon, L., Specht, C. A., and Levitz, S. M. (2011). A nonredundant role for plasmacytoid dendritic cells in host defense against the human fungal pathogen *Aspergillus fumigatus*. *Cell Host Microbe* 9, 415–424. doi: 10.1016/j.chom.2011.04.007

Reese, T. A., Liang, H. E., Tager, A. M., Luster, A. D., Van Rooijen, N., Voehringer, D., et al. (2007). Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 447, 92–96. doi: 10.1038/nature05746

Rivera, A., Hohl, T. M., Collins, N., Leiner, I., Gallegos, A., Saijo, S., et al. (2011). Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. *J. Exp. Med.* 208, 369–381. doi: 10.1084/jem.20100906

Roark, C. L., Simonian, P. L., Fontenot, A. P., Born, W. K., and O'brien, R. L. (2008). gammadelta T cells: an important source of IL-17. *Curr. Opin. Immunol.* 20, 353–357. doi: 10.1016/j.coi.2008.03.006

Roilides, E., Uhlig, K., Venzon, D., Pizzo, P. A., and Walsh, T. J. (1993). Enhancement of oxidative response and damage caused by human neutrophils to *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. *Infect. Immun.* 61, 1185–1193.

Romani, L. (2011). Immunity to fungal infections. *Nat. Rev. Immunol.* 11, 275–288. doi: 10.1038/nri2939

Romani, L., Fallarino, F., De Luca, A., Montagnoli, C., D'angelo, C., Zelante, T., et al. (2008). Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature* 451, 211–215. doi: 10.1038/nature06471

Roy, R. M., Paes, H. C., Nanjappa, S. G., Sorkness, R., Gasper, D., Sterkel, A., et al. (2013). Complement component 3C3 and C3a receptor are required in chitin-dependent allergic sensitization to *Aspergillus fumigatus* but dispensable in chitin-induced innate allergic inflammation. *mBio* 4, pii: e00162-13. doi: 10.1128/mBio.00162-13

Roy, R. M., Wuthrich, M., and Klein, B. S. (2012). Chitin elicits CCL2 from airway epithelial cells and induces CCR2-dependent innate allergic inflammation in the lung. *J. Immunol.* 189, 2545–2552. doi: 10.4049/jimmunol.1200689

Schlosser, A., Thomsen, T., Moeller, J. B., Nielsen, O., Tornoe, I., Mollenhauer, J., et al. (2009). Characterization of FIBCD1 as an acetyl group-binding receptor that binds chitin. *J. Immunol.* 183, 3800–3809. doi: 10.4049/jimmunol.0901526

Shepardson, K. M., Jhingran, A., Caffrey, A., Obar, J. J., Suratt, B. T., Berwin, B. L., et al. (2014). Myeloid derived hypoxia inducible factor 1-α is required for protection against pulmonary *Aspergillus fumigatus* infection. *PLoS Pathog.* 10:e1004378. doi: 10.1371/journal.ppat.1004378

Shepardson, K. M., Ngo, L. Y., Aimanianda, V., Latge, J. P., Barker, B. M., Blosser, S. J., et al. (2013). Hypoxia enhances innate immune activation to *Aspergillus fumigatus* through cell wall modulation. *Microbes Infect.* 15, 259–269. doi: 10.1016/j.micinf.2012.11.010

Shibata, Y., Metzger, W. J., and Myrvik, Q. N. (1997). Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *J. Immunol.* 159, 2462–2467.

Steele, C., Rapaka, R. R., Metz, A., Pop, S. M., Williams, D. L., Gordon, S., et al. (2005). The β-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathog.* 1:e42. doi: 10.1371/journal.ppat.0010042

Sun, W. K., Lu, X., Li, X., Sun, Q. Y., Su, X., Song, Y., et al. (2012). Dectin-1 is inducible and plays a crucial role in *Aspergillus*-induced innate immune responses in human bronchial epithelial cells. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 2755–2764. doi: 10.1007/s10096-012-1624-8

Taniguchi, M., Harada, M., Kojo, S., Nakayama, T., and Wakao, H. (2003). The regulatory role of Vα14 NKT cells in innate and acquired immune response. *Annu. Rev. Immunol.* 21, 483–513. doi: 10.1146/annurev.immunol.21.120601.141057

Taylor, P. R., Leal, S. M. Jr., Sun, Y., and Pearlman, E. (2014). *Aspergillus* and Fusarium corneal infections are regulated by Th17 cells and IL-17-producing neutrophils. *J. Immunol.* 192, 3319–3327. doi: 10.4049/jimmunol.1302235

Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., Latge, J. P., et al. (1994). Rodletless mutants of *Aspergillus fumigatus*. *Infect. Immun.* 62, 4380–4388.

Van Dyken, S. J., Garcia, D., Porter, P., Huang, X., Quinlan, P. J., Blanc, P. D., et al. (2011). Fungal chitin from asthma-associated home environments induces eosinophilic lung infiltration. *J. Immunol.* 187, 2261–2267. doi: 10.4049/jimmunol.1100972

Van Dyken, S. J., Mohapatra, A., Nussbaum, J. C., Molofsky, A. B., Thornton, E. E., Ziegler, S. F., et al. (2014). Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and gammadelta T cells. *Immunity* 40, 414–424. doi: 10.1016/j.immuni.2014.02.003

Verwer, P. E., Van Duijn, M. L., Tavakol, M., Bakker-Woudenberg, I. A., and Van De Sande, W. W. (2012). Reshuffling of *Aspergillus fumigatus* cell wall components chitin and β-glucan under the influence of caspofungin or nikkomycin Z alone or in combination. *Antimicrob. Agents Chemother.* 56, 1595–1598. doi: 10.1128/AAC.05323-11

Wagener, J., Malireddi, R. K., Lenardon, M. D., Koberle, M., Vautier, S., Maccallum, D. M., et al. (2014). Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. *PLoS Pathog.* 10:e1004050. doi: 10.1371/journal.ppat.1004050

Werner, J. L., Gessner, M. A., Lilly, L. M., Nelson, M. P., Metz, A. E., Horn, D., et al. (2011). Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection. *Infect. Immun.* 79, 3966–3977. doi: 10.1128/IAI.05493-11

Werner, J. L., Metz, A. E., Horn, D., Schoeb, T. R., Hewitt, M. M., Schwiebert, L. M., et al. (2009). Requisite role for the dectin-1 β-glucan receptor in pulmonary defense against *Aspergillus fumigatus*. *J. Immunol.* 182, 4938–4946. doi: 10.4049/jimmunol.0804250

Wuthrich, M., Deepe, G. S. Jr., and Klein, B. (2012). Adaptive immunity to fungi. *Annu. Rev. Immunol.* 30, 115–148. doi: 10.1146/annurev-immunol-020711-074958

Zelante, T., De Luca, A., Bonifazi, P., Montagnoli, C., Bozza, S., Moretti, S., et al. (2007). IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* 37, 2695–2706. doi: 10.1002/eji.200737409

Zhu, Z., Zheng, T., Homer, R. J., Kim, Y. K., Chen, N. Y., Cohn, L., et al. (2004). Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 304, 1678–1682. doi: 10.1126/science.1095336

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Amarsaikhan and Templeton. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

MINI REVIEW
published: 11 May 2015
doi: 10.3389/fmicb.2015.00429

Cytokines induce effector T-helper cells during invasive aspergillosis; what we have learned about T-helper cells?

Raman Thakur[1], *Rajesh Anand*[2], *Shraddha Tiwari*[1], *Agam P. Singh*[2], *Bhupendra N. Tiwary*[3] *and Jata Shankar*[1]*

[1] Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, India, [2] Infectious Diseases Laboratory, National Institute of Immunology, New Delhi, India, [3] Department of Biotechnology, Guru Ghasidas Vishwavidyalaya, Bilaspur, India

OPEN ACCESS

Edited by:
Frederic Lamoth, Lausanne University Hospital, Switzerland

Reviewed by:
Nina Khanna, University of Basel, Switzerland
Lars Tramsen, University Hospital Frankfurt, Germany

***Correspondence:**
Jata Shankar, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan 173234, Himachal Pradesh, India
jata.shankar@juit.ac.in, jata_s@yahoo.com

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: 02 January 2015
Accepted: 22 April 2015
Published: 11 May 2015

Citation:
Thakur R, Anand R, Tiwari S, Singh AP, Tiwary BN and Shankar J (2015) Cytokines induce effector T-helper cells during invasive aspergillosis; what we have learned about T-helper cells? Front. Microbiol. 6:429. doi: 10.3389/fmicb.2015.00429

Invasive aspergillosis caused by *Aspergillus* species (*Aspergillus fumigatus*, *A. flavus*, and *A. terreus*) is life-threatening infections in immunocompromised patients. Understanding the innate and adaptive immune response particularly T-helper cells (T_H-cells) against these *Aspergillus* species and how the different sub-set of T_H-cells are regulated by differentiating cytokines at primary target organ site like lung, kidney and brain is of great significance to human health. This review focuses on presentation of *Aspergillus* through Antigen presenting cells (APCs) to the naive $CD4^+$ T-cells in the host. The production of differentiating/effector cytokines that activate following T_H-cells, e.g., T_H1, T_H2, T_H9, and T_H17 has been reported in association or alone in allergic or invasive aspergillosis. Chemokines (CXCL1, CXCL2, CCL1, and CCL20) and their receptors associated to these T_H-cells have also been observed in invasive aspergillosis. Thus, further study of these T_H-cells in invasive aspergillosis and other elements of adaptive immune response with *Aspergillus* species are required in order to have a better understanding of host response for safer and effective therapeutic outcome.

Keywords: cytokines, T-helper cells, dendritic cells, *Aspergillus*, antigen presenting cells, invasive aspergillosis

Introduction

Fungi are the most common microorganisms and have clinical importance. Few of them are pathogenic or opportunistic pathogen and results in morbidity and mortality to human beings. There is a rise in opportunistic fungal infections in recent years due to increased incidence of immunocompromised host (Chamilos et al., 2006; Romani, 2008). After *Candida albicans*, the leading causes of fungal infections in immunocompromised individuals are from *Aspergillus* species. *Aspergillus* is one of the most ubiquitous medically important opportunistic fungi (Weaver et al., 2007). The genus *Aspergillus*, contains about 40 species that can cause infection (Verweij and Brandt, 2007), among them *A. fumigatus*, *A. flavus*, and *A. terreus* are the leading cause of invasive Aspergillosis in immunocompromised individuals. These species produce conidia at a concentration of around 1–100 conidia per m^3 (Barnes and Marr, 2006). Human routinely inhale hundreds of these conidia per day, despite these exposure to *Aspergillus* conidia, human do not develop any disease due to the clearance of conidia from lung by innate immunity especially phagocytic cells (Chamilos et al., 2006; Romani, 2008). However, due to rise in immunocompromised host, e.g., patients receiving organ

transplant, immunosuppressive therapy for autoimmune or neoplastic disease and HIV patients, inhaled conidia if not cleared in these host, colonization of *Aspergillus* occurs (Stevens et al., 2000). The adaptive immune response in human responsible for conidia clearance is not well understood in immunocompetent host as well as where conidia colonize in immunocompromised host. It is worth to note that secondary metabolites (e.g., Gliotoxins, Aflatoxins) excreted by *Aspergillus* especially have been recognized to modulate immunological responses (Shankar, 2013). Thus, we reviewed recent advances made in immune responses against *Aspergillus* species in mice model studies and clinical aspergillosis patients. *A. fumigatus* is the prominent species, which cause 90% of Aspergillosis followed by *A. flavus* and *A. terreus*. Studies have showed that *Aspergillus* species associated with infection after hematopoietic stem cell transplantation include *A. fumigatus* with 56% followed by *A. flavus* (18.7%) and *A. terreus* (16%) (Steinbach et al., 2004; Morgan et al., 2005). The involvement of *Aspergillus* infection in pulmonary tuberculosis and in asthmatic patients has been reported by Denning et al. It has been estimated annually at least 372,385 patients developed chronic pulmonary aspergillosis worldwide following treated pulmonary tuberculosis (Denning et al., 2011). Similarly, around 4,837,000 patients develop Allergic bronchopulmonary aspergillosis out of 193 million adults with active asthma (Denning et al., 2013). However, in a recent study of Indian population by Agarwal et al. (2014) the estimated ABPA burden was 1.38 million out of 27.6 million adults with asthma. This review touches different aspect of antifungal immunity against aspergillosis that include antigen presenting cells (APCs), dendritic cells (DCs), fungal pattern recognition receptors (PRRs), T_H-cells with their subsets profile during infection associated to *Aspergillus* species at different site of infection, e.g., lung, kidney, and brain.

Recognition of *Aspergillus* by the Host

Presentation of Pathogen via Soluble Receptors and Cell Bound Receptors

After the inhalation of *A. fumigatus* conidia, they are entrapped by the lung alveoli and if they are not efficiently cleared from lung, they germinate and establish lung infection termed invasive pulmonary aspergillosis and it may also disseminates to other organs if not treated (Park and Mehrad, 2009). The recognition of *A. fumigatus* conidia and hyphae occurs by PRRs those include soluble receptors and cell-bound receptors. Conidial germination starts with hydrophobic layer degradation and exposure of inner cell wall components mainly polysaccharides, which includes chitin, β-glucan, mannan, and galactomannan. These are termed as pathogen associated molecular patterns (PAMP), are recognized by PRRs (Netea et al., 2006; Inoue and Shinohara, 2014). PRRs soluble receptor such as pulmonary collectins, family of C-type lectins, pentraxin-3, pulmonary surfactant proteins-A and D have been reported in aspergillosis. Further, the cell-bound receptors in association with aspergillosis include Toll like receptor-2 (TLR), TLR-4 and TLR-9, which potentially induce the production of pro-inflammatory cytokines and reactive oxygen species through MyD88 signaling pathway (Willment and Brown, 2008).

Antigen Presenting Cells and T-cell Differentiation

Antigen Presenting Cells Triggers Cytokines Production

The activation of the innate immunity through PRRs present on the APCs that regulate the development of T-cell. APCs express wide-array of PRRs that provides the link between adaptive and innate immunity (Park and Mehrad, 2009). APCs, dominantly DCs, are responsible for antigen monitoring and then shaping T-cell response by secreting cytokines and chemokines. DCs express PRRs on their cell surface and endosomal compartments, which serve to recognize PAMPs. After interaction with DCs, naive T-cells are activated. The activation of T-cell response is regulated by the cytokines milieu predominantly framed by DCs (Akdis et al., 2011). Chemokines secreted by DCs recruit the phagocytic cells to infected areas to clear the *Aspergillus* components. APC cells, e.g., monocytes differentiate into distinct sub-populations of $CD14^+$ and $CD16^+$ cells after *A. fumigatus* conidia infection (Serbina et al., 2009). Monocytes interact with *Aspergillus* antigens resulting in maturation of monocytes into macrophages or DCs. Macrophages and DCs interact with antigens and secrete effector cytokines (Osugi et al., 2002; Ramirez-Ortiz and Means, 2012). Major sub-populations of DCs are myeloid DCs, plasmacytoid DC (pDCs) and monocyte-derived DCs (Bozza et al., 2002; Osugi et al., 2002). pDC recognize the nucleic acids from *A. fumigatus* via TLR-9 and lead to resistance to *A. fumigatus* infection in mice (Ramirez-Ortiz et al., 2008). Further, monocytes migrate toward the lung to differentiate into either DCs or alveolar macrophages during invasive aspergillosis (Cramer et al., 2011; Morton et al., 2012). Monocytes express different chemokines receptor predominantly CCR2, which help in migration of monocytes from bone marrow toward lung in response to *A. fumigatus* infection (Serbina et al., 2009). It has been shown that monocytes expressing CCR2 in the lung involved in conidial uptake and killing (Espinosa et al., 2014). Furthermore, alveolar macrophages induce APCs to release IL -1β in pulmonary invasive infection (Park and Mehrad, 2009). IL-18 has also been observed in lung during invasive aspergillosis mice model (Akdis et al., 2011). Recently, it has been observed that *A. fumigatus* pulmonary challenge induces expression of the inflammasome-dependent cytokines IL-1β and IL-18 within the first 12 h, while IL-1α expression continually increases over at least the first 48 h (Caffrey et al., 2015). Moretti et al. (2014) showed in a pulmonary invasive aspergillosis model that mice injected with IL-37 prior to *A. fumigatus* infection has significant reduction in IL-β production and recruitment of neutrophils and resulted in diminution in lung inflammation and damage. The anti-inflammatory activity of IL-37 has been observed as an inhibitor of the innate response. Thus, cytokines play a vital role in modulation of immune response and coordinate the innate as well as adaptive responses. APCs secrete cytokines that act on naïve T-cells leading to the differentiation of naïve T-cells. These differentiated T-cells further secrete effector cytokines and regulate the function of T_H-cells. The profile of cytokine depends on the type of *Aspergillus* antigens, route of infection, immunological status of the host and cytokines milieu present during the interaction (Romani, 2008; Chai et al., 2010b). $CD4^+$

T-cells can be divided into distinct subtypes according to cytokine profile, and they can differentiate to T_H1, T_H2 T_H17, T_H9, and T-follicular effector cells (Kerzerho et al., 2013; Kara et al., 2014). On the basis of the cytokine profile, these T_H-cells perform distinct functions. However, it is not clear how T-follicular effector cells respond during *Aspergillus* infection (Wüthrich et al., 2012).

Cytokines Associated with T_H1 Type of Response

Aspergillus fumigatus challenged intranasally in mice interacts with DCs and alveolar macrophages in the lung. Secretion of T_H1 associated pro-inflammatory cytokines IL-12, IFN-γ, TNF-α, IL-18 has been observed after the challenge (Chotirmall et al., 2013). Among these, IL-12 is the prominent cytokine released from activated monocytes and macrophages in lung that help in shaping T-cell immune response. IL-12 is a heterodimeric cytokine composed of IL-12p35 and IL-12p40 polypeptides that form the bioactive IL-12p70. The heterodimer binds to the IL-12 receptor composed of IL-12Rβ1 and IL-12Rβ2 chains and signals through STAT-4 (Shao et al., 2005). IL-12 acts on NK cells to promote IFN-γ secretion and differentiate $CD4^+$ T-cells into T_H1-cells, once $CD4^+$ cells differentiates to T_H1-cells, they increase the secretion of IFN-γ, which suppress T_H17 and T_H2 response in the lung (Espinosa and Rivera, 2012; Camargo and Husain, 2014). IL-12, hence, is the most important regulator of T_H1 response during lung infection. IL-12 deficient mice failed to generate a T_H1 response, leading to increased secretion of IL-4 and IL-10 cytokines, which shifts the immune response toward T_H2 pathway (Cenci et al., 1998). In *A. fumigatus* induced neutropenic aspergillosis in mice, NK cells can be the primary source of IFN-γ responsible for activating phagocytic cells and direct antifungal effectors cells against *A. fumigatus* (Park et al., 2009). Further, patients with invasive *Candida* and/or *Aspergillus* infections, recombinant treatment of IFN-γ in combination with antifungal drug partially restored immune function (Delsing et al., 2014). In intravenous infection of *A. flavus* mice model studies, lung and brain homogenate showed pro-inflammatory cytokines IL-12 and IFN-γ and relative absence of IL-4, IL-23, and IL-17 suggesting a T_H1 response (Anand et al., 2013, 2015). *A. terreus* induced invasive aspergillosis showed the presence of IL-1β, IL-6, and reduced level of IL-10 in mice model studies. Although there is activation of T_H17 type of adaptive immune response through IL-1β but the later is suppressed by T_H1 cytokines particularly IFN-γ (Vyas, 2011; Lass-Florl, 2012). The T_H1 response is thus also mounted by *A. terreus* and there is a lack of T_H2 response in contrast to *A. fumigatus* infection where T_H2 promoting cytokines are observed.

Cytokines Associated with T_H17 Type Response

Aspergillus fumigatus mediated infections in lung induce T_H17 and T_H1-cells. These cells play an important role in protection and induction of inflammation (Chai et al., 2010b). Activation of T_H17-cell depends on Dectin-1 signaling pathway. Various studies have suggested that dectin-1 deficient mice entirely activate T_H1-cells. So Dectin-1 signaling not only serves as a positive factor to promote T_H17 differentiation but rather act to balance T_H1 versus T_H17 differentiation. Activation of the APCs by Dectin-1, release the proinflammatory cytokines IL-1β, IL-6, IL-23, and IL-22 which differentiates $CD4^+$ T-cells to T_H17-cells, which further secretes IL-17A and IL-17F cytokines and maintain T_H17 response (Werner et al., 2011). IL-23 is a member of IL-12 family, produced by phagocytic cells, macrophages and activated DCs in lung. IL-23 contains two subunits IL-12p40 and IL-23p19 and it binds to heterodimeric receptors IL-12Rβ1, expressed by activated T-cells (Zelante et al., 2007). IL-6 is another important cytokine involved in regulation of T_H17 response. IL-6 is a multifunctional cytokine, promote T_H17-cells differentiation, inflammation and acute response (Akdis et al., 2011). During T_H17 differentiation, human naïve T-cells are exposed to IL-1β, IL-6, and IL-23 (Zelante et al., 2009; Gresnigt and van de Veerdonk, 2014). T_H17 promoting cytokine IL-17 binds to IL-17RA and IL-17RC receptors expressed in lung cells, like fibroblast, epithelial cells and T-cells. After release of IL-17 from T_H17-cells, it activates the neutrophils migration toward infected area and increases inflammation (Wilson et al., 2007). In *A. flavus* and *A. terreus*, the role of T_H17-cells during lung infection is yet to be established.

Cytokines Associated with T_H2 Type of Response

Aspergillus fumigatus is associated with both invasive and allergic form of aspergillosis. In case, if conidia are not cleared, they germinate to produce hyphae, which are responsible for invasion in host tissues that leads to inflammation. In a healthy human T-cells response, *A. fumigatus* not only evoke pro-inflammatory type of immune response via T_H1 and T_H17-cells but also anti-inflammatory type of immune response mediated by T_H2-cells (Chaudhary et al., 2010). Immune response initiated by IL-4 and IL-10 inhibits T_H1 and T_H17 response and increased secretion of IL-4 and IL-10 inhibits IFN-γ and IL-12 production. T_H2-cells differentiation depends on IL-4 and IL-10 and after differentiation in to T_H2 cells, these cells further secretes IL-5 and IL-13 which maintain T_H2 response. T_H2 immune response is triggered in acute bronchopulmonary aspergillosis and also in invasive pulmonary infection during some time point of infection. IL-4 and IL-10 deficient mice show lower *A. fumigatus* burden and increased survival rates compared to wild type mouse in invasive pulmonary aspergillosis (Cenci et al., 2000). It has been shown that ESTs (L3 ribosomal protein, L7A ribosomal protein, Histone -H2A) have high sequence similarity with human counter parts suggesting molecular mimicry between human and pathogen protein (Shankar et al., 2004). However, role of these genes in eliciting allergic immune response needs investigations. In *A. flavus* mediated infection, lung homogenate showed the absence of T_H2 response in a limited cytokine profile study (Anand and Tiwary, 2010). However, T_H2 type response may get activated in later stages of infection in lung due to rise in IL-4 and IL-10, which suppress the T_H1 response but consistent expression IFN-γ overcomes T_H2 response. In addition to T_H2, the role of T_H9-cells has been shown during infection with a Virus, bacteria, parasites and fungi. T_H9 response contributes to allergic inflammation during allergic aspergillosis due to *A. fumigatus* in mice model (Kerzerho et al., 2013). T_H9-cells develop in the presence of IL-1α and TGF-β along with T_H2-cells. However, role of T_H9 and T_H2 response during invasive aspergillosis remains unclear.

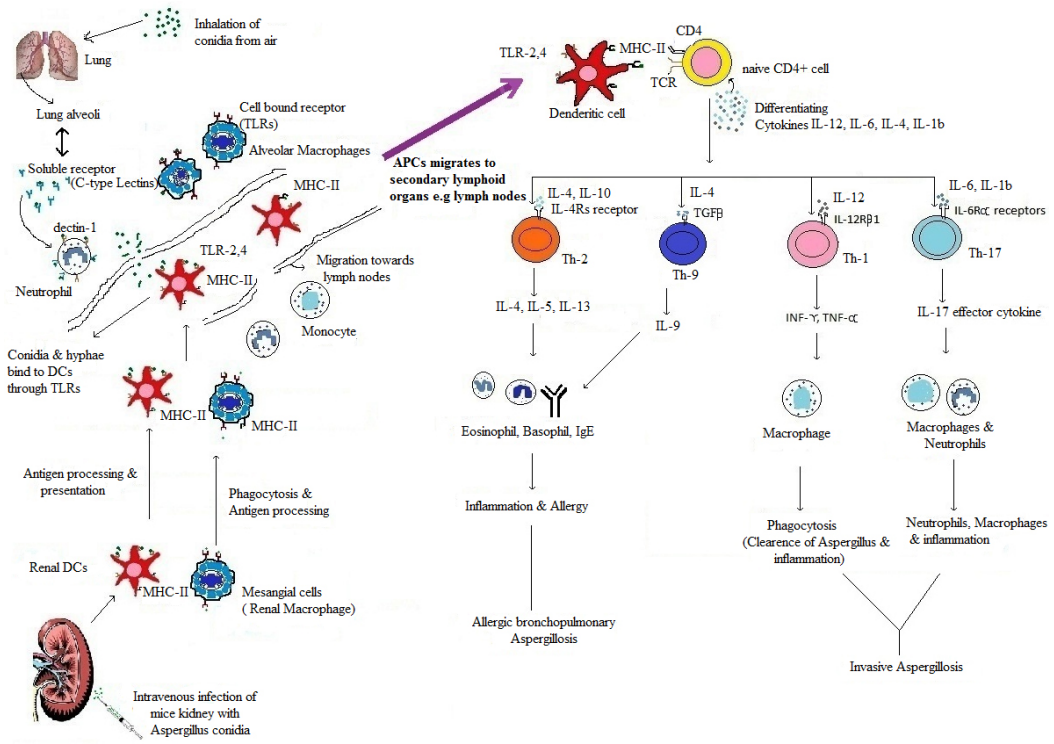


FIGURE 1 | The recognitions, processing and presentation of *Aspergillus* antigens to naïve CD4$^+$ T_H-cells and Production of effector T_H-cells. In infected Lung and Kidney, *Aspergillus* antigens (conidia & germinating conidia) are recognized by PRRs, i.e., soluble (C-type lectins) and cell bound receptors; TLR2, TLR4, and TLR9 (Netea et al., 2006). They are present on antigen presenting cells (DCs and macrophages). After recognition, antigens recognized by APCs, process and present to naïve CD4$^+$ T-cells in secondary lymphoid organs (Chai et al., 2010a). After interaction of APCs and naïve CD4$^+$ T-cells, differential cytokines release (IL-12, IL-6, IL-4, IL-1β) (Korn et al., 2009; Chai et al., 2010a) act upon CD4$^+$ T-cells and differentiate them into effector T_H-cells. IL- 12 give rise to T_H1, IL-6, IL-1β give rise to T_H17 and IL-4 give rise to T_H2 effector T_H-cells (Murdock et al., 2011). These effector cells further secrets effector cytokines (IFN-γ, TNF-α, IL-10, IL-5, IL-17, and IL-23) (Zelante et al., 2007) which maintain effector T_H-cells response. Figure shows summary of development of effector T_H-cells response during Lung and Kidney infection of *Aspergillus*. The figure is not to the scale.

Is Their Co-evolution of T-helper Cells During Invasive Aspergillosis?

In a immunocompromised mice model studies, repeated exposure of *A. fumigatus* conidia in hosts lead to co-evolution of T_H1, T_H2, and T_H17 response in infected lung (Murdock et al., 2011). They have observed the presence of IFN-γ and IL-17 in infected lung of mice along with T_H2 response. These mixed responses might be occurring at different time points during progression of infection and leads to either protection or infection. T_H1 and T_H17 response probably leads to protection where as T_H2 response further complicates the disease. T_H2 response help in evasion of *A. fumigatus* from immune cells and further increase the IgE level, which leads to high inflammation at the site of infection. Mice model of ABPA demonstrated the T_H2 cytokine profile consisting of IL-4, IL-10, and IL-5 (Latge, 1999).

Interplay of Cytokines; T_H1 or T_H2 or T_H17 Type of Response

T_H-cells response during invasive *Aspergillus* depends on differentiating cytokines. T_H1 response is activated by differentiating cytokine IL-12 followed by secretion of IFN-γ. Secretion of IFN-γ further stimulates T_H1 cells, if IFN-γ dominates initially it suppress the other cytokines of T_H2 and T_H17, i.e., IL-4 and IL-17 (Harrington et al., 2005). If IL-4 dominates during initial period of *Aspergillus* infection, it suppresses the protective T_H1 type immune response by inhibiting differential cytokine IL-12 and IFN-γ (Harrington et al., 2005). Recognition of *Aspergillus* antigens by Dectin-1 signaling pathway inhibit the production of IFN-γ and IL-12 receptors suppressing T_H1, which leads to differentiation of T_H17-cells and production of IL-17. In this way Dectin-1 signal balances the T_H1 and T_H17 response through the regulation of their respective cytokines (Rivera et al., 2011; **Figure 1**). The development of effective CD4$^+$ T_H-cells response not only depends upon cytokines, but also on chemokines and their receptors. Chemokines help in recruitment of leukocytes, i.e., neutrophils, monocytes and NK cells toward lung during *Aspergillus* infection. These cells express chemokine receptors; neutrophils contain CXCR2 chemokine receptor for ligand CXCL1 and CXCL2, monocytes contain CCR2 and CCR6 receptor for CCL2 and CCL20 ligands, where as NK cells contain CCR2 receptor for CCL2 ligand. So these chemokine ligands attract monocytes, neutrophils and NK cells to clear the *Aspergillus* hyphae during

lung infection (Park and Mehrad, 2009). These chemokines receptors are also present on DCs, regulatory T-cells (Tregs) and T_H-cells and help in their trafficking (Bendall, 2005). In this way, there is an interdependent relationship between chemokines and cytokines that help in evolution of effector T_H-cells response. CCL17, a chemokine, help in trafficking of DCs, Tregs and T_H1-cells toward infected area during invasive aspergillosis in response to CCR4 chemokine receptor present on these cells. Further, CCR6 receptor present on DCs and T_H17-cells help in migration of these cells in response to chemokine CCL20 (Bendall, 2005; Wüthrich et al., 2012).

Conclusion

Cytokines are important in the development of $CD4^+$ T_H-cells. Understanding of trafficking of $CD4^+$ T_H-cells and their regulation through differentiating/effector cytokines during invasive aspergillosis will be crucial for the targeted immunotherapy. Overall, cytokines and chemokines may serve as prognostic biomarkers that could be followed to assess the effectiveness of treatment response during invasive aspergillosis. Measurement of selected cytokines in the blood samples of aspergillosis patients may be a promising tool for the monitoring of treatment responses. Also, manipulation of cytokine response e. g, IFN-γ or IFN-γ in combination with antifungal drug, IL-37, may be a future avenue for the development of better therapeutic against invasive aspergillosis.

Acknowledgments

Authors are thankful to Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh, India, for financial support to Ph.D. students RT and ST.

References

Agarwal, R., Denning, D. W., and Chakrabarti, A. (2014). Estimation of the burden of chronic and allergic pulmonary aspergillosis in India. *PLoS ONE* 9:e114745. doi: 10.1371/journal.pone.0114745

Akdis, M., Burgler, S., Crameri, R., Eiwegger, T., Fujita, H., Gomez, E., et al. (2011). Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. *J. Allergy Clin. Immunol.* 127, 701–721 e701–e770. doi: 10.1016/j.jaci.2010.11.050

Anand, R., Shankar, J., Singh, A. P., and Tiwary, B. N. (2013). Cytokine milieu in renal cavities of immunocompetent mice in response to intravenous challenge of *Aspergillus flavus* leading to aspergillosis. *Cytokine* 61, 63–70. doi: 10.1016/j.cyto.2012.08.024

Anand, R., Shankar, J., Tiwary, B. N., and Singh, A. P. (2015). *Aspergillus flavus* induces granulomatous cerebral aspergillosis in mice with display of distinct cytokine profile. *Cytokine* 72, 166–172. doi: 10.1016/j.cyto.2015.01.006

Anand, R., and Tiwary, B. N. (2010). Th1 and Th2 cytokines in a self-healing primary pulmonary *Aspergillus flavus* infection in BALB/c mice. *Cytokine* 52, 258–264. doi: 10.1016/j.cyto.2010.07.428

Barnes, P. D., and Marr, K. A. (2006). Aspergillosis: spectrum of disease, diagnosis, and treatment. *Infect. Dis. Clin. North Am.* 20, 545–561, vi. doi: 10.1016/j.idc.2006.06.001

Bendall, L. (2005). Chemokines and their receptors in disease. *Histol. Histopathol.* 20, 907–926.

Bozza, S., Gaziano, R., Spreca, A., Bacci, A., Montagnoli, C., Di Francesco, P., et al. (2002). Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J. Immunol.* 168, 1362–1371. doi: 10.4049/jimmunol.168.3.1362

Caffrey, A. K., Lehmann, M. M., Zickovich, J. M., Espinosa, V., Shepardson, K. M., Watschke, C. P., et al. (2015). IL-1alpha signaling is critical for leukocyte recruitment after pulmonary *Aspergillus fumigatus* challenge. *PLoS Pathog.* 11:e1004625. doi: 10.1371/journal.ppat.1004625

Camargo, J. F., and Husain, S. (2014). Immune correlates of protection in human invasive aspergillosis. *Clin. Infect. Dis.* 59, 569–577. doi: 10.1093/cid/ciu337

Cenci, E., Mencacci, A., Bacci, A., Bistoni, F., Kurup, V. P., and Romani, L. (2000). T cell vaccination in mice with invasive pulmonary aspergillosis. *J. Immunol.* 165, 381–388. doi: 10.4049/jimmunol.165.1.381

Cenci, E., Mencacci, A., Fe D'ostiani, C., Del Sero, G., Mosci, P., Montagnoli, C., et al. (1998). Cytokine- and T helper-dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. *J. Infect. Dis.* 178, 1750–1760. doi: 10.1086/314493

Chai, L., Netea, M. G., Teerenstra, S., Earnest, A., Vonk, A. G., Schlamm, H. T., et al. (2010a). Early proinflammatory cytokines and C-reactive protein trends as predictors of outcome in invasive Aspergillosis. *J. Infect. Dis.* 202, 1454–1462. doi: 10.1086/656527

Chai, L. Y., Van De Veerdonk, F., Marijnissen, R. J., Cheng, S. C., Khoo, A. L., Hectors, M., et al. (2010b). Anti-*Aspergillus* human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity. *Immunology* 130, 46–54. doi: 10.1111/j.1365-2567.2009.03211.x

Chamilos, G., Luna, M., Lewis, R. E., Bodey, G. P., Chemaly, R., Tarrand, J. J., et al. (2006). Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989–2003). *Haematologica* 91, 986–989.

Chaudhary, N., Staab, J. F., and Marr, K. A. (2010). Healthy human T-Cell Responses to *Aspergillus fumigatus* antigens. *PLoS ONE* 5:e9036. doi: 10.1371/journal.pone.0009036

Chotirmall, S. H., Al-Alawi, M., Mirkovic, B., Lavelle, G., Logan, P. M., Greene, C. M., et al. (2013). *Aspergillus*-associated airway disease, inflammation, and the innate immune response. *Biomed. Res. Int.* 2013, 723129. doi: 10.1155/2013/723129

Cramer, R. A., Rivera, A., and Hohl, T. M. (2011). Immune responses against *Aspergillus fumigatus*: what have we learned? *Curr. Opin. Infect. Dis.* 24, 315–322. doi: 10.1097/QCO.0b013e328348b159

Delsing, C. E., Gresnigt, M. S., Leentjens, J., Preijers, F., Frager, F. A., Kox, M., et al. (2014). Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series. *BMC Infect. Dis.* 14:166. doi: 10.1186/1471-2334-14-166

Denning, D. W., Pleuvry, A., and Cole, D. C. (2011). Global burden of chronic pulmonary aspergillosis as a sequel to pulmonary tuberculosis. *Bull. World Health Organ.* 89, 864–872. doi: 10.2471/blt.11.089441

Denning, D. W., Pleuvry, A., and Cole, D. C. (2013). Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults. *Med. Mycol.* 51, 361–370. doi: 10.3109/13693786.2012.738312

Espinosa, V., Jhingran, A., Dutta, O., Kasahara, S., Donnelly, R., Du, P., et al. (2014). Inflammatory monocytes orchestrate innate antifungal immunity in the lung. *PLoS Pathog.* 10:e1003940. doi: 10.1371/journal.ppat.1003940

Espinosa, V., and Rivera, A. (2012). Cytokines and the regulation of fungus-specific CD4 T cell differentiation. *Cytokine* 58, 100–106. doi: 10.1016/j.cyto.2011.11.005

Gresnigt, M. S., and van de Veerdonk, F. L. (2014). The role of interleukin-1 family members in the host defence against *Aspergillus fumigatus*. *Mycopathologia* 178, 395–401. doi: 10.1007/s11046-014-9776-y

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., et al. (2005). Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6, 1123–1132. doi: 10.1038/ni1254

Inoue, M., and Shinohara, M. L. (2014). Clustering of pattern recognition receptors for fungal detection. *PLoS Pathog.* 10:e1003873. doi: 10.1371/journal.ppat.1003873

Kara, E. E., Comerford, I., Fenix, K. A., Bastow, C. R., Gregor, C. E., Mckenzie, D. R., et al. (2014). Tailored immune responses: novel effector helper T cell

subsets in protective immunity. *PLoS Pathog.* 10:e1003905. doi: 10.1371/journal.ppat.1003905

Kerzerho, J., Maazi, H., Speak, A. O., Szely, N., Lombardi, V., Khoo, B., et al. (2013). Programmed cell death ligand 2 regulates TH9 differentiation and induction of chronic airway hyperreactivity. *J. Allergy Clin. Immunol.* 131, 1048–1057, 1057 e1041–e1042. doi: 10.1016/j.jaci.2012.09.027

Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V. K. (2009). IL-17 and Th17 Cells. *Annu. Rev. Immunol.* 27, 485–517. doi: 10.1146/annurev.immunol.021908.132710

Lass-Florl, C. (2012). *Aspergillus terreus*: how inoculum size and host characteristics affect its virulence. *J. Infect. Dis.* 205, 1192–1194. doi: 10.1093/infdis/jis185

Latge, J. P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12, 310–350.

Moretti, S., Bozza, S., Oikonomou, V., Renga, G., Casagrande, A., Iannitti, R. G., et al. (2014). IL-37 inhibits inflammasome activation and disease severity in murine aspergillosis. *PLoS Pathog.* 10:e1004462. doi: 10.1371/journal.ppat.1004462

Morgan, J., Wannemuehler, K. A., Marr, K. A., Hadley, S., Kontoyiannis, D. P., Walsh, T. J., et al. (2005). Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. *Med. Mycol.* 43(Suppl. 1), S49–S58. doi: 10.1080/13693780400020113

Morton, C. O., Bouzani, M., Loeffler, J., and Rogers, T. R. (2012). Direct interaction studies between *Aspergillus fumigatus* and human immune cells; what have we learned about pathogenicity and host immunity? *Front. Microbiol.* 3:413. doi: 10.3389/fmicb.2012.00413

Murdock, B. J., Shreiner, A. B., Mcdonald, R. A., Osterholzer, J. J., White, E. S., Toews, G. B., et al. (2011). Coevolution of TH1, TH2, and TH17 responses during repeated pulmonary exposure to *Aspergillus fumigatus* conidia. *Infect. Immun.* 79, 125–135. doi: 10.1128/iai.00508-10

Netea, M. G., Ferwerda, G., Van Der Graaf, C. A., Van Der Meer, J. W., and Kullberg, B. J. (2006). Recognition of fungal pathogens by toll-like receptors. *Curr. Pharm. Des.* 12, 4195–4201. doi: 10.2174/138161206778743538

Osugi, Y., Vuckovic, S., and Hart, D. N. (2002). Myeloid blood $CD11c^+$ dendritic cells and monocyte-derived dendritic cells differ in their ability to stimulate T lymphocytes. *Blood* 100, 2858–2866. doi: 10.1182/blood.V100.8.2858

Park, S. J., Hughes, M. A., Burdick, M., Strieter, R. M., and Mehrad, B. (2009). Early NK cell-derived IFN-{gamma} is essential to host defense in neutropenic invasive aspergillosis. *J. Immunol.* 182, 4306–4312. doi: 10.4049/jimmunol.0803462

Park, S. J., and Mehrad, B. (2009). Innate immunity to *Aspergillus* species. *Clin. Microbiol. Rev.* 22, 535–551. doi: 10.1128/cmr.00014-09

Ramirez-Ortiz, Z. G., and Means, T. K. (2012). The role of dendritic cells in the innate recognition of pathogenic fungi (*A. fumigatus*, *C. neoformans*, and *C. albicans*). *Virulence* 3, 635–646. doi: 10.4161/viru.22295

Ramirez-Ortiz, Z. G., Specht, C. A., Wang, J. P., Lee, C. K., Bartholomeu, D. C., Gazzinelli, R. T., et al. (2008). Toll-like receptor 9-dependent immune activation by unmethylated CpG motifs in *Aspergillus fumigatus* DNA. *Infect. Immun.* 76, 2123–2129. doi: 10.1128/iai.00047-08

Rivera, A., Hohl, T. M., Collins, N., Leiner, I., Gallegos, A., Saijo, S., et al. (2011). Dectin-1 diversifies *Aspergillus fumigatus*-specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. *J. Exp. Med.* 208, 369–381. doi: 10.1084/jem.20100906

Romani, L. (2008). Cell mediated immunity to fungi: a reassessment. *Med. Mycol.* 46, 515–529. doi: 10.1080/13693780801971450

Serbina, N. V., Cherny, M., Shi, C., Bleau, S. A., Collins, N. H., Young, J. W., et al. (2009). Distinct responses of human monocyte subsets to *Aspergillus fumigatus* conidia. *J. Immunol.* 183, 2678–2687. doi: 10.4049/jimmunol.0803398

Shankar, J. (2013). An overview of toxins in *Aspergillus* associated with pathogenesis. *Int. J. Life Sci. Biotechnol. Pharm. Res.* 2, 16–31.

Shankar, J., Nigam, S., Saxena, S., Madan, T., and Sarma, P. U. (2004). Identification and assignment of function to the genes of *Aspergillus fumigatus* expressed at 37 degrees C. *J. Eukaryot. Microbiol.* 51, 428–432. doi: 10.1111/j.1550-7408.2004.tb00390.x

Shao, C., Qu, J., He, L., Zhang, Y., Wang, J., Zhou, H., et al. (2005). Dendritic cells transduced with an adenovirus vector encoding interleukin-12 are a potent vaccine for invasive pulmonary aspergillosis. *Genes Immun.* 6, 103–114. doi: 10.1038/sj.gene.6364167

Steinbach, W. J., Benjamin, D. K. Jr., Kontoyiannis, D. P., Perfect, J. R., Lutsar, I., et al. (2004). Infections due to *Aspergillus terreus*: a multicenter retrospective analysis of 83 cases. *Clin. Infect. Dis.* 39, 192–198. doi: 10.1086/421950

Stevens, D. A., Kan, V. L., Judson, M. A., Morrison, V. A., Dummer, S., Denning, D. W., et al. (2000). Practice guidelines for diseases caused by *Aspergillus*. Infectious Diseases Society of America. *Clin. Infect. Dis.* 30, 696–709. doi: 10.1086/313756

Verweij, P. E., and Brandt, M. E. (2007). *Aspergillus, Fusarium and Other Opportunistic Moniliaceous Fungi*. Washington, DC: ASM Press.

Vyas, J. M. (2011). The duality of *Aspergillus terreus*: differential immune responses to distinct conidia. *Virulence* 2, 181–184. doi: 10.4161/viru.2.3.16613

Weaver, C. T., Hatton, R. D., Mangan, P. R., and Harrington, L. E. (2007). IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25, 821–852. doi: 10.1146/annurev.immunol.25.022106.141557

Werner, J. L., Gessner, M. A., Lilly, L. M., Nelson, M. P., Metz, A. E., Horn, D., et al. (2011). Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection. *Infect. Immun.* 79, 3966–3977. doi: 10.1128/IAI.05493-11

Willment, J. A., and Brown, G. D. (2008). C-type lectin receptors in antifungal immunity. *Trends Microbiol.* 16, 27–32. doi: 10.1016/j.tim.2007.10.012

Wilson, N. J., Boniface, K., Chan, J. R., Mckenzie, B. S., Blumenschein, W. M., Mattson, J. D., et al. (2007). Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat. Immunol.* 8, 950–957. doi: 10.1038/ni1497

Wüthrich, M., Deepe, G. S., Jr., and Klein, B. (2012). Adaptive immunity to fungi. *Annu. Rev. Immunol.* 30, 115–148. doi: 10.1146/annurev-immunol-020711-074958

Zelante, T., Bozza, S., De Luca, A., D'angelo, C., Bonifazi, P., Moretti, S., et al. (2009). Th17 cells in the setting of *Aspergillus* infection and pathology. *Med. Mycol.* 47(Suppl. 1), S162–S169. doi: 10.1080/13693780802140766

Zelante, T., De Luca, A., Bonifazi, P., Montagnoli, C., Bozza, S., Moretti, S., et al. (2007). IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* 37, 2695–2706. doi: 10.1002/eji.200737409

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Thakur, Anand, Tiwari, Singh, Tiwary and Shankar. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Microbiology

MINI REVIEW
published: 05 May 2015
doi: 10.3389/fmicb.2015.00411

Paving the way for predictive diagnostics and personalized treatment of invasive aspergillosis

*Ana Oliveira-Coelho [1,2], Fernando Rodrigues [1,2], António Campos Jr. [3], João F. Lacerda [4,5], Agostinho Carvalho [1,2] and Cristina Cunha [1,2]**

[1] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal, [2] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal, [3] Serviço de Transplantação de Medula Óssea, Instituto Português de Oncologia do Porto, Porto, Portugal, [4] Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Lisboa, Portugal, [5] Serviço de Hematologia e Transplantação de Medula, Hospital de Santa Maria, Lisboa, Portugal

OPEN ACCESS

Edited by:
Frederic Lamoth, Lausanne University Hospital, Switzerland

Reviewed by:
Oliver Kurzai, Friedrich-Schiller-University Jena, Germany
Pierre-Yves Bochud, Centre Hospitalier Universitaire Vaudois, Switzerland

***Correspondence:**
Cristina Cunha, Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
cristinacunha@ecsaude.uminho.pt

Specialty section:
This article was submitted to Fungi and Their Interactions, a section of the journal Frontiers in Microbiology

Received: 11 February 2015
Accepted: 20 April 2015
Published: 05 May 2015

Citation:
Oliveira-Coelho A, Rodrigues F, Campos A Jr., Lacerda JF, Carvalho A and Cunha C (2015) Paving the way for predictive diagnostics and personalized treatment of invasive aspergillosis. Front. Microbiol. 6:411. doi: 10.3389/fmicb.2015.00411

Invasive aspergillosis (IA) is a life-threatening fungal disease commonly diagnosed among individuals with immunological deficits, namely hematological patients undergoing chemotherapy or allogeneic hematopoietic stem cell transplantation. Vaccines are not available, and despite the improved diagnosis and antifungal therapy, the treatment of IA is associated with a poor outcome. Importantly, the risk of infection and its clinical outcome vary significantly even among patients with similar predisposing clinical factors and microbiological exposure. Recent insights into antifungal immunity have further highlighted the complexity of host-fungus interactions and the multiple pathogen-sensing systems activated to control infection. How to decode this information into clinical practice remains however, a challenging issue in medical mycology. Here, we address recent advances in our understanding of the host-fungus interaction and discuss the application of this knowledge in potential strategies with the aim of moving toward personalized diagnostics and treatment (theranostics) in immunocompromised patients. Ultimately, the integration of individual traits into a clinically applicable process to predict the risk and progression of disease, and the efficacy of antifungal prophylaxis and therapy, holds the promise of a pioneering innovation benefiting patients at risk of IA.

Keywords: invasive aspergillosis, stem cell transplantation, antifungal immunity, single nucleotide polymorphism, personalized medicine

Introduction

Aspergillosis includes an extensive spectrum of diseases caused by fungi of the genus *Aspergillus* with clinical manifestations that range from colonization to allergic bronchopulmonary aspergillosis and disseminated forms of infection (Segal, 2009). The prevalence of invasive aspergillosis (IA) has steadily increased in the last decades, mostly due to the advent of solid organ and hematopoietic stem cell transplantation (HSCT), and the increased use of chemotherapy and immunosuppression (Kontoyiannis et al., 2010; Pagano et al., 2010). Although the diagnosis of IA has improved, namely because of the introduction of biomarkers such as the detection of galactomannan in the clinical practice (Morrissey et al., 2013), successful treatment is still a challenging endeavor. Indeed, established infection is difficult to eradicate, resulting in associated mortality rates ranging from 40 to 90% (Walsh et al., 2008).

The extensive use of empiric antifungal therapy is a major concern given the potential development of resistance in the pathogen. The emergence of azole-resistant strains of *Aspergillus fumigatus* (Steinmann et al., 2015) underscores the need for personalized diagnostic and risk prediction approaches based on individual traits that may allow targeting antifungal therapy and improving outcomes without unnecessary empiric therapy. Several host genetic variants have been proposed as suitable markers to categorize patients with the highest risk of infection (Cunha et al., 2013). However, many challenges confront the development of these predictive tools. Among them, our insufficient understanding of the critical immune defects that predispose to the infection and the lack of validation of genetic diagnostics in well-designed clinical trials.

The purpose of this review is to reposition novel findings on the host-fungus interaction and to discuss the challenges in exploiting this knowledge to the design of patient-tailored diagnostic or therapeutic approaches to deliver to specific target populations.

The Host-fungus Interaction: What's New?

The physical barriers afforded by the respiratory mucosa confer the first line of innate resistance to fungal infection. Because of their small size, conidia may reach the lung alveoli, where they are phagocytosed and killed through the production of reactive oxygen species (ROS) by resident macrophages, whereas neutrophils are instead proficient at handling hyphae germinating from conidia that escape macrophage surveillance through an array of extracellular killing mechanisms (Ibrahim-Granet et al., 2003; Cunha et al., 2014b; Heinekamp et al., 2015). This morphotype preference may be in part due to the ability of neutrophils to sense microbe size and selectively release neutrophil extracellular traps in response to larger structures (Branzk et al., 2014). Importantly, recent findings also suggest that lung epithelial cells act as an active extension of the innate immune system, operating as a surveillance mechanism sensing fungal spores and prompting antifungal effector responses (Osherov, 2012).

Innate immune cells are equipped with pattern recognition receptors (PRRs) able to discriminate pathogen-associated molecular patterns (PAMPs; Bourgeois and Kuchler, 2012; Plato et al., 2015). Stimulation of antigen-presenting cells, including macrophages and dendritic cells (DCs), leads to the activation and recruitment of lymphocytes, and the development of adaptive antifungal immune responses. Once committed, T cells monitor the host for infection and mobilize appropriate effector functions by inducing cytokines and cytolytic molecules, which are instrumental in rallying and activating professional phagocytes to the site of microbial deposition, thus providing a prompt and effective control of infection (Romani, 2011).

The fungal cell wall is the main source of PAMPs owing to its dynamic composition and structural properties according to morphotype, growth stage and environmental conditions (Latge, 2010). Toll-like receptor (TLR)-2 (in cooperation with TLR1 and TLR6), TLR3, TLR4, and TLR9, and the C-type lectin receptors dectin-1, dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) and mannose receptor are the most important PRRs recognizing fungal PAMPs including mannan, β-glucan and nucleic acids (Romani, 2011).

The Host Perspective

The immune response to *A. fumigatus* is determined not only by the relative degree of stimulation of the individual PRRs but also by the level of receptor cooperation and cellular localization. Indeed, a sequential activation of distinct signal transduction pathways through the PRR adapters myeloid differentiation primary response 88 (MyD88) and caspase-associated recruitment domain 9 (CARD9) in the respiratory epithelium and hematopoietic compartment in response to infection was recently reported (Jhingran et al., 2015). By means of a partial overlap, these signals ensure optimal chemokine induction, neutrophil recruitment, and fungal clearance within the respiratory tract.

Patients undergoing immunosuppressive regimens based on calcineurin inhibitors such as cyclosporine A or corticosteroids are highly susceptible to disseminated fungal infections. Previous work demonstrated that calcineurin is an important regulator of dectin-1-mediated signaling and activation of immunity to *Candida albicans* (Greenblatt et al., 2010), and that corticosteroid immunosuppression blocks dectin-1-mediated signaling required for maturation of *A. fumigatus* phagosomes (Kyrmizi et al., 2013). More recently, the calcineurin inhibitor tacrolimus was found to impair primary alveolar macrophage activation in response to *A. fumigatus* by preventing a signaling pathway involving a TLR9-Bruton's tyrosine kinase-calcineurin-nuclear factor of activated T-cells axis required for proinflammatory cytokine production (Herbst et al., 2015). Taken together, these findings suggest that disseminated fungal infections seen in these patients are not just a general consequence of systemic suppression of adaptive immunity but are, rather, a result of the specific blockade of evolutionarily conserved innate pathways for fungal resistance.

Another important feature of dectin-1-mediated signaling in response to *A. fumigatus* is the activation of the nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome leading to the production of bioactive interleukin (IL)-1β (Said-Sadier et al., 2010). Indeed, members of the IL-1 receptor family of cytokines are critical effector molecules in antifungal immunity (Gresnigt and van de Veerdonk, 2014). By preventing activation of the NLRP3 inflammasome and reducing IL-1β secretion, IL-37 was recently found to act as a broad spectrum inhibitor of innate responses to fungal infection-mediated inflammation (Moretti et al., 2014). Along this line, the increased IL-1β release intrinsically associated to chronic granulomatous disease (CGD) was found to be reverted by the use of IL-1 receptor antagonist (IL-1Ra), leading to restrained neutrophil recruitment and T helper 17 responses, thereby protecting from IA (de Luca et al., 2014).

Soluble PRRs found in the fluids lining the epithelial surfaces support fungal sensing by binding to conidia and enhancing their uptake by phagocytes. Among these, the long pentraxin 3 (PTX3) was demonstrated to play a non-redundant role in antifungal host defense (Garlanda et al., 2002) by enhancing recognition and phagocytosis through mechanisms that depend on Fcγ receptor, CD11b and complement activation (Moalli et al., 2010). Engagement of myeloid differentiation protein 2 (MD-2) during uptake

of PTX3-opsonized conidia was also revealed to activate TLR4 signaling converging on the production of type I interferons (Bozza et al., 2014). This suggests that in addition to pro-phagocytic properties, PTX3 is able to elicit antifungal effector mechanisms associated with limited immunopathology, thereby highlighting potential mechanisms of action underlying its favorable synergism with antifungal therapy against IA (Lo Giudice et al., 2012; Marra et al., 2014).

The Pathogen Perspective

Fungi have evolved their own elaborate mechanisms to escape innate immunity. By masking dectin-1 and dectin-2-dependent recognition, the hydrophobin layer of *A. fumigatus* conidia restrains neutrophil infiltration and cytokine production (Aimanianda et al., 2009; Carrion Sde et al., 2013). Galactosaminogalactan (GAG), a polysaccharide of the fungal cell wall produced by glucose epimerases (Lee et al., 2014), also hampers neutrophil recruitment (Fontaine et al., 2011). GAG functions as an adhesin, mediating adherence and suppressing host inflammatory responses, in part through masking cell wall β-glucan from recognition by dectin-1 (Gravelat et al., 2013). The immunosuppressive properties of GAG have also been attributed to its potent ability to induce IL-1Ra (Gresnigt et al., 2014), a finding further highlighting a possible therapeutic option targeting IL-1Ra in IA.

Genomic and transcriptomic approaches have revealed that fungal pathogenicity depends also on mechanisms regulating fungal metabolism and response to stress in adaptation to the host environment. In particular, the ability of *A. fumigatus* to adapt to hypoxic microenvironments has been found to involve the production of secondary metabolites that promote lung inflammation, exacerbate infection and influence subsequent host immune responses (Grahl et al., 2011). Given the need of myeloid cells to adapt to hypoxic and inflamed microenvironments that develop during infection, the hypoxia-inducible factor 1-alpha (HIF-1α) has been found to be essentially required for chemokine production and maintenance of neutrophil numbers in the lungs of infected animals (Shepardson et al., 2014).

Effector T cell responses are also targeted by fungi. For example, mucosal vaccination was found to subvert T cell priming by impairing chemokine signals on egress of inflammatory monocytes from the bone marrow and their recruitment to the lung (Wuthrich et al., 2012). This finding is even more significant considering the role of inflammatory monocytes in orchestrating antifungal immunity in the lung by regulating the conidiocidal activity of neutrophils and their own differentiation to DCs (Espinosa et al., 2014). Importantly, inflammatory monocytes are required for optimal IL-1α expression in the lungs, which in turn regulates the early accumulation of neutrophils in the lung (Caffrey et al., 2015).

Host Genetic Determinants of Risk of IA

The inborn deficiency of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase leading to a defective production of ROS and underlying CGD is the best known example of primary immunodeficiency associated with a distinctive predisposition to IA (Vinh, 2011). Patients with autosomal-dominant hyper-IgE syndrome (AD-HIES) are also at risk for IA (Vinh et al., 2010); however, the susceptibility of AD-HIES patients classically results from concurrent anatomical lung defects from previous bacterial infections and defective STAT3-dependent epithelial immunity (Holland et al., 2007). For most individuals however, the genetic propensity to IA has a polygenic source that acts in combination with other remarkable predisposing variants (e.g., the profound immunosuppression typical of many clinical settings), and that may translate into further immunological dysfunction, ultimately increasing the proneness to infection.

Our increasing ability to analyze human variability at the DNA level has made possible the identification of genetic factors implicated in the development of IA in hematological patients (Cunha et al., 2013), providing important insights into the mechanisms of human diversity underlying increased susceptibility to IA. Although genetic profiling is nowadays regarded as a promising methodology to exploit in the future toward improved diagnosis and therapy of fungal diseases (Cunha and Carvalho, 2012), this field is still bedeviled by difficulties, mostly related to heterogeneity of cohorts, sample size, selection bias, and statistical flaws (van der Velden et al., 2011), which compromise the clinical applicability of this knowledge.

The identification of a donor haplotype in *TLR4* increasing risk of IA after HSCT was one of the first solid reports illustrating the remarkable influence of host genetics on the susceptibility to IA (Bochud et al., 2008). This phenotype was associated with a delayed T cell and natural killer T cell immune reconstitution after transplant (Koldehoff et al., 2013). However, despite *TLR4* polymorphisms have also been linked with chronic aspergillosis in immunocompetent individuals (Carvalho et al., 2008) and fungal colonization in HSCT recipients (Carvalho et al., 2009), their prognostic significance remains unclear, since the exact mechanism(s) through which TLR4 deficiency impacts human antifungal immunity remain to be identified. Along this line, the early finding that genetic variants in *TLR1* and *TLR6* predisposed to IA among HSCT recipients (Kesh et al., 2005) was recently supported by evidence demonstrating the defective production of crucial antifungal cytokines and chemokines by TLR1- and TLR6-deficient mouse cells after stimulation with *A. fumigatus* (Rubino et al., 2012).

The discovery that sensing of fungal RNA by TLR3 was required for the activation of protective memory $CD8^+$ T cells responses in experimental aspergillosis was complemented by the identification of a regulatory variant impairing the expression of the human receptor in human DCs and hampering the efficient priming of memory $CD8^+$ responses (Carvalho et al., 2012b). These findings suggest that, by interpreting immunogenetic signatures and identifying subtle differences in immune profiles, response efficiencies to potential antifungal vaccination strategies are likely to be discriminated. In fact, the measurement of *A. fumigatus*-specific immune responses in hematological patients was confirmed as a promising immunodiagnostic approach (Potenza et al., 2013) amenable to combination with other diagnostic tools. Finally, a stop codon in recipient TLR5 was also disclosed as an important prognostic factor for the development of IA among HSCT recipients (Grube et al., 2013), a finding warranting further studies into the function of this receptor in antifungal immunity.

Dectin-1 deficiency was also consistently reported to contribute to susceptibility to IA (Cunha et al., 2010; Chai et al., 2011; Sainz et al., 2012). The finding that by compromising the surface expression and dectin-1-mediated cytokine production, the presence of the Y238X polymorphism in HSCT donors and recipients displayed a cumulative effect toward risk for infection (Cunha et al., 2010) emphasizes the contribution of non-hematopoietic dectin-1 to antifungal immunity. In addition, although damage perception is fundamental for resolution of fungal infection (Cunha et al., 2012), genetic variants triggering hyperactive danger signaling, and presumably leading to uncontrolled inflammatory responses to the fungus, were also recently found to increase risk for IA (Cunha et al., 2011).

A number of positive associations between genetic variants in cytokine and chemokine genes and vulnerability to IA has also been reported (Sainz et al., 2007a,b, 2008, 2010; Mezger et al., 2008; Carvalho et al., 2010). One recent example regards the identification of polymorphisms in the genes encoding for IL-1β and beta-defensin 1 (*DEFB1*) that, by affecting production of *A. fumigatus*-induced proinflammatory cytokines by mononuclear cells, influenced susceptibility to mold infection after solid organ transplantation (Wojtowicz et al., 2014).

A number of unconventional strategies have been employed to uncover additional candidate genes for susceptibility to IA (Zaas et al., 2008; Durrant et al., 2011). For example, genetic mapping analysis of survival data of infected mice allowed the identification of plasminogen, a regulatory molecule with opsonic properties, as a fitting contestant for susceptibility (Zaas et al., 2008). Consequently, a non-synonymous polymorphism in human plasminogen was found to increase risk for IA in HSCT recipients. Genetic and functional deficiency of other molecules with opsonic activity—e.g., mannose-binding lectin (MBL; Lambourne et al., 2009) and PTX3 (Cunha et al., 2014a)—has also been disclosed as a major determinant of susceptibility to IA, pointing to a key contribution of innate humoral responses to antifungal immunity. Indeed, a donor haplotype in *PTX3* associated

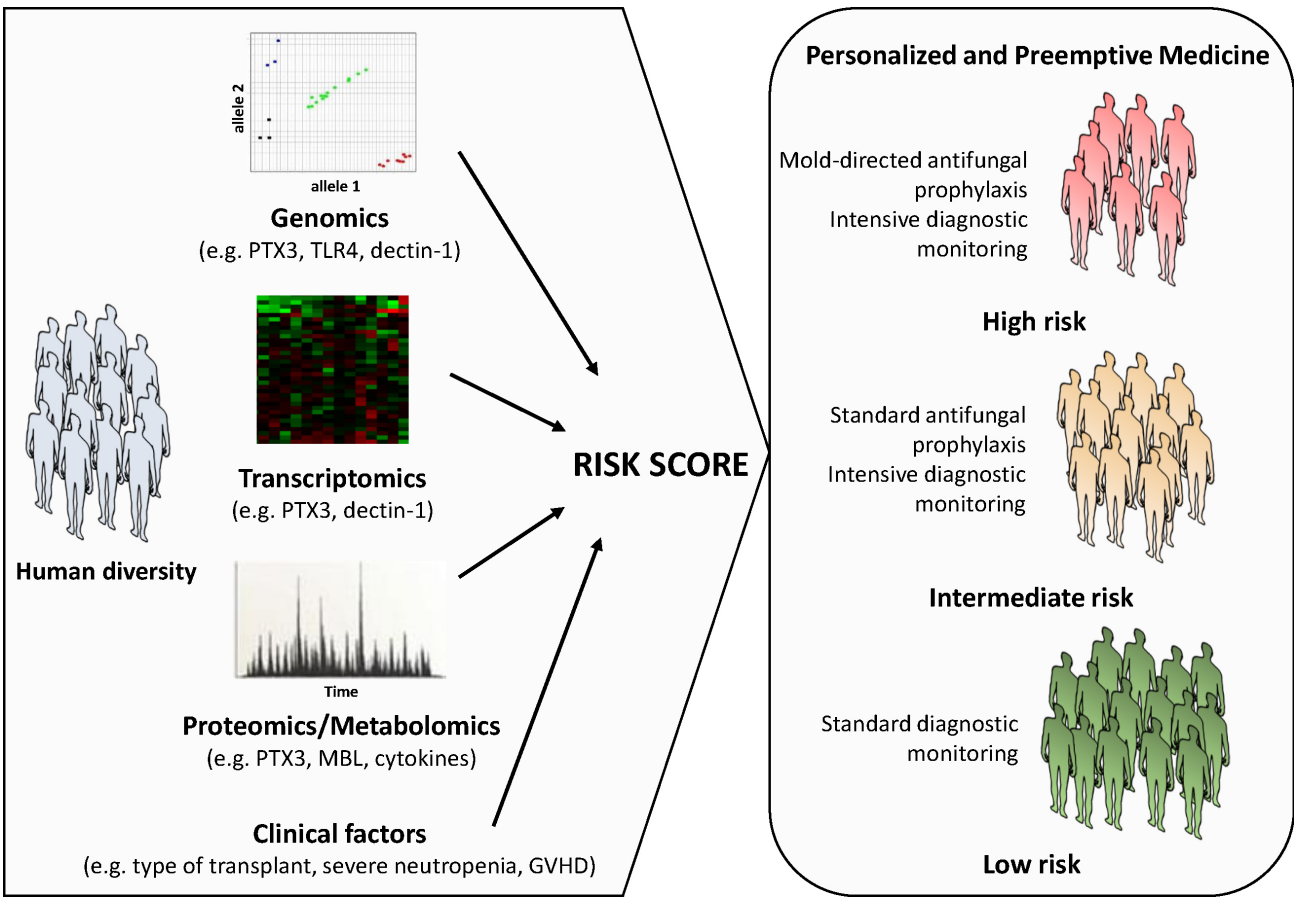


FIGURE 1 | Schematic representation of a personalized medicine approach to the management of IA. A prognostic score calculated using information on host biomarkers and clinical factors is used to determine the risk of IA associated to a given patient profile. The individual aspects of the patient (and donor), including the genetic make-up and downstream activated transcriptomic and proteomic or metabolomic networks, as well as inherent clinical factors are directly considered to guide treatment planning. Although the risk category is defined at initiation of treatment (or before stem cell transplantation), it may be updated in the course of treatment according to the clinical status of the patient (e.g., development of graft-versus-host-disease, prolonged neutropenia, etc.). A number of host genetic variants in innate immunity genes (e.g., PTX3, TLR4, dectin-1, MBL, and several cytokines) have been disclosed as promising targets to use in patient-tailored strategies to optimize and target the diagnostic workup, and the antifungal prophylaxis and therapy, thereby improving patient outcome.

with increased risk of IA in the corresponding HSCT recipient was found to compromise PTX3 expression during the developmental programming of neutrophils in the bone marrow, leading to a defective antifungal capacity of newly reconstituted neutrophils (Cunha et al., 2014a). Importantly, this association was recently replicated in a cohort of solid organ transplant recipients (Wojtowicz and Bochud, 2015). The fact that exogenous administration of PTX3 is able to revert the genetic defect (Cunha et al., 2014a) further highlights the potential of PTX3-based immunotherapies to treat (or prevent) IA (Carvalho et al., 2012a).

Decoding the Host-fungus Interaction into Clinical Strategies

Early diagnosis is crucial to a favorable outcome of IA. However, the existing diagnostic tools are often compromised by slowness, invasiveness, lack of standardization and insufficient understating of their kinetics (Hope et al., 2005). The introduction of molecular and serological diagnostic techniques into clinical practice has undoubtedly improved our capacity to diagnose IA. Nonetheless, the broad applicability of both techniques is hampered by considerable variability in performance. Given these technical barriers, the search for tools to diagnose IA that are more efficient and reliable is an active field of research. One example was recently provided by a study demonstrating the usefulness of direct detection of exogenous fungal metabolites in the breath to the identification of the underlying microbial etiology of pneumonia (Koo et al., 2014).

Although the interaction of the fungus with the immune system is being exploited to project novel and improved fungal diagnostics, efforts have on the other hand been also devoted to the implementation of clinical models aimed at the prediction of infection in high-risk patients. In this regard, interpretation of individual genomic, transcriptomic, proteomic or metabolomic profiles associated with impaired antifungal immune responses and their integration with clinical data is regarded as a promising approach (**Figure 1**). Indeed, next-generation sequencing technologies now provide exciting possibilities to pin down essential steps in host-fungus interaction at a level of complexity previously unanticipated. The first genome-wide association studies (GWAS) exploring host susceptibility to IA are underway and are expected to provide unbiased insights into the genetic defects contributing to development of IA, thereby laying the foundation for clinical trials aimed at the validation of medical interventions based on individual genomics. These efforts are nonetheless centered on the fairly "static" role played by the genetic variants. Physiological responses to fungal infection require the coordinated regulation of gene expression, which may vary markedly between individuals and influence phenotypes such as protein levels, the cell morphology and function, and ultimately the immunity to infection. Thus, genetic analysis of molecular traits such as the gene expression represents a powerful approach enabling insights into the human genomic landscape by generating expression maps useful for the functional interpretation of non-coding variants likely to arise from ongoing genome-wide initiatives (Fairfax and Knight, 2014).

Conclusions and Perspectives

The discovery of accurate and reliable genetic markers of susceptibility may be a turning point toward innovative stratification strategies based on genetic screening or immune profiling to predict risk and severity of disease, efficacy of antifungal prophylaxis and therapy, and eventually contribute to the successful design of antifungal vaccines. As shown for PTX3 deficiency (Cunha et al., 2014a), targeting cell function (e.g., exogenous administration of lacking or deficient factors) may prove an interesting approach to be validated in the future. Indeed, engineering of T cell function to target carbohydrates was demonstrated as a potentially exploitable strategy for the treatment of IA (Kumaresan et al., 2014). Ultimately, approaches based on individual genomics (and with influence on multiple functional transcriptomic, proteomic and metabolomic networks) may warrant important clinical tools allowing discrimination of patients that require enhanced surveillance for fungal disease or alternative antifungal therapies.

Acknowledgement

CC is supported by the Fundação para a Ciência e Tecnologia, Portugal (SFRH/BPD/96176/2013).

References

Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S. R., et al. (2009). Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 460, 1117–1121. doi: 10.1038/nature08264

Bochud, P. Y., Chien, J. W., Marr, K. A., Leisenring, W. M., Upton, A., Janer, M., et al. (2008). Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. *N. Engl. J. Med.* 359, 1766–1777. doi: 10.1056/NEJMoa0802629

Bourgeois, C., and Kuchler, K. (2012). Fungal pathogens-a sweet and sour treat for toll-like receptors. *Front. Cell Infect. Microbiol.* 2:142. doi: 10.3389/fcimb.2012.00142

Bozza, S., Campo, S., Arseni, B., Inforzato, A., Ragnar, L., Bottazzi, B., et al. (2014). PTX3 binds MD-2 and promotes TRIF-dependent immune protection in aspergillosis. *J. Immunol.* 193, 2340–2348. doi: 10.4049/jimmunol.1400814

Branzk, N., Lubojemska, A., Hardison, S. E., Wang, Q., Gutierrez, M. G., Brown, G. D., et al. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* 15, 1017–1025. doi: 10.1038/ni.2987

Caffrey, A. K., Lehmann, M. M., Zickovich, J. M., Espinosa, V., Shepardson, K. M., Watschke, C. P., et al. (2015). IL-1alpha signaling is critical for leukocyte recruitment after pulmonary *Aspergillus fumigatus* challenge. *PLoS Pathog.* 11:e1004625. doi: 10.1371/journal.ppat.1004625

Carrion Sde, J., Leal, S. M. Jr., Ghannoum, M. A., Aimanianda, V., Latge, J. P., and Pearlman, E. (2013). The RodA hydrophobin on *Aspergillus fumigatus* spores masks dectin-1- and dectin-2-dependent responses and enhances fungal survival *in vivo*. *J. Immunol.* 191, 2581–2588. doi: 10.4049/jimmunol.1300748

Carvalho, A., Cunha, C., Bistoni, F., and Romani, L. (2012a). Immunotherapy of aspergillosis. *Clin. Microbiol. Infect.* 18, 120–125. doi: 10.1111/j.1469-0691.2011.03681.x

Carvalho, A., De Luca, A., Bozza, S., Cunha, C., D'angelo, C., Moretti, S., et al. (2012b). TLR3 essentially promotes protective class I-restricted memory $CD8^+$ T-cell responses to *Aspergillus fumigatus* in hematopoietic transplanted patients. *Blood* 119, 967–977. doi: 10.1182/blood-2011-06-362582

Carvalho, A., Cunha, C., Carotti, A., Aloisi, T., Guarrera, O., Di Ianni, M., et al. (2009). Polymorphisms in Toll-like receptor genes and susceptibility to infections in allogeneic stem cell transplantation. *Exp. Hematol.* 37, 1022–1029. doi: 10.1016/j.exphem.2009.06.004

Carvalho, A., Cunha, C., Di Ianni, M., Pitzurra, L., Aloisi, T., Falzetti, F., et al. (2010). Prognostic significance of genetic variants in the IL-23/Th17 pathway for the outcome of T cell-depleted allogeneic stem cell transplantation. *Bone Marrow Transplant.* 45, 1645–1652. doi: 10.1038/bmt.2010.28

Carvalho, A., Pasqualotto, A. C., Pitzurra, L., Romani, L., Denning, D. W., and Rodrigues, F. (2008). Polymorphisms in toll-like receptor genes and susceptibility to pulmonary aspergillosis. *J. Infect. Dis.* 197, 618–621. doi: 10.1086/526500

Chai, L. Y., De Boer, M. G., Van Der Velden, W. J., Plantinga, T. S., Van Spriel, A. B., Jacobs, C., et al. (2011). The Y238X stop codon polymorphism in the human beta-glucan receptor dectin-1 and susceptibility to invasive aspergillosis. *J. Infect. Dis.* 203, 736–743. doi: 10.1093/infdis/jiq102

Cunha, C., Aversa, F., Lacerda, J. F., Busca, A., Kurzai, O., Grube, M., et al. (2014a). Genetic PTX3 deficiency and aspergillosis in stem-cell transplantation. *N. Engl. J. Med.* 370, 421–432. doi: 10.1056/NEJMoa1211161

Cunha, C., Kurzai, O., Loffler, J., Aversa, F., Romani, L., and Carvalho, A. (2014b). Neutrophil responses to aspergillosis: new roles for old players. *Mycopathologia* 178, 387–393. doi: 10.1007/s11046-014-9796-7

Cunha, C., Aversa, F., Romani, L., and Carvalho, A. (2013). Human genetic susceptibility to invasive aspergillosis. *PLoS Pathog.* 9:e1003434. doi: 10.1371/journal.ppat.1003434

Cunha, C., and Carvalho, A. (2012). Host genetics and invasive fungal diseases: towards improved diagnosis and therapy? *Expert Rev. Anti Infect Ther.* 10, 257–259. doi: 10.1586/eri.12.3

Cunha, C., Carvalho, A., Esposito, A., Bistoni, F., and Romani, L. (2012). DAMP signaling in fungal infections and diseases. *Front. Immunol.* 3:286. doi: 10.3389/fimmu.2012.00286

Cunha, C., Di Ianni, M., Bozza, S., Giovannini, G., Zagarella, S., Zelante, T., et al. (2010). Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. *Blood* 116, 5394–5402. doi: 10.1182/blood-2010-04-279307

Cunha, C., Giovannini, G., Pierini, A., Bell, A. S., Sorci, G., Riuzzi, F., et al. (2011). Genetically-determined hyperfunction of the S100B/RAGE axis is a risk factor for aspergillosis in stem cell transplant recipients. *PLoS ONE* 6:e27962. doi: 10.1371/journal.pone.0027962

de Luca, A., Smeekens, S. P., Casagrande, A., Iannitti, R., Conway, K. L., Gresnigt, M. S., et al. (2014). IL-1 receptor blockade restores autophagy and reduces inflammation in chronic granulomatous disease in mice and in humans. *Proc. Natl. Acad. Sci. U.S.A.* 111, 3526–3531. doi: 10.1073/pnas.1322831111

Durrant, C., Tayem, H., Yalcin, B., Cleak, J., Goodstadt, L., De Villena, F. P., et al. (2011). Collaborative Cross mice and their power to map host susceptibility to *Aspergillus fumigatus* infection. *Genome Res.* 21, 1239–1248. doi: 10.1101/gr.118786.110

Espinosa, V., Jhingran, A., Dutta, O., Kasahara, S., Donnelly, R., Du, P., et al. (2014). Inflammatory monocytes orchestrate innate antifungal immunity in the lung. *PLoS Pathog.* 10:e1003940. doi: 10.1371/journal.ppat.1003940

Fairfax, B. P., and Knight, J. C. (2014). Genetics of gene expression in immunity to infection. *Curr. Opin. Immunol.* 30C, 63–71. doi: 10.1016/j.coi.2014.07.001

Fontaine, T., Delangle, A., Simenel, C., Coddeville, B., Van Vliet, S. J., Van Kooyk, Y., et al. (2011). Galactosaminogalactan, a new immunosuppressive polysaccharide of *Aspergillus fumigatus*. *PLoS Pathog.* 7:e1002372. doi: 10.1371/journal.ppat.1002372

Garlanda, C., Hirsch, E., Bozza, S., Salustri, A., De Acetis, M., Nota, R., et al. (2002). Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* 420, 182–186. doi: 10.1038/nature01195

Grahl, N., Puttikamonkul, S., Macdonald, J. M., Gamcsik, M. P., Ngo, L. Y., Hohl, T. M., et al. (2011). *In vivo* hypoxia and a fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. *PLoS Pathog.* 7:e1002145. doi: 10.1371/journal.ppat.1002145

Gravelat, F. N., Beauvais, A., Liu, H., Lee, M. J., Snarr, B. D., Chen, D., et al. (2013). *Aspergillus* galactosaminogalactan mediates adherence to host constituents and conceals hyphal beta-glucan from the immune system. *PLoS Pathog.* 9:e1003575. doi: 10.1371/journal.ppat.1003575

Greenblatt, M. B., Aliprantis, A., Hu, B., and Glimcher, L. H. (2010). Calcineurin regulates innate antifungal immunity in neutrophils. *J. Exp. Med.* 207, 923–931. doi: 10.1084/jem.20092531

Gresnigt, M. S., Bozza, S., Becker, K. L., Joosten, L. A., Abdollahi-Roodsaz, S., Van Der Berg, W. B., et al. (2014). A polysaccharide virulence factor from *Aspergillus fumigatus* elicits anti-inflammatory effects through induction of Interleukin-1 receptor antagonist. *PLoS Pathog.* 10:e1003936. doi: 10.1371/journal.ppat.1003936

Gresnigt, M. S., and van de Veerdonk, F. L. (2014). The role of interleukin-1 family members in the host defence against *Aspergillus fumigatus*. *Mycopathologia* 178, 395–401. doi: 10.1007/s11046-014-9776-y

Grube, M., Loeffler, J., Mezger, M., Kruger, B., Echtenacher, B., Hoffmann, P., et al. (2013). TLR5 stop codon polymorphism is associated with invasive aspergillosis after allogeneic stem cell transplantation. *Med. Mycol.* 51, 818–825. doi: 10.3109/13693786.2013.809630

Heinekamp, T., Schmidt, H., Lapp, K., Pahtz, V., Shopova, I., Koster-Eiserfunke, N., et al. (2015). Interference of *Aspergillus fumigatus* with the immune response. *Semin. Immunopathol.* 37, 141–152. doi: 10.1007/s00281-014-0465-1

Herbst, S., Shah, A., Mazon Moya, M., Marzola, V., Jensen, B., Reed, A., et al. (2015). Phagocytosis-dependent activation of a TLR9-BTK-calcineurin-NFAT pathway co-ordinates innate immunity to *Aspergillus fumigatus*. *EMBO Mol. Med.* 7, 240–258. doi: 10.15252/emmm.201404556

Holland, S. M., Deleo, F. R., Elloumi, H. Z., Hsu, A. P., Uzel, G., Brodsky, N., et al. (2007). STAT3 mutations in the hyper-IgE syndrome. *N. Engl. J. Med.* 357, 1608–1619. doi: 10.1056/NEJMoa073687

Hope, W. W., Walsh, T. J., and Denning, D. W. (2005). Laboratory diagnosis of invasive aspergillosis. *Lancet Infect. Dis.* 5, 609–622. doi: 10.1016/S1473-3099(05)70238-3

Ibrahim-Granet, O., Philippe, B., Boleti, H., Boisvieux-Ulrich, E., Grenet, D., Stern, M., et al. (2003). Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. *Infect. Immun.* 71, 891–903. doi: 10.1128/IAI.71.2.891-903.2003

Jhingran, A., Kasahara, S., Shepardson, K. M., Junecko, B. A., Heung, L. J., Kumasaka, D. K., et al. (2015). Compartment-specific and sequential role of MyD88 and CARD9 in chemokine induction and innate defense during respiratory fungal infection. *PLoS Pathog.* 11:e1004589. doi: 10.1371/journal.ppat.1004589

Kesh, S., Mensah, N. Y., Peterlongo, P., Jaffe, D., Hsu, K., VAN DEN Brink, M., et al. (2005). TLR1 and TLR6 polymorphisms are associated with susceptibility to invasive aspergillosis after allogeneic stem cell transplantation. *Ann. N. Y. Acad. Sci.* 1062, 95–103. doi: 10.1196/annals.1358.012

Koldehoff, M., Beelen, D. W., and Elmaagacli, A. H. (2013). Increased susceptibility for aspergillosis and post-transplant immune deficiency in patients with gene variants of TLR4 after stem cell transplantation. *Transpl. Infect. Dis.* 15, 533–539. doi: 10.1111/tid.12115

Kontoyiannis, D. P., Marr, K. A., Park, B. J., Alexander, B. D., Anaissie, E. J., Walsh, T. J., et al. (2010). Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin. Infect. Dis.* 50, 1091–1100. doi: 10.1086/651263

Koo, S., Thomas, H. R., Daniels, S. D., Lynch, R. C., Fortier, S. M., Shea, M. M., et al. (2014). A breath fungal secondary metabolite signature to diagnose invasive aspergillosis. *Clin. Infect. Dis.* 59, 1733–1740. doi: 10.1093/cid/ciu725

Kumaresan, P. R., Manuri, P. R., Albert, N. D., Maiti, S., Singh, H., Mi, T., et al. (2014). Bioengineering T cells to target carbohydrate to treat opportunistic fungal infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, 10660–10665. doi: 10.1073/pnas.1312789111

Kyrmizi, I., Gresnigt, M. S., Akoumianaki, T., Samonis, G., Sidiropoulos, P., Boumpas, D., et al. (2013). Corticosteroids block autophagy protein recruitment in *Aspergillus fumigatus* phagosomes via targeting dectin-1/Syk kinase signaling. *J. Immunol.* 191, 1287–1299. doi: 10.4049/jimmunol.1300132

Lambourne, J., Agranoff, D., Herbrecht, R., Troke, P. F., Buchbinder, A., Willis, F., et al. (2009). Association of mannose-binding lectin deficiency with acute invasive aspergillosis in immunocompromised patients. *Clin. Infect. Dis.* 49, 1486–1491. doi: 10.1086/644619

Latge, J. P. (2010). Tasting the fungal cell wall. *Cell. Microbiol.* 12, 863–872. doi: 10.1111/j.1462-5822.2010.01474.x

Lee, M. J., Gravelat, F. N., Cerone, R. P., Baptista, S. D., Campoli, P. V., Choe, S. I., et al. (2014). Overlapping and distinct roles of *Aspergillus fumigatus* UDP-glucose 4-epimerases in galactose metabolism and the synthesis of galactose-containing cell wall polysaccharides. *J. Biol. Chem.* 289, 1243–1256. doi: 10.1074/jbc.M113.522516

Lo Giudice, P., Campo, S., De Santis, R., and Salvatori, G. (2012). Effect of PTX3 and voriconazole combination in a rat model of invasive pulmonary aspergillosis. *Antimicrob. Agents Chemother.* 56, 6400–6402. doi: 10.1128/AAC.01000-12

Marra, E., Sousa, V. L., Gaziano, R., Pacello, M. L., Arseni, B., Aurisicchio, L., et al. (2014). Efficacy of PTX3 and posaconazole combination in a rat model of invasive pulmonary aspergillosis. *Antimicrob. Agents Chemother.* 58, 6284–6286. doi: 10.1128/AAC.03038-14

Mezger, M., Steffens, M., Beyer, M., Manger, C., Eberle, J., Toliat, M. R., et al. (2008). Polymorphisms in the chemokine (C-X-C motif) ligand 10 are associated with invasive aspergillosis after allogeneic stem-cell transplantation and influence CXCL10 expression in monocyte-derived dendritic cells. *Blood* 111, 534–536. doi: 10.1182/blood-2007-05-090928

Moalli, F., Doni, A., Deban, L., Zelante, T., Zagarella, S., Bottazzi, B., et al. (2010). Role of complement and Fcγ receptors in the protective activity of the long pentraxin PTX3 against *Aspergillus fumigatus*. *Blood* 116, 5170–5180. doi: 10.1182/blood-2009-12-258376

Moretti, S., Bozza, S., Oikonomou, V., Renga, G., Casagrande, A., Iannitti, R. G., et al. (2014). IL-37 inhibits inflammasome activation and disease severity in murine aspergillosis. *PLoS Pathog.* 10:e1004462. doi: 10.1371/journal.ppat.1004462

Morrissey, C. O., Chen, S. C., Sorrell, T. C., Milliken, S., Bardy, P. G., Bradstock, K. F., et al. (2013). Galactomannan and PCR versus culture and histology for directing use of antifungal treatment for invasive aspergillosis in high-risk haematology patients: a randomised controlled trial. *Lancet Infect. Dis.* 13, 519–528. doi: 10.1016/S1473-3099(13)70076-8

Osherov, N. (2012). Interaction of the pathogenic mold *Aspergillus fumigatus* with lung epithelial cells. *Front. Microbiol.* 3:346. doi: 10.3389/fmicb.2012.00346

Pagano, L., Caira, M., Candoni, A., Offidani, M., Martino, B., Specchia, G., et al. (2010). Invasive aspergillosis in patients with acute myeloid leukemia: a SEIFEM-2008 registry study. *Haematologica* 95, 644–650. doi: 10.3324/haematol.2009.012054

Plato, A., Hardison, S. E., and Brown, G. D. (2015). Pattern recognition receptors in antifungal immunity. *Semin Immunopathol.* 37, 97–106. doi: 10.1007/s00281-014-0462-4

Potenza, L., Vallerini, D., Barozzi, P., Riva, G., Forghieri, F., Beauvais, A., et al. (2013). Characterization of specific immune responses to different *Aspergillus* antigens during the course of invasive Aspergillosis in hematologic patients. *PLoS ONE* 8:e74326. doi: 10.1371/journal.pone.0074326

Romani, L. (2011). Immunity to fungal infections. *Nat. Rev. Immunol.* 11, 275–288. doi: 10.1038/nri2939

Rubino, I., Coste, A., Le Roy, D., Roger, T., Jaton, K., Boeckh, M., et al. (2012). Species-specific recognition of *Aspergillus fumigatus* by Toll-like receptor 1 and Toll-like receptor 6. *J. Infect. Dis.* 205, 944–954. doi: 10.1093/infdis/jir882

Said-Sadier, N., Padilla, E., Langsley, G., and Ojcius, D. M. (2010). *Aspergillus fumigatus* stimulates the NLRP3 inflammasome through a pathway requiring ROS production and the Syk tyrosine kinase. *PLoS ONE* 5:e10008. doi: 10.1371/journal.pone.0010008

Sainz, J., Hassan, L., Perez, E., Romero, A., Moratalla, A., Lopez-Fernandez, E., et al. (2007a). Interleukin-10 promoter polymorphism as risk factor to develop invasive pulmonary aspergillosis. *Immunol. Lett.* 109, 76–82. doi: 10.1016/j.imlet.2007.01.005

Sainz, J., Perez, E., Hassan, L., Moratalla, A., Romero, A., Collado, M. D., et al. (2007b). Variable number of tandem repeats of TNF receptor type 2 promoter as genetic biomarker of susceptibility to develop invasive pulmonary aspergillosis. *Hum. Immunol.* 68, 41–50. doi: 10.1016/j.humimm.2006.10.011

Sainz, J., Lupianez, C. B., Segura-Catena, J., Vazquez, L., Rios, R., Oyonarte, S., et al. (2012). Dectin-1 and DC-SIGN polymorphisms associated with invasive pulmonary Aspergillosis infection. *PLoS ONE* 7:e32273. doi: 10.1371/journal.pone.0032273

Sainz, J., Perez, E., Gomez-Lopera, S., and Jurado, M. (2008). IL1 gene cluster polymorphisms and its haplotypes may predict the risk to develop invasive pulmonary aspergillosis and modulate C-reactive protein level. *J. Clin. Immunol.* 28, 473–485. doi: 10.1007/s10875-008-9197-0

Sainz, J., Salas-Alvarado, I., Lopez-Fernandez, E., Olmedo, C., Comino, A., Garcia, F., et al. (2010). TNFR1 mRNA expression level and TNFR1 gene polymorphisms are predictive markers for susceptibility to develop invasive pulmonary aspergillosis. *Int. J. Immunopathol. Pharmacol.* 23, 423–436.

Segal, B. H. (2009). Aspergillosis. *N. Engl. J. Med.* 360, 1870–1884. doi: 10.1056/NEJMra0808853

Shepardson, K. M., Jhingran, A., Caffrey, A., Obar, J. J., Suratt, B. T., Berwin, B. L., et al. (2014). Myeloid derived hypoxia inducible factor 1-alpha is required for protection against pulmonary *Aspergillus fumigatus* infection. *PLoS Pathog.* 10:e1004378. doi: 10.1371/journal.ppat.1004378

Steinmann, J., Hamprecht, A., Vehreschild, M. J., Cornely, O. A., Buchheidt, D., Spiess, B., et al. (2015). Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *J. Antimicrob. Chemother.* 70, 1522–1526. doi: 10.1093/jac/dku566

van der Velden, W. J., Blijlevens, N. M., and Donnelly, J. P. (2011). Genetic variants and the risk for invasive mould disease in immunocompromised hematology patients. *Curr. Opin. Infect. Dis.* 24, 554–563. doi: 10.1097/QCO.0b013e32834ab1f4

Vinh, D. C. (2011). Insights into human antifungal immunity from primary immunodeficiencies. *Lancet Infect. Dis.* 11, 780–792. doi: 10.1016/S1473-3099(11)70217-1

Vinh, D. C., Sugui, J. A., Hsu, A. P., Freeman, A. F., and Holland, S. M. (2010). Invasive fungal disease in autosomal-dominant hyper-IgE syndrome. *J. Allergy Clin. Immunol.* 125, 1389–1390. doi: 10.1016/j.jaci.2010.01.047

Walsh, T. J., Anaissie, E. J., Denning, D. W., Herbrecht, R., Kontoyiannis, D. P., Marr, K. A., et al. (2008). Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clin. Infect. Dis.* 46, 327–360. doi: 10.1086/525258

Wojtowicz, A., and Bochud, P. Y. (2015). Host genetics of invasive *Aspergillus* and Candida infections. *Semin Immunopathol.* 37, 173–86. doi: 10.1007/s00281-014-0468-y

Wojtowicz, A., Gresnigt, M. S., Lecompte, T., Bibert, S., Manuel, O., Joosten, L. A., et al. (2014). IL1B and DEFB1 polymorphisms increase susceptibility to invasive mold infection after solid-organ transplantation. *J. Infect. Dis.* doi: 10.1093/infdis/jiu636 [Epub ahead of print].

Wuthrich, M., Ersland, K., Sullivan, T., Galles, K., and Klein, B. S. (2012). Fungi subvert vaccine T cell priming at the respiratory mucosa by preventing chemokine-induced influx of inflammatory monocytes. *Immunity* 36, 680–692. doi: 10.1016/j.immuni.2012.02.015

Zaas, A. K., Liao, G., Chien, J. W., Weinberg, C., Shore, D., Giles, S. S., et al. (2008). Plasminogen alleles influence susceptibility to invasive aspergillosis. *PLoS Genet.* 4:e1000101. doi: 10.1371/journal.pgen.1000101

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Oliveira-Coelho, Rodrigues, Campos Jr., Lacerda, Carvalho and Cunha. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

ADVANTAGES OF PUBLISHING IN FRONTIERS

FAST PUBLICATION
Average 90 days from submission to publication

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

RESEARCH NETWORK
Our network increases readership for your article

OPEN ACCESS
Articles are free to read, for greatest visibility

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

GLOBAL SPREAD
Six million monthly page views worldwide

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

IMPACT METRICS
Advanced metrics track your article's impact

SUPPORT
By our Swiss-based editorial team

frontiers

EPFL Innovation Park · Building I · 1015 Lausanne · Switzerland
T +41 21 510 17 00 · info@frontiersin.org · frontiersin.org