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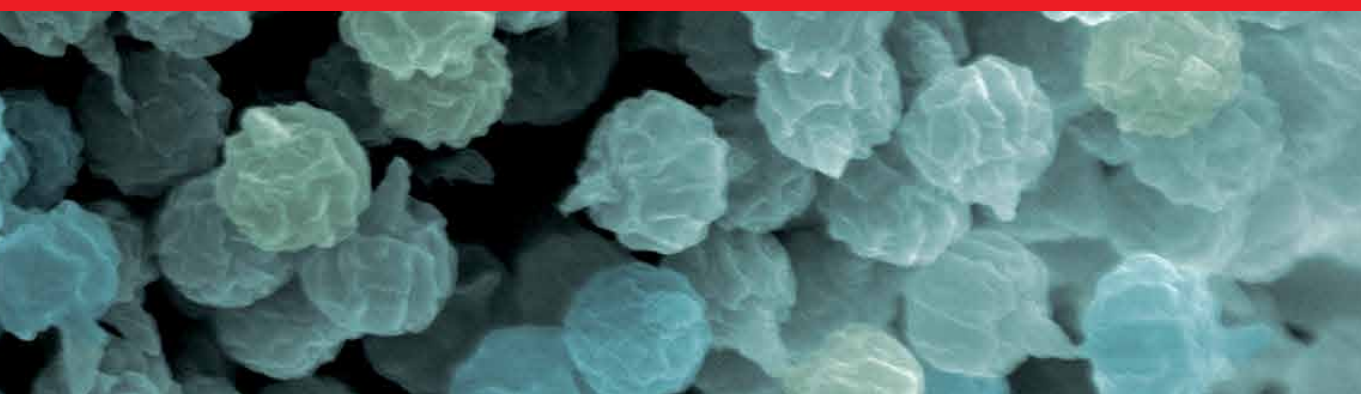
The Genus *Aspergillus*

Pathogenicity, Mycotoxin Production and Industrial Applications

Edited by Mehdi Razzaghi-Abyaneh and Mahendra Rai



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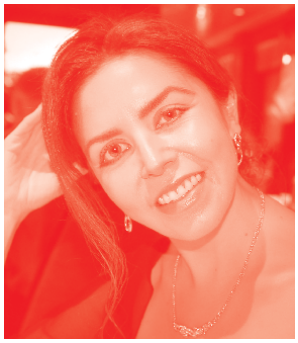

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Meet the editor

Professor Mehdi Razzaghi-Abyaneh obtained his Ph.D. in Mycology from Tarbiat Modares University, Tehran-Iran. In 2006/2007, he completed a training course on the identification of antifungal compounds from bioactive plants at the laboratory of the Department of Applied Biological Chemistry, Graduate School of Agriculture, Tokyo University, Japan. He is currently a full professor and head of the Mycology Department, Pasteur Institute of Iran, where he is working on mycotoxins and mycotoxigenic fungi as well as antifungal nanomaterial, and biologically active antifungals of plant, fungal, and bacterial origin. He has supervised numerous Ph.D. and MSc theses and published more than 150 papers in peer-reviewed international journals, 10 books, and several book chapters. He is studying the chemical basis of plant-fungal interactions and determining the mode of action of antifungal peptides and other small and macromolecules at cellular and molecular levels.

Professor Mahendra Rai is a visiting scientist at Nicolaus Copernicus University, Torun, Poland. He has published 380 research papers, more than 102 journal articles, and more than 60 books. He is a member of several scientific societies and has been a national scholar for five years. He received several prestigious awards, including the father T.A. Mathias award (1989) from the All India Association for Christian Higher Education, and the Medini award (1999) from the Department of Environment and Forest, Government of India. He also received a SERC visiting fellowship from the Department of Science and Technology (1996), INSA visiting fellowship from the Indian National Science Academy (1998), and TWAS-UNESCO Associateship (2002), Italy. Dr. Rai was also awarded a UGC-BSR faculty fellowship by the University Grants Commission, New Delhi. He serves as a referee for twenty international journals and is an editorial board member for ten national and international journals. He has approximately three decades of teaching and research experience. The main focus of his research is plant and nano-based bioactive against human pathogenic microbes.

Contents

Preface

The genus *Aspergillus* consists of a diverse group of airborne species with environmental and public health impacts. The members of this genus are cosmopolitan fungi frequently found in soil as the main reservoir and they are responsible for food spoilage, mycotoxin contamination, and various types of human and animal mycoses. Moreover, they are rich sources of beneficial metabolites such as antibiotics, organic acids, enzymes, and additives. At present, there are more than 350 identified species in the genus, of which around 20 species are known to be involved in the etiology of *Aspergillus*-related diseases under the common name "aspergillosis." Mode of infection is the inhalation of airborne conidia, exposure to contaminated water, and nosocomial infections. This book, which includes six chapters over two sections, discusses different aspects of the genus *Aspergillus*, including *Aspergillus*–host interactions to immunopathogenesis of aspergillosis, mycotoxin production, and industrial applications of the beneficial species.

Chapter 1 is an introductory chapter that contains useful information about all sections and chapters of the book. Chapter 2 discusses the environmental and clinical importance of *Aspergillus*–human interactions with a special focus on host immune status and previous underlying diseases as important determinants of clinical outcomes and disease spectra of aspergillosis. Chapter 3 examines the immunopathogenesis of aspergillosis with emphasis on the route of entry of etiologic *Aspergillus* species, and the function of pulmonary host defense in the clearance of infective conidia. In this context and in conditions of poor host immune response, where the neutrophils and macrophages fail to recognize the etiologic fungus, *Aspergillus* conidia attack and destroy airway epithelium. Chapter 4 reviews the role of aflatoxin in *A. flavus* resilience to stress with special attention to *Aspergillus* section *Flavi* in relation to producing the aflatoxins, secondary metabolites toxic to humans and animals. Chapter 5 examines why these fungi produce aflatoxins and the role of this mycotoxin in pathogenicity or in niche competition of producing fungus. The chapter also addresses another amazing aspect of *Aspergillus* research, the relationship between mycovirus-containing *A. flavus* and acute lymphoblastic leukemia as carcinogenesis beyond mycotoxin production. Finally, Chapter 6 discusses the industrial importance of members belonging to the genus *Aspergillus* with a focus on the ability of important species to green synthesis of functional nanomaterials with potential application in agriculture and medicine.

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Section 1

Pathogenicity

Chapter 1

Introductory Chapter: The Genus *Aspergillus* - Pathogenicity, Mycotoxin Production and Industrial Applications

Mehdi Razzaghi-Abyaneh and Mahendra Rai

1. Introduction

Aspergillus infections in humans were firstly reported in the eighteenth century [1, 2]. *Aspergillus* was first described in 1729 by Micheli, an Italian priest and biologist, who was the first person to attempt the scientific study of fungi [3]. *A. flavus* was named and reported by Link in 1809. John Hughes Bennett (1812–1875) was the first to describe aspergillosis. In his seminal paper published in 1842, he made the very first description of *Aspergillus* growing in the lung tissue of humans [4]. Paranasal sinus mycosis in 1893 and since then numerous cases have been reported from different parts of the world. In 1926, the genus *Aspergillus* was first classified and accepted in 69 *Aspergillus* species in 11 groups. By the year 1965, the previous classification of *Aspergillus* was declared outdated, and detailed 151 species in 18 different groups were introduced. Additional research led to further refined species designations with the use of new technologies such as thin-layer chromatography of secondary metabolites and DNA hybridization.

The genus *Aspergillus* consists of numerous species gathered in a diverse group with environmental and public health importance [5, 6]. The members of this genus are cosmopolitan fungi frequently found in various natural habitats especially in soil as the main reservoir and they are responsible for food spoilage, mycotoxin contamination, and various types of human and animal mycoses [7, 8]. Moreover, they are rich sources of beneficial metabolites such as antibiotics, organic acids, enzymes, and additives. At present, there are more than 300 species which are now accepted, and new species continue to be described and added to this list. The taxonomy of species within the *Aspergillus* genus is gradually undergoing emendation with the use of molecular methods and is not yet complete. Of the known *Aspergillus* species, only 20 have been confirmed to cause human infections and three of them are consistently and regularly encountered as etiological agents of over 95% of diseases caused by members of the genus including *A. fumigatus, A. niger,* and *A. flavus* [9]. The other species of this genus related to human lesions are *A. terreus*, *A. glaucus*, *A. nidulans*, *A. oryzae,* and *A. clavatus*. Mode of infection is the inhalation of airborne conidia, exposure to contaminated water (contact with conidia during showering), and nosocomial infections (hospital fabrics and plastics may serve as important sources of *Aspergillus* species). The incubation period is between 2 days and 3 months.

Aspergillosis is a common term used to describe infections caused by different species of *Aspergillus* [10]. Aspergillosis was described as a clinical human disease

under the name of bronchopulmonary *Aspergillus.* The species *A. fumigatus*, with *A. flavus* and *A. niger* are responsible for more than 90% of aspergillosis worldwide. A wide array of clinical forms from allergic reactions (allergic bronchopulmonary aspergillosis, rhinitis, Farmer's lung) to superficial and cutaneous infections, localized aspergilloma, and invasive infections have been reported. Invasive life-threatening aspergillosis occurs mainly in immunocompromised individuals who have undergone widespread antibiotics, cancers, or autoimmune underlying disorders. Invasive infections initiate by entering air-borne conidia to lungs with clinical entities such as invasive sinusitis, fever, facial pain, headache, cough, and dyspnea with subsequent spread to the central nervous system (CNS), leading to seizures or death.

2. Description

In the current book which comprises five distinct chapters, different aspects of the genus *Aspergillus* from *Aspergillus*-host interactions to the immunopathogenesis of aspergillosis, mycotoxin production, and industrial applications of the beneficial species have gained special attention.

It has been shown that host immune status and previous underlying diseases act as important determinants of clinical outcomes and disease spectra of aspergillosis which is life-threatening in the invasive form where the etiologic fungus affects lung tissue and disseminates to different organs with high morbidity and mortality. The role of influenza and COVID-19 infections in ICU patients has been noticed as the new risk factors of invasive aspergillosis. In relation to the immunopathogenesis of aspergillosis, documents demonstrated that following entry of causative *Aspergillus* species, fungal elements are affected by pulmonary host defense in order to clearance of infective conidia. In conditions of poor host immune response, where the neutrophils and macrophages fail to recognize the etiologic fungus, *Aspergillus* conidia attack and destroy airway epithelium and neutrophils play an important role in the clearance of fungal hyphae via oxidative and non-oxidative mechanisms. As an amazing topic in mycotoxin research, the relationship between mycovirus-containing *Aspergillus flavus* and acute lymphoblastic leukemia as carcinogenesis beyond mycotoxin production has been noticed. The role of aflatoxin in *Aspergillus flavus* resistance to stress conditions is a very interesting subject in the importance of members of *Aspergillus* section *Flavi*. In this context, it has been shown that *Aspergillus* employs a considerable amount of energy to synthesize aflatoxins which are not so obviously linked to an enhancement of population fitness. Another important aspect of the genus *Aspergillus* is the industrial application of nanomaterials produced by *Aspergillus* species. These fungi produce a large number of beneficial metabolites enabling the producing fungus to the successful synthesis of nanoparticles.

In conclusion, we would like to thank all authors for their invaluable contribution and hard work to make the successful endeavor on the goals of the present book. We are also grateful to the "In-Tech" Publisher personnel, especially Ms. Karmen Đaleta, who kindly assisted us in the arrangement of the book and scheduling our activities.

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Chapter 2

Aspergillus-Human Interactions: From the Environment to Clinical Significance

Arsa Thammahong

Abstract

Aspergillus species are ubiquitous fungi found in the environment worldwide. The most common *Aspergillus* species causing diseases in humans are *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus.* However, species causing human infections are also depending on human immune status. Host immune status and previous underlying diseases are important factors leading to different clinical manifestations and different disease spectra of *Aspergillus* infections. The most severe form of *Aspergillus* infections is invasive aspergillosis in human tissue, especially invasive pulmonary aspergillosis (IPA), which has high morbidity and mortality in immunocompromised patients. ICU patients with influenza infections and COVID-19 infections are recently risk factors of invasive pulmonary aspergillosis. New diagnostic criteria include galactomannan antigen assays, nucleic acid amplification assays, and lateral flow assays for early and accurate diagnosis. Voriconazole and the newest azole, isavuconazole, are antifungals of choice in IPA. Nevertheless, azole-resistant *Aspergillus* strains are increasing throughout the world. The etiology and spreading of azole-resistant *Aspergillus* strains may originate from the widespread use of fungicides in agriculture, leading to the selective pressure of azole-resistant strains. Therefore, there is a necessity to screen *Aspergillus* antifungal susceptibility patterns for choosing an appropriate antifungal agent to treat these invasive infections. In addition, mutations in an ergosterol-producing enzyme, i.e., lanosterol 14-α demethylase, could lead to azole-resistant strains. As a result, the detection of these mutations would predict the resistance to azole agents. Although many novel azole agents have been developed for invasive *Aspergillus* infections, the rate of novel antifungal discovery is still limited. Therefore, better diagnostic criteria and extensive antifungal resistant *Aspergillus* screening would guide us to better manage invasive *Aspergillus* infections with our existing limited resources.

Keywords: *Aspergillus*, *Aspergillus*-human interactions, invasive aspergillosis, antifungal susceptibility test, azole, voriconazole, amphotericin B, influenza-associated pulmonary aspergillosis, COVID-19-associated pulmonary aspergillosis

1. Introduction

Aspergillus species are saprophytic ubiquitous filamentous fungi [1]. They are in Phylum Ascomycota with both sexual and asexual forms [1]. In their sexual form, they produce asci and ascospores within the appropriate environment, while they produce conidia, or asexual spores, on phialides surrounding their

vesicles at the tip of conidiophores in their asexual form [1]. *Aspergillus* conidia are different in size and shape depending on *Aspergillus* species, which affects the dispersion and infectivity properties of *Aspergillus* [1]. Their conidia can be found in the soil, decomposed piles, air, animals, and humans. They cause diseases in immunocompromised hosts, e.g., patients with acquired immunodeficiency syndrome (AIDS), allogenic hematopoietic stem cell transplant or solid organ transplant candidates, patients with immunosuppressive drugs, patients with prolonged neutropenia, and patients with other underlying diseases [2]. The common pathogenic *Aspergillus* species are *A. fumigatus, A. flavus, A. niger,* and *A. terreus* [3]. There are a wide variety of disease spectra of *Aspergillus* infections, i.e., invasive aspergillosis, chronic aspergillosis, and allergic forms of aspergillosis [1, 2]. The most severe form causing high morbidity and mortality rate, especially in immunocompromised hosts, is invasive aspergillosis (IA) [2, 4]. An increase of immunocompromised hosts would also increase patients with IA with a high mortality rate [4–14].

Invasive aspergillosis (IA) is recently increasing in patients with allogenic hematopoietic stem cell transplantation (HSCT) and solid organ transplantation [5, 8, 13, 15–22]. Underlying conditions of patients with IA are hematological malignancies, e.g., leukemia or lymphoma, bone marrow transplant, and solid-organ transplant patients [5, 8, 13, 15–22]. Recently, not only neutropenic patients are at risk for IA, but non-neutropenic patients with immunosuppressive agents, e.g., biologics, small-molecule kinase inhibitors (SMKIs), Chimeric Antigen Receptor (CAR) T cells, are also at risk [23–28]. In developing countries, poor-controlled diabetes mellitus is one of the critical risk factors of IA [10, 12]. Therefore, risk factors of IA are now patients with malignancy, autoimmune, inflammatory diseases, complex immune-metabolic diseases from aging, immunosuppressive treatment, previous septic conditions, novel biologic treatment, including patients with hematological malignancies receiving SMKIs, patients in ICU, patients with a cytokine storm syndrome from CAR-T cells treated with high-dose corticosteroids, patients in ICU with severe influenza or other viral infections [23–36]. In an era of Coronavirus Disease 2019 (COVID-19) infections, IA was recognized as a severe complication of patients with COVID-19 infections in ICU [37–46].

2. Pathogenesis of *Aspergillus* and its virulence factors

Among thousands of *Aspergillus* species, only less than twenty species could cause diseases in humans [47]. The pathogenic species usually possess virulence factors that help them survive and cause infections inside hosts. *Aspergillus fumigatus* was utilized as a model to study virulence factors in many studies (**Table 1**) [1].

To survive inside the host environment, *Aspergillus* species need to adapt to heat and hypoxic conditions inside hosts. For the heat stress, the trehalose pathway was shown to have a role in heat tolerance and virulence of *A. fumigatus* [47]. Heat shock proteins (HSPs), especially Hsp90, are chaperone proteins associated with stress tolerance, not only for heat [48–50]. In mammalians, HIF1α, as a common transcription factor, controls cellular homeostasis in hypoxic conditions [51]. In fungi, a homolog of HIF1α, called the sterol regulatory element-binding protein (SREBP) or SrbA in *A. fumigatus,* is induced by hypoxia and iron starvation conditions [52–56]. SrbA protein is also associated with the virulence of *A. fumigatus in vivo* [52–54].

A. fumigatus possesses enzymes to protect itself against host reactive oxygen species (ROS), e.g., catalase, superoxide dismutases, thioredoxin, glutathione, including mitochondrial electron transport chain [57–62]. In some animal

Virulence factors	Characteristics
Stress tolerance	• Thermotolerance • Hypoxic adaptation • pH/Reactive oxygen species (ROS) resistance • Secondary metabolites • Light response
Metabolism and nutrient uptake	• siderophores, Zinc Magnesium Copper transporter, calmodulin, calcineurin, phosphate permeases
Cell components	• Cell wall: β-glucan, chitin, rodlet • Galactosaminogalactan (GAG) • Melanin
Others	• Biofilm • Cellular heterogeneity

Table 1.
Essential virulence factors in Aspergillus fumigatus requiring for causing infections inside humans [1].

models, e.g., an eye infection model, demonstrated that these fungal enzymes were essential for fungal virulence [63]. Secondary metabolites are also playing a role in fungal virulence [64–66]. *A. fumigatus* secondary metabolites are gliotoxin, fumigaclavine, trypacidin, helvolic acid, fumitremorgin, fumagillin, and pseurotin, associated with host cellular toxicity [67–71]. However, the mechanisms behind this toxicity is still unclear and need to be further investigated *in vivo* [71]. *A. flavus* produces aflatoxins, which are important carcinogenic secondary metabolites, and other secondary metabolites, called Velvet complex, as environmental response mechanisms [72, 73]. Circadian rhythms or light response, which were studied thoroughly in the *Neurospora* model system, are essential to react with the environment [74]. Light-induced mycelial pigmentation and germination acted as a stress signaling pathway in *A. fumigatus* via transcription factor LreA and FphA, respectively [75–77].

For nutrient acquisition, exoenzymes or proteases are major enzymes produced by *A. fumigatus,* especially the alkaline protease Alp1 and the metalloprotease Mep1 [1, 78]. In *A. fumigatus,* a transcriptional repressor called CreA has a vital role in carbon catabolite repression. *Af*CreA regulates growth on different nitrogen, carbon, and lipid sources and has a role in amino acid transportation, nitrogen, and carbon assimilation, including glycogen and trehalose metabolism [79, 80]. Although CreA is not required for virulence, it is required for disease progression in invasive pulmonary aspergillosis (IPA) mouse models [79–81]. For nitrogen utilization, *Af*RhbA, a Ras-related protein in a nitrogen-regulated signaling pathway, and *Af*AreA, a GATA transcription factor requiring the expression of genes involving nitrogen utilization, are related to virulence in *A. fumigatus* [82–84]. *A. fumigatus* still needs divalent cations, i.e., iron, copper, magnesium, zinc, calcium, for its growth and virulence inside hosts via siderophores, calmodulin, calcineurin, specific importers, and exporters [85, 86].

Additionally, cell wall components of *Aspergillus fumigatus* are also essential virulence factors for fungal survival inside hosts and are important for host immune response [87–92]. Cell wall components consist of β-1,3-glucan, chitin, galactomannan, α-1,3-glucan, and melanin depending on different stages of *A. fumigatus*, i.e., conidial, or hyphal stage [91–95]. β-1,3-glucan, a central component of *Aspergillus* cell wall polysaccharide, is a pathogen-associated molecular

pattern (PAMP) recognized by host pattern recognition receptors (PRR), e.g., dectin-1 [88]. During its conidial stage, rodlet, or hydrophobins, and dihydroxynapthalene (DHN) melanin are present to protect fungal conidia against host immune response by evading host pathogen-associated molecular patterns (PAMPs) recognition, including protecting fungi from unfavorable stress conditions [93–97]. Furthermore, in its hyphal stage, galactosaminogalactan (GAG), which is a water-insoluble polymer consisting of a pyranose-form galactose, galactosamine, and N-acetylgalactosamine (GalNAc), is present as an extracellular matrix on an outer layer of the cell wall [98]. GAG is associated with biofilm formation and immunosuppression properties by masking PAMP exposure and resisting neutrophil killing via neutrophil extracellular traps (NETs) [99–102]. The linkage between cell wall components and metabolic pathways is still unclear. Nevertheless, these components share the same building blocks, e.g., UDP-glucose, glucose 6-phosphate, with specific metabolic pathways, e.g., glycolysis, trehalose biosynthesis pathway [81, 103–105]. It is possible that the homeostasis of cell wall biosynthesis is involved with some metabolic pathways, e.g., the trehalose biosynthesis pathway. Disruption of one of these trehalose enzymes or building blocks would result in decreased virulence due to changes in cell wall compositions [81, 103–105]. Understanding this homeostasis would lead to the discovery of novel antifungal targets in the future.

3. Diagnosis of invasive *Aspergillus* infections: challenge in the field

Aspergillus infections are associated closely with host immune status [106, 107]. Severe asthma with fungal sensitization and allergic bronchial pulmonary aspergillosis (ABPA) are found in immunocompetent hosts with hypersensitivity, while aspergilloma and chronic pulmonary aspergillosis are found in immunocompetent hosts with previous structural diseases, such as lung cavity from previous tuberculosis infections [108]. In immunocompromised hosts, invasive aspergillosis is common and severe, causing high morbidity and mortality in patients [108, 109].

For invasive pulmonary aspergillosis, early diagnosis and prompt treatment are the keys to decrease the disease burden. Differentiation between *Aspergillus* colonization and invasive infections is still challenging [25, 92, 93]. Recently, the revised EORTC guideline for diagnosis of invasive fungal infections, including *Aspergillus* infections, recommended the diagnostic criteria including host factors, clinical, radiological, and microbiological criteria with new diagnostic methods (**Table 2**) [109]. Proven invasive aspergillosis is confirmed with histopathologic, cytopathologic, microscopic analysis, or nucleic acid analysis of sterile specimens or tissue or formalin-fixed paraffin-embedded tissue (FFPE), including culture recovered from sterile sites [109]. Species of common *Aspergillus* recovered from cultures are differentiated using macroscopic and microscopic morphology, but the nucleic acid analysis is necessary for the species complex (**Table 3**) [110]. For probable and possible invasive aspergillosis, host factors, clinical features, and mycological evidence are including for the diagnosis of invasive aspergillosis. Host factors include the history of neutropenia, which is less than 500 neutrophils/mm^3, for more than ten days, hematological malignancy, allogenic stem cell transplantation, solid organ transplantation, therapeutic-dose corticosteroids at not less than 0.3 mg/kg for not less than three weeks during the previous 60 days, treatment with T-cell or B-cell immunosuppressants, inherited immunodeficiency, or acute graft-versus-host disease grade III or IV [109]. For clinical evidence of pulmonary aspergillosis, a chest high-resolution CT scan is recommended to observe any halo

Diagnosis of invasive aspergillosis.	Criteria
Proven	• Microscopic analysis: from needle aspiration or biopsy OR • Culture: from sterile sites except for BAL fluid, paranasal sinuses, and urine OR • Tissue nucleic acid analysis from formalin-fixed paraffin-embedded tissue
Probable: 1 host factor + 1 clinical feature+1 mycological evidence	Host factors • Recent neutropenia • Hematological malignancy • Receipt of an allogenic stem cell transplant • Receipt of a solid organ transplant • Prolonged use of corticosteroids • Use of T-cell immunosuppressants • Use of B-cell immunosuppressants • Inherited severe immunodeficiency • Acute GVHD grade III or IV Clinical features: pulmonary aspergillosis • One of the following CT Chest patterns: ○ Dense well-circumscribed lesion with or without a halo sign ○ Air crescent sign ○ Cavity ○ Wedge-shaped and segmental or lobar consolidation Mycological evidence • Culture positive from sputum, BAL, bronchial brush, or aspirate • Direct examination positive from sputum, BAL, bronchial brush, or aspirate • Galactomannan antigen: plasma serum BAL CSF: any of: ○ Single serum or plasma >/= 1 ○ BAL fluid >/= 1 ○ Single serum or plasma >/= 0.7 and BAL fluid >/= 0.8 ○ CSF >/= 1 • *Aspergillus* PCR: any of: ○ Plasma, serum, or whole blood 2 or more consecutive PCR ○ BAL fluid 2 or more duplicate PCR ○ At least 1 PCR from plasma serum or whole blood & 1 PCR from BAL fluid
Possible: 1 host factor + 1 clinical feature	

Table 2.
Diagnosis of invasive aspergillus infections from revised EORTC/MSG criteria 2020 (BAL: bronchoalveolar lavage; CT: computed tomography; CSF: cerebrospinal fluid; GVHD: graft versus host disease; PCR: polymerase chain reaction) [109].

sign, air-crescent sign, cavity, or wedge-shaped and segmental or lobar consolidation [109, 111]. Probable invasive aspergillosis still needs at least one mycological evidence to support the diagnosis. Mycological evidence is including cultures recovered from sputum, bronchoalveolar lavage (BAL), bronchial brush, or

***Aspergillus* species**	**Macroscopic features**	**Microscopic features**
Aspergillus fumigatus	Typical blue-green colony with suede-like surface	Columnar uniseriate conidial heads with phialides limited to upper two-thirds of its vesicles; short and smooth conidiophores; basipetal green, rough-walled globose to subglobose conidia
Aspergillus flavus	Bright to dark yellow-green colony with a granular, flat surface	Radiate biseriate conidial heads with phialides over the surface of mature vesicles; coarsely rough conidiophores; pale green, globose to subglobose conidia
Aspergillus niger	Dark brown to the black colony with white to yellow color at the reverse side of the colony	Globose, large, dark brown, biseriate, radiate conidial head with long metulae; smooth, hyaline conidiophores; dark brown, rough conidia
Aspergillus terreus	Cinnamon-brown colony with suede-like surface and yellow to deep brown color at the reverse side of the colony	Compact, columnar, biseriate conidial heads; hyaline, smooth conidiophores; hyaline to yellow, smooth-walled conidia

Table 3.
Macroscopic and microscopic features of clinical-relevant Aspergillus species (colony on Czapek Dox agar at 30°C) [110].

aspirate [109]. *Aspergillus* galactomannan antigen assays with different thresholds depending on specimens, including serum, BAL fluid, plasma, and cerebrospinal fluid (CSF), support the diagnosis of invasive aspergillosis [112–115]. However, decreased sensitivity of galactomannan antigen assay is observed in patients with anti-mold therapy [115]. In addition, *Aspergillus* PCR from blood and BAL fluid is introduced to confirm the diagnosis and identify specific *Aspergillus* species with certain mutations related to triazole resistance [109, 116–124].

Nonetheless, revised EORTC/MSG criteria for diagnosing invasive fungal infections may be applied mainly for neutropenic patients or immunocompromised patients. Therefore, specific guidelines for the diagnosis of invasive aspergillosis in non-neutropenic patients in ICU (Invasive pulmonary aspergillosis in ICU, AspICU) or patients with influenza (Influenza-associated pulmonary aspergillosis, IAPA) or Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (COVID-19 associated pulmonary aspergillosis, CAPA) co-infections were developed and published for early and accurate diagnosis (**Table 4**) [31, 125–127].

4. Treatment of *Aspergillus* infections

IA also includes the infections of the lower respiratory system, sinuses, and skin as entry routes. In addition, the cardiovascular system, central nervous system, and other tissues could be infected from hematogenous dissemination or direct extension from adjacent infected tissues [2]. Infectious Diseases Society of America (IDSA, 2016) and ESCMID-ECMM-ERS (2017) recommended voriconazole (6 mg/kg, intravenous route every 12 hours for one day, and then 4 mg/kg every 12 hours; 200–300 mg every 12-hour, oral route) as a first-line treatment for invasive pulmonary aspergillosis (IPA) [2, 128]. For alternative treatment, liposomal amphotericin B (3–5 mg/kg/day, intravenous route) and isavuconazole (200 mg every 8 hours for three days and then 200 mg daily) [2]. For other invasive aspergillosis syndromes, i.e., invasive sinus aspergillosis, tracheobronchial aspergillosis, invasive aspergillosis of the central nervous system or cardiovascular system, *Aspergillus* osteomyelitis,

Diagnostic criteria of IPA	**AspICU [125]**	**IPA with influenza (IAPA) [126]**	**IPA with SARS-CoV-2 (CAPA) [127]**
Host factors	One of the following: • Neutropenia (<500/mm^3) before or at ICU admission • Hematological or oncological malignancy with cytotoxic therapy • Glucocorticoid treatment with prednisolone equivalent >20 mg/day • Immunodeficiency	Entry criteria: influenza-like illness + positive influenza PCR or antigen + timing (7 days before and 96 hours after ICU admission)	Entry criteria: patients with COVID-19 infections (RT-PCR) in ICU with a temporal relationship to suspected IPA
Clinical features	One of the following: • Fever with appropriate antibiotic treatment for at least three days • Recurrent fever after a fever-free period for at least 48 hours with antibiotics and without other apparent cause • Dyspnea • Hemoptysis • Pleuritic chest pain or pleural friction rub • Worsening respiratory failure with appropriate antibiotics and ventilator support	None	None
Radiological evidence	• Any medical imaging by conventional chest X-ray or CT scan of lungs	• Pulmonary infiltrate OR • Cavitating infiltrate (not from other causes)	• Pulmonary infiltrate OR • Cavitating infiltrate (not from other causes)

Diagnostic criteria of IPA	**AspICU [125]**	**IPA with influenza (IAPA) [126]**	**IPA with SARS-CoV-2 (CAPA) [127]**
Microbiological evidence	• *Aspergillus* recovered from the lower respiratory tract (LRT) (entry criterion) • *Aspergillus*-positive culture of BAL fluid without bacterial growth together with a positive microscopic analysis showing branching hyphae (if no host factor)	• If pulmonary infiltrate presents, at least one of the following: ○ Galactomannan (GM) antigen assay: serum >0.5 or BAL ≥ 1.0 or ○ positive culture from BAL • If lung cavity presents, at least one of the following: positive sputum culture or tracheal aspirate culture	Probable CAPA: at least one of the following: • Microscopic detection of septate hyphae in BAL • Positive BAL culture • Serum GM >0·5 or serum LFA index >0·5 • BAL GM ≥1·0 or BAL LFA index ≥1·0 • Two or more positive *Aspergillus* PCR in plasma, serum, or whole blood or a single positive *Aspergillus* PCR in BAL (<36 cycles); or a single positive *Aspergillus* PCR in plasma, serum, or whole blood with a single positive in BAL fluid (any threshold cycle) Possible CAPA: at least one of the following: • Microscopic detection of septate hyphae in non-BAL • Positive non-BAL culture • Single non-BAL GM >4·5 • Non-BAL GM >1·2 twice or more • Non-BAL GM >1·2 plus another non-BAL PCR or LFA positive

Diagnostic criteria of IPA	**AspICU [125]**	**IPA with influenza (IAPA) [126]**	**IPA with SARS-CoV-2 (CAPA) [127]**
Categories	• Proven IPA: similar to EORTC/MSG 2020 criteria • Putative IPA: *Aspergillus*-positive from LRT + Clinical evidence + Radiological evidence + (Host factors or *Aspergillus* culture from BAL with positive microscopic analysis) • Colonization: ≥ 1 criterion for a diagnosis of putative IPA is not fulfilled	• Proven IAPA: entry criteria with tissue diagnosis similar to EORTC/MSG 2020 criteria • Putative IAPA: entry criteria + Radiological evidence + Microbiological evidence • Colonization: ≥ 1 criterion for a diagnosis of putative IPA is not fulfilled	• Proven CAPA: entry criteria with tissue diagnosis similar to EORTC/MSG 2020 criteria • Probable CAPA: entry criteria + radiological evidence + probable criteria of microbiological evidence • Possible CAPA: entry criteria + radiological evidence + possible criteria of microbiological evidence

Table 4.
Diagnostic criteria for invasive pulmonary aspergillosis (IPA) of patients in ICU (AspICU) or with influenza (IAPA) or SARS-CoV-2 (CAPA) coinfections (PCR: polymerase chain reaction; ICU: intensive care unit; RT-PCR: Real-time polymerase chain reaction; BAL: bronchoalveolar lavage; GM: galactomannan; LFA: lateral flow assay) [31, 125–127].

Aspergillus endophthalmitis and keratitis, cutaneous aspergillosis, and *Aspergillus* peritonitis, intravenous voriconazole is still the first-line therapy [2]. For IPA in ICU patients, patients with hematological malignancies, or solid organ transplants, IAPA, and CAPA, voriconazole and isavuconazole are still recommended as the first-line treatment (**Table 5**).

Voriconazole is metabolized at the liver via CYP2C19 and CYP3A4 [135]. Medications with CYP2C19 and CYP3A4-dependent metabolism, antacids, proton pump inhibitors may affect serum voriconazole levels [136]. Adverse reactions and toxicity of voriconazole are associated with higher serum voriconazole levels [137]. Common adverse reactions include reversible visual disturbances, hepatotoxicity, photosensitivity, reversible visual or auditory hallucinations, tachyarrhythmias, and QT interval prolongations [137, 138]. Isavuconazole is a second-generation broad-spectrum triazole requiring a loading dose with a five-day half-life [139]. Isavuconazole has fewer adverse reactions in photosensitivity, hepatotoxicity, visual abnormality, and less drug–drug interaction [140–142]. Isavuconazole is a CYP3A4 inhibitor and can decrease the metabolism of sirolimus, tacrolimus, cyclosporine, and digoxin, leading to increased levels of these agents [142]. Furthermore, isavuconazole can induce dose-dependent QTc shortening [143]. Isavuconazole was shown to be non-inferior to voriconazole to treat invasive mold disease from the SECURE trial [144]. Posaconazole is also a broad-spectrum triazole used mainly for prophylaxis and salvage treatment of invasive fungal infections [145]. A suspension form of posaconazole has unpredictable bioavailability and needs a high-fat meal for better absorption [146]. However, tablet and IV formulations overcome this limitation. Posaconazole strongly inhibits CYP3A4 and is metabolized through UGT1A4 [145]. Using CYP3A4 substrates with posaconazole should be cautious [145]. The common adverse effects of posaconazole are gastrointestinal disturbances, hepatotoxicity, rashes, fever, hypokalemia, hypomagnesemia, and QTc prolongation [145].

Amphotericin B, a polyene antifungal agent binding to ergosterol in the fungal cell membrane, has many forms, i.e., conventional with deoxycholate and lipid-based form [2, 147]. Conventional amphotericin B has common adverse effects, including acute reactions after infusion (fever, chills, nausea), phlebitis, hypokalemia, hypomagnesemia, and nephrotoxicity (usually from renal tubular acidosis). The lipid-based form has less nephrotoxicity than the conventional form [2]. Nevertheless, acute infusion reactions may still present in liposomal amphotericin B [148]. In addition, hypokalemia, hypomagnesemia, mild bilirubin, alkaline phosphatase elevations are also present occasionally in lipid-based amphotericin B [2]. Lipid-based amphotericin B is recommended for alternative treatment of invasive aspergillosis in case that azoles cannot be used [2].

Echinocandins, e.g., caspofungin, micafungin, is a non-competitive β-1,3 D-glucan synthase inhibitor leading to loss of fungal cell wall's strength and integrity [149, 150]. Echinocandins have fewer adverse reactions and fewer drug–drug interactions [149, 150]. Echinocandins are recommended for salvage therapy or in azole-resistant *Aspergillus* infections combined with azoles for invasive aspergillosis treatment (**Table 5**) [2, 151–153].

Therapeutic drug monitoring (TDM) of azoles, e.g., voriconazole, posaconazole, isavuconazole, is necessary, especially in elderly patients, obese patients, critically ill patients, and patients with potential azole drug–drug interactions [2]. For treatment of IA, IDSA recommended TDM of voriconazole at a trough level of >1–1.5 μg/mL but less than 5–6 μg/mL to prevent neurotoxicity [2]. American Society of Transplantation Infection Diseases Community of Practice (AST) recommended TDM of posaconazole (suspension and tablet form) and isavuconazole at a trough level of >1–1.25 μg/mL and 2–3 μg/mL, respectively [154]. Timing

Condition	First-line treatment	Prophylaxis
IPA in ICU patients [129, 130]	Voriconazole (6 mg/kg, intravenous route every 12 hours for one day, and then 4 mg/kg every 12 hours; 200–300 mg every 12 hours oral route) or Isavuconazole (200 mg every 8 hours for 3 days and then 200 mg daily) (Liposomal amphotericin B, 3–5 mg/kg/day, intravenous route, in ICU patients with severe liver insufficiency, cirrhosis Child-Pugh scores B, C)	In immunocompetent patients in ICU, prophylaxis is not recommended for IPA
IPA in patients with hematological malignancies [131–133]	Voriconazole or Isavuconazole (Liposomal amphotericin B as alternative treatment)	Posaconazole (oral solution 200 mg every eight hours or tablet/intravenous route 300 mg every 12 h on day one then 300 mg daily) (in AML and MDS undergoing intensive chemotherapy with the incidence of invasive mold diseases >8% or in graft-versus-host disease) Voriconazole (200 mg orally every 12 h) (in HSCT)
IPA in patients with solid organ transplantation [134]	Voriconazole or Isavuconazole (Liposomal amphotericin B in hepatotoxicity, drug–drug interaction, ≥10% environment azole-resistant isolates found)	Kidney and heart transplantation are not recommended Lung transplantation: voriconazole, nebulized liposomal amphotericin B
IAPA [31, 126]	Voriconazole or Isavuconazole	No current recommendation Need further studies
CAPA [127]	For azole sensitive: Voriconazole or Isavuconazole (for 6–12 weeks) For azole-resistant: - Suspected: voriconazole + echinocandin (Caspofungin 70 mg first day followed by 50 mg daily) or isavuconazole + echinocandin - Proven: Liposomal amphotericin B	No current recommendation Need further studies

Table 5.
Treatment of invasive pulmonary aspergillosis (IPA) in ICU patients, patients with hematological malignancies, or solid organ transplants, influenza-associated pulmonary aspergillosis (IAPA), and COVID-19 associated pulmonary aspergillosis (CAPA) (AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; HSCT: hematological stem cell transplantation).

for measuring serum trough concentration of voriconazole, posaconazole, and isavuconazole is at 5–7 days, after 5 days, and within 7 days, respectively [154]. For prophylaxis, International Society for Heart and Lung Transplantation (ISHLT) recommended TDM of voriconazole and posaconazole at a trough level of ≥1 μg/mL and > 0.7 μg/mL, respectively [155]. Additionally, in CAPA, ECMM/ISHAM

recommended weekly TDM of voriconazole and posaconazole at a trough level of 2–6 μg/mL and 1–3.75 μg/mL, respectively [127].

5. Azole-resistant *Aspergillus*

5.1 Etiology and clinical significance

Voriconazole and isavuconazole are the first-line therapy of invasive aspergillosis [2, 129, 130]. Furthermore, azoles, i.e., posaconazole and voriconazole, are also used as prophylaxis of invasive aspergillosis in patients with hematological malignancies and solid organ transplantation [131–134]. Therefore, azoles are important antifungal agents to combat invasive aspergillosis. Unfortunately, azole-resistant *Aspergillus fumigatus* strains are emerging and increasing, leading to increased treatment failure and mortality [156, 157]. The etiology of these emerging azole-resistant *A. fumigatus* (ARAF) may be from the environmental selective pressure associated with azole fungicides in the agricultural area, including Europe, Asia, Latin America, the Midwest, and Southeast states of the USA [158–161]. The supporting evidence of environment-derived ARAF is that ARAF strains were recovered from azole-naive patients [158, 162–165]. In addition, the most common mutations at the *cyp51A* gene (encoding lanosterol 14-α demethylase) causing azole resistance in ARAF strains, which are TR_{34}/L98H and TR_{46}/Y121F/T289A mutations, were also recovered from patients' homes and surroundings [166–171].

Azole fungicides, i.e., bromuconazole, difenoconazole, epoxiconazole, enilconazole, metconazole, prochloraz, propiconazole, prothioconazole-desthio, and tebuconazole, play an important role in the development of environment-derived azole-resistant *Aspergillus* isolates leading to cross resistance to medical azoles [169, 172, 173].

Antifungal susceptibility tests (AST) of *Aspergillus* species are essential for screening azole-resistant *Aspergillus* isolates. The indications to perform *Aspergillus* AST are that the fungus is recovered from sterile sites in regions with high azole-resistant rates, including long-term azole treatment in chronic bronchopulmonary aspergillosis and breakthrough *Aspergillus* infections or recurrent or persistent infections [2, 128, 174].

The standard antifungal susceptibility testing of filamentous fungi to observe the minimum inhibitory concentration (MIC) using broth microdilution assays was described by the Clinical and Laboratory Standards Institute (CLSI) and the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) [175, 176]. To determine antifungal resistance of *Aspergillus* species, e.g., *A. flavus, A. fumigatus, A. niger, A. terreus,* CLSI and EUCAST utilized two values, which are epidemiological cutoff values (ECVs or ECOFFs) and clinical breakpoints (BP) (**Table 6**). ECVs for CLSI and ECOFFs for EUCAST of each antifungal agent against each *Aspergillus* originate from MIC distribution of the wild-type *Aspergillus* population [175–178]. These values can divide *Aspergillus* strains into two groups, which are wild-type and non-wild-type strains. Non-wild-type strains may resist those antifungal agents [175, 176, 178]. Clinical breakpoints are based on antifungal pharmacodynamics, pharmacokinetics, data from clinical trials, and patient outcomes [175, 176, 178]. Resistance is determined by the MICs over R (resistant) (**Table 6**). For EUCAST, another value is the area of technical uncertainty (ATU), which is the value that needs to be addressed before reporting these results, i.e., repeating the test, using a genotypic test, changing the susceptibility category, or including ATU as a part of the report [176].

Aspergillus species	Antifungal agents	CLSI M59 & M61, 2020 (μg/mL)				EUCAST BP_ECOFF v2.0, 2020 (μg/mL)			
		ECV	S	I	R	ECV	S≤	R>	ATU
A. flavus	Amphotericin B	4	—	—	—	4	—	—	—
	Caspofungin	0.5	—	—	—	—	—	—	—
	Isavuconazole	1	—	—	—	2	1	2	2
	Itraconazole	1	—	—	—	1	1	1	2
	Posaconazole	0.5	—	—	—	0.5	—	—	—
	Voriconazole	2	—	—	—	2	—	—	—
A. fumigatus	Amphotericin B	2	—	—	—	1	1	1	—
	Caspofungin	0.5	—	—	—	—	—	—	—
	Isavuconazole	1	—	—	—	2	1	2	2
	Itraconazole	1	—	—	—	1	1	1	2
	Posaconazole	—	—	—	—	0.25	0.125	0.25	0.25
	Voriconazole	1	≤0.5	1	≥2	1	1	1	2
A. niger	Amphotericin B	2	—	—	—	0.5	1	1	—
	Caspofungin	0.25	—	—	—	—	—	—	—
	Isavuconazole	4	—	—	—	4	—	—	—
	Itraconazole	4	—	—	—	4	—	—	—
	Posaconazole	2	—	—	—	0.5	—	—	—
	Voriconazole	2	—	—	—	2	—	—	—
A. terreus	Amphotericin B	4	—	—	—	8	—	—	—
	Caspofungin	0.12	—	—	—	—	—	—	—
	Isavuconazole	1	—	—	—	1	1	1	—
	Itraconazole	2	—	—	—	0.5	1	1	2
	Posaconazole	1	—	—	—	0.25	0.125	0.25	0.25
	Voriconazole	2	—	—	—	2	—	—	—

Table 6.
Interpretation of antifungal susceptibility tests and epidemiological cutoff values (ECVs) of Aspergillus species according to CLSI M59 and M61, 2020 and EUCAST BP ECOFF version 2, 2020 (S: susceptible, I: intermediate, R: resistant, ATU: Area of Technical Uncertainty) [175–177].

Molecular methods to detect *CYP51A* mutations, e.g., TR_{34}/L98H, TR_{46}/Y121F, are established by using classic PCRs with sequencing, real-time PCRs, loop-mediated isothermal amplification (LAMP), or whole-genome sequencing (WGS) [179]. These molecular methods have a high negative predictive value to rule out these resistant strains' infections [179]. However, they had narrow coverage and mutations at this point depending on association data between mutations and antifungal resistance property. Furthermore, commercial tools are still not approved by the US FDA [179].

5.2 Management of azole-resistant *Aspergillus* and novel antifungal candidates

Overexpression with a tandem repeat in the promoter area (TR_{34} or TR_{46}) and point mutations (L98H or Y121F/T289A) in the *cyp51A* gene, encoding azole's target called lanosterol 14-α demethylase, would lead to azole resistance in *Aspergillus*

fumigatus including voriconazole and isavuconazole [156, 178]. To treat these azole-resistant *Aspergillus* infections, monotherapy of each azole should be avoided, especially in areas with more than 10% of azole resistance prevalence [180]. In areas with high rates of azole resistance, liposomal amphotericin B and a combination of voriconazole and echinocandin should be considered [2, 127, 128, 156, 180]. Therefore, the prevalence of azole-resistant *Aspergillus* strains using conventional culturing methods together with broth microdilution assays or using molecular biology (RT-PCR) is essential to decide the optimal treatment and to choose suitable antifungal agents to get rid of these infections [156, 179].

From the increased speed of azole-resistant *Aspergillus* strains, novel antifungal agents with high efficacy and fewer side effects are crucial to combat these infections with very high mortality [156]. However, discovering these novel antifungal agents has many steps and methods to evaluate both *in vitro* and *in vivo* analyses for both antifungal activity and toxicity [181, 182]. The first step for screening antifungal activity has many methods depending on the screening purpose [181]. To observe the antifungal activity of novel antifungal candidates, the broth microdilution method is the standard method to provide the MICs [183]. This method is perfect for various compounds requiring high throughput assays [181]. Furthermore, this method requires a small number of compounds and can apply to different *Aspergillus* species simultaneously [181]. To observe combinatorial effects between novel antifungal candidates and current antifungal agents, checkerboard assays are used to determine the fractional inhibitory concentration index (FICI) [184, 185]. The FICI is calculated using the sum of the fractional inhibitory concentration (FIC_1) of the first compound, which is MIC_{1+2} of the combination of the first and the second compounds divided by MIC_1 of the first compound alone, and the FIC_2 of the second compound [184, 185]. Synergistic, additive, indifferent, and antagonistic effects are defined by FICI $\leq$0.5; >0.5–1; >1–4; and > 4, respectively [184–186]. For the cytotoxicity effects on human epithelial cells, many *in vitro* colorimetric assays, including mammalian tissue culture systems and vital dyes, are used, such as Alamar blue, MTT, XTT (tetrazolium) assays [181]. Next steps after *in vitro* studies to prove the antifungal activity and toxicity, *in vivo* animal models are used to study pharmacodynamics and pharmacokinetics, including *in vivo* antifungal activity and *in vivo* toxicity [181]. Then, these antifungal candidates would follow through the clinical trial phase I (safety), phase II (checking effectiveness), phase III (confirming effectiveness, side effects), and get approved [181, 182, 187].

Many novel antifungal compounds against both classical targets and novel targets are in clinical trials (**Table 7**) [262]. Novel targets against *Aspergillus* species include glycosylphosphatidylinositol (GPI) anchor protein, dihydroorotate dehydrogenase in pyrimidine synthesis, fungal mitochondrial respiration chain, siderophore iron transporter, Heat shock protein 90 (Hsp90), calcineurin, histone deacetylase (HDAC), inositol phosphorylceramide (IPC) synthase, chitin synthase, and sphingolipid pathway (**Table 7**). Nevertheless, more clinical trials are on the way for these agents before using them in the clinical practice against antifungal-resistant *Aspergillus*/fungal strains.

In addition, enzymes in the *Aspergillus* trehalose biosynthesis pathway, i.e., trehalose-6-phosphate synthase, trehalose-6-phosphate phosphatase, trehalase enzymes, were identified as important virulence factors, including proteins related to the trehalose pathway, i.e., *Af*SsdA, *Af*TslA [103, 105, 263, 264]. The trehalose pathway in *A. fumigatus* is associated with cell wall integrity and fungal virulence *in vivo* [103, 264, 265]. However, inhibitors of this pathway are still lacking and under-investigated. Validamycin A is one of the inhibitors of trehalase enzymes and was first demonstrated its strong antifungal activity against a plant fungal

Name.	Target	Mechanism	Advantage	Administration	Clinical trial
Classical targets					
Encochleated amphotericin B (CAmB) [188–192]	Ergosterol	Renovated structure of amphotericin B with cochleated lipid-crystal nanoparticles	Oral administration, broad-spectrum, less toxicity	Oral	Phase I
Rezafungin (CD101) [192–203]	1,3-β-D-glucan synthase (FKS)	1,3-β-D-glucan synthase inhibitor	Improved stability, long half-life (once a week), activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger*	Intravenous	Phase III
Ibrexafungerp (SCY-078) [204–210]	1,3-β-D-glucan synthase (FKS)	1,3-β-D-glucan synthase inhibitor	activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger,* including itraconazole-resistant *Aspergillus*	Oral and intravenous	Phase III
VT-1598 [211, 212]	Lanosterol demethylase (CYP51)	Tetrazole, inhibiting lanosterol demethylase	Less drug–drug interactions, long half-life, broad-spectrum: *Candida, Aspergillus*	Oral	Phase I
VT-1161 (oteseconazole) [213, 214]	Lanosterol demethylase (CYP51)	Tetrazole, inhibiting lanosterol demethylase	Less drug–drug interactions, long half-life: activity against azole-resistant *Candida,* onychomycosis	Oral	Phase III
VT-1129 (quilseconazole) [215–220]	Lanosterol demethylase (CYP51)	Tetrazole, inhibiting lanosterol demethylase	Less drug–drug interactions, long half-life, brain penetration, activity against *Cryptococcus, Candida*	Oral	Phase I
PC945 [221–227]	Lanosterol demethylase (CYP51)	Triazole, inhibiting lanosterol demethylase	Fungicidal, high lung exposure, activity against *A. fumigatus*	Inhalation	Phase II
Novel targets					
Fosmanogepix (APX001) [228–236]	Glycosylphosphatidylinositol (GPI) anchor protein synthesis (GWT1)	Inhibiting GPI	Fungal-specific target, broad-spectrum, activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger*	Intravenous and oral	Phase II
APX2096 [236]	Glycosylphosphatidylinositol (GPI) anchor protein synthesis (GWT1)	Inhibiting GPI	Strong activity against *Cryptococcus,* effective blood–brain barrier penetration	Intraperitoneal and oral	—
Olorofim (F901318) [237–239]	Dihydroorotate dehydrogenase in pyrimidine synthesis	Inhibiting pyrimidine synthesis	Activity against *A. fumigatus, A. terreus, A. flavus,* and *A. nidulans,* including azole-resistant *A. fumigatus*	Intravenous and oral	Phase III

Name.	Target	Mechanism	Advantage	Administration	Clinical trial
T-2307 [240–242]	Intracellular mitochondrial membrane respiration potential	Inhibiting mitochondrial respiration chain (arylamidine)	Uptaking more by fungal cells, fungicidal activity against *A. fumigatus, A. terreus, A. flavus, A. nidulans,* and *A. niger*	Subcutaneous	Phase I
VL-2397 (ASP2397) [243–245]	Unknown	Uptaking by siderophore iron transporter (Sit1)	Fungicidal, activity against *A. fumigatus, A. terreus, A. flavus,* and *A. niger*	Intravenous	Phase II
Geldanamycin [246–248]	Heat shock protein 90 (Hsp90)	Inhibiting Hsp90	Synergy to caspofungin	Intravenous	—
Tacrolimus (FK506) [249–251]	Calcineurin	Inhibiting calcineurin	Synergy to caspofungin, activity against *A. fumigatus*	Intravenous and oral	—
Cyclosporin A [249, 252]	Calcineurin	Inhibiting calcineurin	Activity against *A. fumigatus*	Intravenous, oral, and topical	—
FK506 analogs (9D31OD-FK506) [251]	Calcineurin	Inhibiting calcineurin	Synergy to azoles, decrease T-cell toxicity and host immunosuppression	Intravenous	—
Trichostatin A [253]	Histone deacetylase (HDAC)	Inhibiting HDAC	Synergy to caspofungin, activity against *A. fumigatus*	Intravenous	—
MGCD290 [254]	Histone deacetylase (HDAC)	Inhibiting HDAC	Synergy to caspofungin, azole, broad spectrum	Oral	Phase II
Aureobasidin A [255–258]	Inositol phosphorylceramide (IPC) synthase	Inhibiting IPC synthase	Synergy to caspofungin	Intravenous and oral	—
Nikkomycin [259]	Chitin synthase	Inhibiting chitin synthase	Broad-spectrum	Intravenous	—
BHBM D13 [260, 261]	Sphingolipid pathway	Acylhydrazone, inhibiting fungal sphingolipid glucosylceramide (GlcCer) synthesis	Broad-spectrum, specific to fungi, fungicidal, blood–brain barrier penetration, less toxicity	Intraperitoneal and oral	—

Table 7.
Summary of novel antifungal agents against classical targets and novel targets for Aspergillus infections.

pathogen, *Rhizoctonia solani* [266–269]. Furthermore, validamycin A has antifungal activity against *Candida albicans* and *Aspergillus flavus* [186, 270]. Validamycin A also possesses combinatorial effects with conventional amphotericin B against *A. flavus* [186]. Nevertheless, *in vivo* experiments are still necessary to verify an antifungal activity of validamycin A. Additionally, the high-osmolarity glycerol (HOG)-mitogen-activated protein kinase (MAPK) signaling pathway is associated with trehalose production and stress response in *A. fumigatus* [271–274]. This signaling pathway may be another good antifungal target to be developed in the future. Therefore, there are many more pathways involved with *Aspergillus* virulence, and there are so many unexplored areas in *Aspergillus* pathogenesis to develop novel antifungal candidates. With this knowledge, we could overcome the shortage of antifungal agents against many more antifungal-resistant *Aspergillus* strains to emerge very soon.

6. Conclusion

Aspergillus species are common fungi found everywhere around humans. They adapt and express many virulence factors to survive inside hosts and cause infections in immunocompromised hosts. Recently, new risk factors that cause severe invasive pulmonary aspergillosis are ICU patients with influenza infections or COVID-19 infections. The diagnosis of invasive aspergillosis, especially without proven tissue or culture evidence, is still challenging. New molecular methods, i.e., nucleic acid assays, lateral flow assays, are introduced for supporting the diagnosis of probable and possible invasive aspergillosis. Nevertheless, voriconazole and isavuconazole are the first-line therapy in IPA in ICU patients, patients with hematological malignancies, patients with IAPA, and CAPA. Furthermore, posaconazole is the principal antifungal agent for the prophylactic treatment of IPA in patients with hematological malignancies. Additionally, emerging azole-resistant *Aspergillus* strains are increasing, and the management against these azole-resistant *Aspergillus* strains is the combination therapy between azoles and echinocandins, including liposomal amphotericin B. Although novel antifungal agents against *Aspergillus* species are on their way, antimicrobial stewardship of existing antifungal agents is also crucial to prevent further breakthrough antifungal-resistant strains in the future. With our better understanding of *Aspergillus* pathogenesis, the shortage of antifungal agents against *Aspergillus* and its resistant strains would no longer be for the better lives of patients suffering from *Aspergillus* infections.

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Conflict of interest

The author declares no conflict of interest.

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Chapter 3

Immunopathogenesis of Aspergillosis

Shreya Singh, Rimjhim Kanaujia and Shivaprakash M. Rudramurthy

Abstract

Aspergillus species are ubiquitous saprophytes and opportunistic pathogens causing wide spectrum of diseases in humans depending on the host immune status. Following pathogen entry, various soluble bronchopulmonary factors enhance conidial clearance. However, due to virulence factors and poor host immune response *Aspergillus* conidia bind and damage the airway epithelium. The host immune cells like neutrophils and macrophages recognise *Aspergillus* spp. through various pathogen recognition receptors and form reactive oxygen species which mediate conidial killing. Neutrophils also attack extracellular hyphae by oxidative attack, non-oxidative granule proteins and neutrophil extracellular traps. In case of adaptive immunity, Th1 cells are crucial sources of IFN-γ mediated protective immunity. The Th17 also display a highly pro-inflammatory which is counterbalanced by a Treg cell. B cells and antibodies also enhance fungal clearance although excessive IgE production may result in atopy. The immune responses are influenced by changes in production of short-chain fatty acids by the gut microbiome which primes cells toward Th2 responses, and this is synchronized by the Innate lymphoid cells. This review provides comprehensive knowledge of various virulence factors of *Aspergillus*, antifungal host defences including innate and humoral immune response and regulation of host immunity by microbiome.

Keywords: Immunity, pathogenesis, aspergillus, genetic polymorphism, virulence

1. Introduction

Aspergillus species are globally ubiquitous saprophytes and are also opportunistic pathogens which have evolved in the environment and adapted to invade and proliferate within the human host. It can cause serious invasive infections. Invasive aspergillosis (IA) is associated with high mortality and morbidity which makes it essential to understand the factors involved in disease pathogenesis. The interplay between *Aspergillus* spp. and various components of the host immune system influences disease progression. Agent factors such as conidia size, temperature tolerance, hydrophobin /melanin expression etc. which contribute to virulence must be studied. Additionally, comprehensive knowledge of the host defenses, innate and humoral immune response, genetic susceptibility to *Aspergillus* and the role of microbiome in modulating immune response is important to study the disease immunopathogenesis.

In the genus *Aspergillus, Aspergillus fumigatus* is most commonly reported from human infections, followed by *A. flavus*, *A. terreus* and other uncommon species like *A. niger* and *A. nidulans* [1, 2]. It can cause plethora of infections, depending

on the immune status of the host as immunocompetent individuals with asthma or cystic fibrosis are predisposed to a hypersensitive response while Invasive aspergillosis (IA) is seen in severely immunocompromised patients.

A better understanding of the interplay between the host immune system and *Aspergillus* is important to understand disease pathology and can provide us with useful insights regarding potential therapeutic targets. In this review, we will thus discuss the pathogen related virulence factors, clinical spectrum of diseases caused by it, its interaction with various components of the host immune system, factors involved in regulating the anti-fungal immune response and will also give an overview of the genetic polymorphisms in immune pathways that predispose to aspergillosis. *Aspergillus* and disease pathology and progression are the result of both fungal growth and the host response.

2. Virulence factors

The various virulence factors involved in the pathogenesis of aspergillosis are summarized in **Table 1.**

	Function	**Gene(s) involved**	**Reference**
Enzymes			
Superoxide dismutases (SODs)	Oxidative stress defense	*SOD* genes	[3]
Protease	Degradation of host structural barriers		
1. Serine protease 2. Metalloproteinase 3. Aspartic (acid) proteinase	Degrades elastin. Degrades fibrinogen and laminin. Assist in host cell invasion of the hyphae.	36-kDa 23-kDa	[4] [5] [6, 7]
Catalase	ROS scavengers. Breakdown hydrogen peroxide ($H_2 O_2$) to oxygen and water.	*catA* - conidium-specific gene *cat2* - mycelium-specific gene	[8, 9]
Toxins			
1. Gliotoxin 2. Restrictocin 3. Aflatoxin	Inhibits macrophage phagocytosis. Induces fragmentation and apoptosis of DNA in macrophages. Inhibition of T-cell activation. RNA nuclease activity by cleavage of the phosphodiester bond in the 28S rRNA of eukaryotic ribosomes Induces DNA adducts causing genetic changes in cells responsible for carcinogenic potential *in vitro*. Also, epidemiologically to hepatocellular carcinoma.	18-kDa cytotoxin gene cluster of aflatoxin biosynthesis regulated by *AflCDC14*	[10, 11] [12, 13] [14]
Others			
1. Melanin	Masking of beta (1,3)-glucan. Delay macrophage activation. ROS scavengers.	pksP - polyketide synthase gene	[15, 16]
2. Rodlets	Rodlet proteins form hydrophobic layer around *Aspergillus* conidia and helps in its dispersal. ROS scavengers.	*rodA* gene	[17]

Table 1.
Virulence factors of Aspergillus *species.*

3. Risk factors and clinical spectrum

An elaborate range of diseases can be caused by *Aspergillus* species and the clinical spectrum depends on the immune status of the infected host. Correlation of clinical spectrum of aspergillosis and immune status in various condition has been depicted in **Figure 1**.

Immunocompetent Patient*:* In immunocompetent individuals *Aspergillus* spp. remain colonized as a saprophytic fungus. *Aspergillus* spp. can colonize in pre-existing cavities due to bronchiectasis, tuberculosis, cavitary neoplasia or sarcoidosis and cause chronic non-invasive infections like chronic pulmonary aspergillosis (CPA) [18, 19].

Hyper responsive or Atopic Patient: A hypersensitive response in these individuals in various forms like Allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitization (SAFS) and allergic rhinitis [20]. This is commonly seen in patients with cystic fibrosis (CF) and poorly controlled or steroid-refractory asthma [20]. In cases of CF, inflammation of bronchial mucosa and abnormal mucus can result in fungal colonization and up to 10% patients develop sensitization to *A. fumigatus* [21]. This can further progress to ABPA suggesting the importance of testing such patients with markers of immune hyper-reactivity.

Immunocompromised Patient: IA is a dreaded, life-threatening disease with a high mortality ranging from 40–80% [22, 23]. It is commonly seen in are individuals with hematological malignancies such as acute leukemia; solid-organ and hematopoietic stem cell transplant patients; patients on prolonged corticosteroid or chemotherapy. Invasive pulmonary aspergillosis (IPA) is also reported in patients with history of influenza or coronavirus disease and those receiving broad-spectrum antibiotics [24, 25]. Genetic susceptibly to IA is also seen in patients

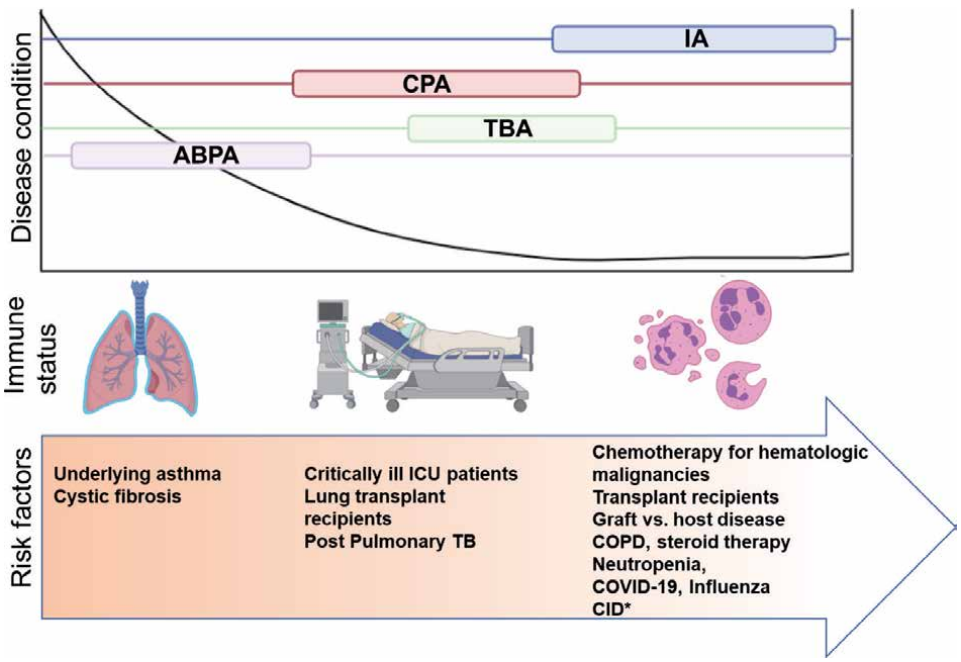

Figure 1.
Correction of clinical spectrum of Aspergillosis *and immune status in various condition. *CID: Congenital immunodeficiency disorders includes chronic granulomatous disease, CARD9 deficiency, leukocyte adhesion deficiency, Job's syndrome, pulmonary alveolar proteinosis.*

with congenital immune deficiencies like Caspase recruitment domain-containing protein-9 (CARD-9) deficiency and Chronic granulomatous disease [26, 27].

4. Pathogenesis

The range of ailments caused by *Aspergillus* depends on the host immune status. In atopic individuals the T helper 2 lymphocyte leads to hypersensitive response with increase in eosinophil counts and serum IgE levels. Formation of non-invasive aspergillomas is seen in CPA following repeated exposure to conidia in pre-existing cavitary lesions. IA is a destructive form of *Aspergillus*-related disease seen commonly in immunocompromised and critically ill patients.

5. Pathogen entry

The mode of reproduction in *Aspergillus* is predominantly asexual by formation of conidia (2–5 μm in size) which are ubiquitously present in the environment. These dormant conidia disperse in air easily due to their small size and common occurrence in soil, seeds and grains, decaying vegetation etc. and humans can inhale several hundred conidia per day. *Aspergillus* spp. are also found indoors in moisture damaged buildings both at homes and healthcare facilities [28] . There are therefore recommendations to avoid known sources of fungal proliferation (plants and flowers) in indoor places as they can serve as natural niches for fungal growth [29].

Conidia being small bypass the natural host nasal and bronchial defenses. The rodlet layer forms a hydrophobic layer outside conidia and protects it from host defenses and reach the lung alveoli. Natural defenses like mucociliary clearance and cough reflex are further compromised in intubated and mechanically ventilated patients. Also, the tracheal and bronchial epithelium is injured and provides easier passage for fungal conidia to the lower respiratory tract. Among healthy hosts, neutrophils and macrophages effectively clear the *Aspergillus* conidia. However, in immunocompromised patients, few conidia start swelling and become metabolically active after losing the outermost rodlet layer. These conidia, then germinate to produce fungal hyphae and cause a spectrum of invasive diseases.

6. Interaction with the innate immune system

The interaction of *Aspergillus* with cells of the innate immune system is depicted in **Figure 2**.

6.1 Soluble lung components

Various soluble factors found in the bronchopulmonary fluid are involved in *Aspergillus* defense including pathogen recognition receptors (PRRs) like C-type lectins, mannose binding ligand (MBL), Surfactant proteins (SP) – A and –D and pentraxin (PTX). These soluble factors enhance complement activation and phagocytosis of conidia, thus contributing to its clearance.

Although components of the complement system are predominant in serum they can also be found at lower levels in bronchial and alveolar fluid. Conidia and hyphae of *Aspergillus* species have been shown to bind to C3 followed by its cleavage to a ligand for phagocytic complement receptors iC3b. It has been reported

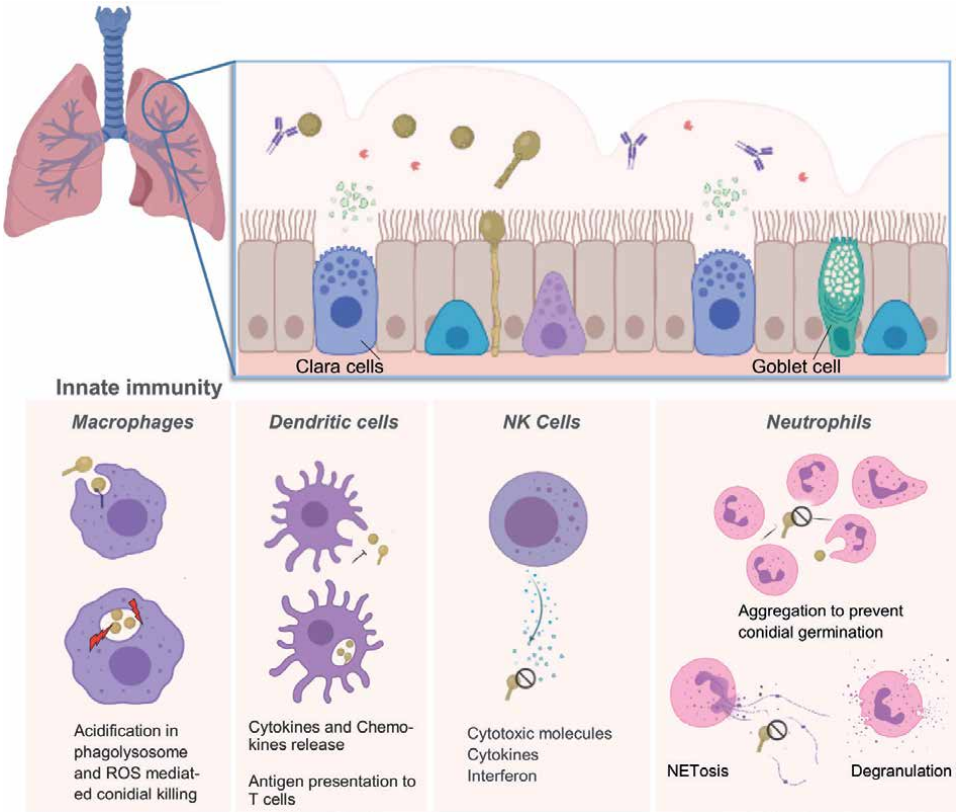

Figure 2.
Innate immune response to Aspergillus *infection. The conidia of* Aspergillus *spp. are inhaled and enter the lung where they encounter various soluble lung components including antibodies, complement factors and antimicrobial compounds. Those conidia which swell and undergoes germination further interact with a variety of innate immune cells including alveolar macrophages, dendritic cells, and NK cells. Conidial germination and development of hyphal forms is also prevented by neutrophils.*

that the common pathogens *A. fumigatus* and *A. flavus* bind to fewer C3 molecules compared to other to species making their complement-mediated phagocytosis and killing, less effective [30]. Hyphae and conidia from various *Aspergillus* spp. bind to alternative complement receptors like complement inhibitor factor H and the factor H family protein FFHL-1 which prevents complement cascade activation thereby protecting the fungus [31]. *A. fumigatus* and *A. flavus* have also been seen to produce a soluble complement-inhibitory factor which inhibits the activation of the alternative complement pathway [32]. This also acts as a defense mechanism of these species contributing to their overall pathogenesis.

6.2 Respiratory epithelial cells

The airway epithelial cells are the first cells to encounter inhaled *Aspergillus* conidia, which bind to it via sialic acid residues and subsequently modulate it. Other conidial proteins also mediate binding to fibrinogen, laminin and fibronectin which are all linked with lung injury indicating a role in adhesion and colonization [30]. A broad range of antimicrobial peptides of the defensin family are produced by the respiratory epithelial cells. Although the contribution of airway epithelial cells is less robust than that of the alveolar macrophages and germinating conidia and hyphae of *Aspergillus* are recognized by various PRRs on epithelial cells and subsequently assist in initiating pro-inflammatory response.

The proteases secreted by *A. fumigatus* cause desquamation and shrinkage of the respiratory epithelial cells along with actin cytoskeletal rearrangement with loss of cellular attachment and focal contact, thus assisting in invasion by germinating hyphae [33]. Secondary metabolites like gliotoxin, fumagillin, helvolic acid, verruculogen also damage airway epithelium and interfere with mucocilliary clearance [30, 34].

6.3 Pathogen recognition by innate immune cells

The recognition of *Aspergillus* by host immune cells is mostly via the PRRs – TLR1, TLR2, TLR4, TLR6 and the C-type lectin receptor i.e. dectin-1 [35]. TLR2 recognizes both hyphal and conidial form, while TLR4 recognizes only the hyphal morphology [36, 37]. The protective role of TLR4 mediated immune recognition has been seen in allogeneic hematopoietic stem cell transplant patients where it is observed that TLR4 polymorphisms are associated with IA [38]. The critical role of TLR6 in regulation of allergic inflammatory response in chronic fungal-induced asthma was studied by Moreira et al. in mice and the absence of TLR6 was found to be associated with less production of IL-23 and Th17 responses causing exacerbation of asthma [39]. Interestingly, the inflammatory response to *A. fumigatus* is intact in alveolar macrophages even in the setting of TLR2 deficiency and mice with defects in TLR2/TLR4 or its downstream effectors (like MyD88) have higher susceptibility to *A. fumigatus* lung infection, only in the setting of neutropenia [40–42].

Dectin – 1 is also an important PRR recognizing beta (1,3)-glucan on *Aspergillus* in both immunosuppressed and immunocompetent hosts. Although beta (1,3)-glucan is usually masked by the rodlet layer on resting conidia, the conidial swelling on entry in host epithelium exposes it, causing dectin – 1 mediated recognition and phagocytosis. Macrophages stimulation by *A. fumigatus* conidia increases intracellular PRR expression as well eg. Nucleotide-binding oligomerization domain (NOD) proteins ((NOD1 and NOD2) followed by production of proinflammatory cytokines which contribute to innate immune response [43].

6.4 Alveolar macrophages

Alveolar macrophages recognize and phagocytose fungal (1,3)-glucan bound to dectin-1. Internalization of conidia occurs within 2 hours and then conidial swelling begins [44]. This is an important requirement for induction of reactive oxygen species (ROS) production by the macrophage. Kinetic studies indicate that maximum ROS production occurs after 3 hours of phagocytosis resulting in fungistatic inhibition of germ tube formation due to which conidia are unable to germinate [44]. In immunosuppressed mice, although corticosteroid intake does not directly affect the internalization of conidia by alveolar macrophages there is impaired killing of *A. fumigatus* conidia due to defective production of ROS thereby increasing susceptibility to IA [44, 45]. The exact mechanisms of conidial killing by ROS are unknown and could be via direct toxicity or by acting as a cofactor for other phagolysosomal toxic molecules like elastase, cathepsins, proteases and chitinases [46]. In addition to phagolysosome acidification, phosphatidylinositol (PI) 3-kinase activity is also an important requirement for proper killing of conidia [47].

Neutrophils and macrophages produce nitric oxide (NO) and reactive nitrogen intermediates (RNI) that can also contribute to conidial killing. However, the expression of nitrogen oxidative species (NOS) which is seen in classically activated or M1 macrophages does not have much effect on conidial killing. A study by Lapp et al., reported that in *A. fumigatus* genes encoding flavohemoglobins (*FhpA* and

FhpB) which converts NO to nitrate and *S*-nitrosoglutathione reductase (*GnoA*) which reduce *S*-nitrosoglutathione to ammonium and glutathione disulphide are observed [48]. Although, these genes play a major role in detoxification of host derived RNI, they were not found to be essential for virulence.

Following macrophage phagocytosis, dihydroxynapthalene-melanin (DHN-melanin) of *A. fumigatus* prevents the phagolysosome acidification allowing conidial germination. However, *A. terreus* conidia lack the genes for DHN-melanin synthesis and instead produce a different type of melanin, i.e., Asp-melanin [49]. Although Asp-melanin does not impede acidification of phagolysosome it hampers phagocytosis and contributes to the survival and long-term persistence of *A. terreus* even in acidic environment.

In a study by Bhatia et al., alveolar macrophages were found to express Arginase 1 (Arg1) a key marker of alternatively activated macrophages (AAMs)/M2 macrophages after infection by *A. fumigatus* [50]. These macrophages efficiently phagocytose conidia and play a crucial role in pathogen clearance. The activation of macrophages is also followed by translocation of mitogen-activated protein kinases (MAPKs) to the nucleus where they phosphorylate the transcription factor NF-kappa B, thus activating a pro-inflammatory immune response.

6.5 Neutrophils

Neutrophils are professional phagocytes playing a pivotal role in innate immunity. Neutrophil recruitment is essential for effective *Aspergillus* clearing as they attack the germinating conidia and extracellular hyphae which have escaped macrophage surveillance. Neutrophils utilize TLR2, TLR4 and dectin-1, to identify and respond to *Aspergillus.* It can also be recognized directly by the complement receptor 3 (CD3, i.e., CD 11b/CD18), antigen–antibody complex detection by the Fcγ receptors (FcγR) or indirectly by opsonisation by various soluble components in lung environment.

In a study by Braem et al., higher deposition of the serum C3b was reported on germ tubes and swollen conidia compared to dormant conidia [51]. Also, patchy deposition of both C3b and immunoglobulin G (IgG) is seen over dormant conidia compared to uniform deposits on other morphotypes.

The release of chemotactic molecules, like C5a, increases migration of neutrophil to the infection site. The soluble mammalian extracellular β-galactose-binding lectin, galectin-3 is released in infected host tissues and facilitates neutrophil recruitment to the site of *A. fumigatus* infection by directly stimulating neutrophil motility in addition to exhibiting with both antimicrobial and immunomodulatory activities [52].

Neutrophil mediated killing involves both oxidative killing by NADPH oxidase which generates superoxide and myeloperoxidase and non-oxidative granule proteins containing various compounds with antimicrobial activity e.g., defensins, serine proteases, lysozyme, pentraxin-3 and lactoferrin [53]. Neutrophils attach to hyphae, spread over their surfaces, and degranulate thereby damaging the fungal hyphae. Neutrophils form aggregates in the lung and restrict conidial germination via lactoferrin mediated sequestration of iron [54]. Also, neutrophils produce lipocalin-1, which sequesters fungal siderophores thereby inhibiting fungal growth [55].

Another neutrophil dependent defense is the formation of neutrophil extracellular traps (NETs). Conidia and germ tubes of the *A. fumigatus* have been shown to trigger the formation of NETs. Pathogens in contact with the NETs become immobilized, limiting the spread of the infection. Calprotectin, a chelator of Zn^{2+} and Mn^{2+} ions is also produced by neutrophils and is associated with the *Aspergillus*-induced

NETs [56, 57]. Thus, in view of the important role that neutrophils play against *Aspergillus*, it is no surprize that patients with qualitative or quantitative defects in the neutrophils experience a greater risk of IA. It is worth mentioning however, that neutrophils may act as double-edged swords, since these are needed for fungal eradication but can also cause further lung injury by release of proteases and ROS. Thus, stringent regulatory mechanisms are essential to balance the protective activity and immunopathological responses for efficient control of the *Aspergillus.*

6.6 Natural killer cells

There is growing evidence suggesting the role of NK cells in immune response against *Aspergillus* spp. Direct antifungal activity via cytotoxic molecules like perforin and NK cell derived cytokines and interferon modulate the activation of other immune cells. *A. fumigatus* activates NK cells resulting in the production of low-levels of TNF-α, IFN-γ and lytic granules and release of fungal DNA [58]. These cells are a major source of early IFN-gamma production in the lungs of neutropenic patient with IA causing higher expression of IFN-inducible chemokines and subsequently enhancing macrophage antimicrobial effects. Studies in mice-models also suggest a critical role of NK cells in the pulmonary clearance of *A. fumigatus* [59].

Interestingly, in a study by Santiago et al., down-regulation of NK cell activating receptors NKG2D and NKp46 and a failure of full granule release was observed on contact of NK cells with *A. fumigatus* hyphae [59]. They also reported *A. fumigatus*-mediated NK cell immune-paresis which reduces cytokine-mediated response causing immune evasion during pulmonary aspergillosis [59]. Characterization of the clinical impact of NK cells in antifungal host immune response is still in its nascent stage as it involves complex interplay between multiple arms of the immune system [60].

6.7 Dendritic cells

Dendritic cells (DCs) bridge the innate and adaptive immune responses. They not only sense and patrol the lung environment but also initiate host response by antigen presentation which primes the T cell responses and causes cytokine secretion. Immature DCs are phagocytic and constantly perform surveillance of the lung environment while expressing PRRs like TLR 1, 2, 3, 4, 6 and Dectin-1 on cell surface that recognize various pathogen-associated molecular patterns (PAMPs). After phagocytosis, *A. fumigatus* conidia have been reported to escape from DCs, whereas some species like *A. terreus* persist with long-term survival, protecting them from anti-fungal action [49].

Typically, DCs are of two types, the plasmacytoid (pDCs) which are IFNα (type I interferon)-producing cells with a significant role in antifungal response and Classical (cDCs) which remain in the lymphoid tissue and cross-present antigens to T cells [61]. There is considerable plasticity in the functional activity of pulmonary DCs depending on the morphology of invading fungus [62].

1. Although DCs internalize both conidial and hyphal form of *A. fumigatus,* internalization of conidia occurs by coiling phagocytosis while entry of hyphae occurs by zipper-type phagocytosis. Also, phagocytosis of conidia is via involvement of a C-type lectin receptor while CR3 together with FcγR mediate the entry of opsonized hyphae.

2. Cytokine production is also variable depending on the fungal morphotype as TNF-α response is seen to any fungal form, but IL-12 is produced on exposure to conidia, while IL-4/IL-10 upon phagocytosis of hyphae.

3. The pulmonary DC transport *Aspergillus* fungal forms to the draining lymph nodes and spleen followed by functional maturation and eventual degradation for efficient antigen presentation.

4. The DCs also direct both local and peripheral T helper cell in response to fungus.

7. Interaction with the adaptive immune system

The adaptive immune response to *Aspergillus* infection is depicted in **Figure 3**.

7.1 Role of T-cells

Antigen-specific Th1 cells are crucial sources of IFN-γ mediated protective immunity to *A. fumigatus* [18, 58]. Peripheral blood of healthy adult donors has been found to have *A. fumigatus* specific effector/memory CD4 T cells with Th1 phenotype [63, 64]. A Th17 phenotype is noted in lung-derived *Aspergillus*-specific T cells [65]. IL-22 is produced by Th17 cells and has shown to play a crucial role in regulating *Aspergillus* induced asthma [66]. Like neutrophils, Th17 responses represent a "double-edged sword". During pulmonary fungal infections, the Th17 cell usually display a highly pro-inflammatory profile, which is detrimental to the infected host.

The Th2 cell-mediated immune responses along with Th1 and Th17 induces chronic pulmonary inflammation and lead to significant lung damage [67, 68]. This

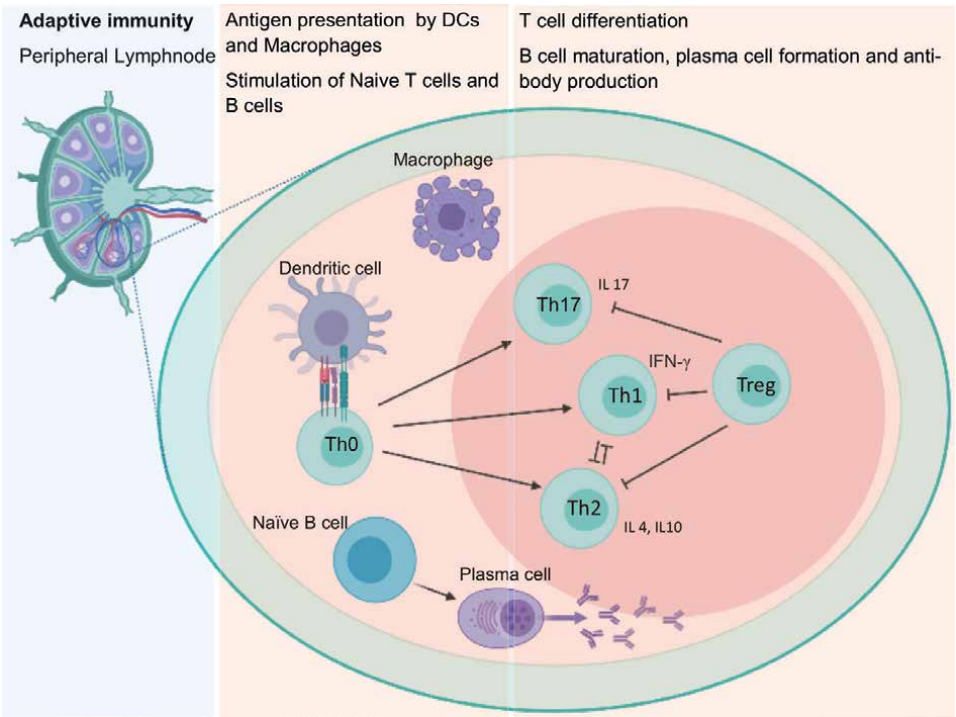

Figure 3.
Adaptive immune response to aspergillus *infection.* Aspergillus *spp. antigens are presented to naive T cells in peripheral lymphoid organs by dendritic cells and macrophages which further induces inflammation with coevolution of Th1, Th2, and Th17 response. B cells are also stimulated resulting in formation of anti-fungal antibody producing plasma cells.*

allows influx of macrophages followed by differentiation of both M1 and M2 subtypes [69]. These macrophages and T cells play a key role subsequently promoting extensive remodeling of medium- and small-sized pulmonary arteries. Pulmonary artery pathology including an increase in intimal area, smooth muscle proliferation, calcification of elastic membrane, and narrowed arterial lumens is seen in those with fatal asthma [70].

In healthy subjects, a strong Treg response has been seen as a part of the normal physiological T-cell repertoire which counterbalances the *A. fumigatus* specific T cells [71]. This intriguing finding raises the possibility that colonizing *A. fumigatus* may selectively promote Treg responses and subsequently limit antifungal immune activity. Activation of indoleamine 2,3- dioxygenase (IDO) as a regulator of infection-linked tissue pathology is now being recognized as it acts via local tryptophan depletion, or generation of immunomodulatory metabolites. Interaction of TLRs with PAMPs induces IDO which regulates the inflammatory/anti-inflammatory status of the innate immune cell and modifies the local tissue microenvironment. There is also activation of GCN2, a T-cell stress-response kinase which senses amino acid starvation and impairs lymphocyte proliferation while enhancing polarization toward a Treg phenotype [72]. In patients of CF with ABPA, dysregulation of the IDO pathway is seen at both the genetic and transcriptional levels, leading to an imbalanced Th17/Treg with high Th2 polarization resulting in chronic inflammation and significant lung damage in response to *A. fumigatus* [73].

7.2 Role of B-cells

In a study by Montagnoli et al., the role of B cells and antibodies in the generation of antifungal immune resistance was studied in B cell-deficient (μMT) mice which were infected with *A. fumigatus* [74]. They reported that, although passive transfer of antibodies helped in fungal clearance, a compensatory increase in both innate and Th1-mediated resistance to infection was seen in μMT mice with aspergillosis. This suggests that in the absence of opsonizing antifungal antibodies, the nature of the interaction between the innate immune cells and with fungi may be modified with subsequent development of long-lasting antifungal immunity [74].

Chen et al., demonstrated that basophil interaction with IgD bound antigens and activation of TLRs induces expression of B-cell-activating factor (BAFF), an important regulator of B-cell activation, proliferation, and immunoglobulin production. This results in IgG and IgE production by B cells, pointing to a role of basophils in adaptive immune responses [75]. In a study by Boita et al. stimulation of basophil membrane by *Aspergillus* resulted in upregulation of BAFF expression in patients with SAFS and ABPA. These patients had high IgE suggesting the role of basophils in polyclonal IgE production [76].

8. Role of the microbiome

Host immune responses are influenced by changes in the gut microbiome. Short-chain fatty acids (SCFAs) produced by the gut microbiome are recognized by innate immune cells like macrophages and neutrophils expressing G-coupled protein receptor GPR43 [77]. The gut microbiome also plays a crucial role in anti-*Aspergillus* host defense by coordinating lymphocyte subsets at the mucosal level in distant organs such as the lungs. Although, fungal microbiome compromise <0.1% of total microbiome, fungal cell components such as β-glucans may influence immune responses as perceived by their role in autoimmune diseases [78]. *In-vivo* studies in mice have revealed that intake of SCFA (propionate/butyrate) or supplementation

of diet with fermentable fibers which increases SCFA producing bacteria, increases the generation of DCs and macrophages in the lung and bone marrow with increased phagocytic capacity [79–81]. These alterations also reduce the ability to prime cells toward Th2 responses lowering DC ability to induce *Aspergillus*-allergic inflammation [82].

The intestinal segmented filamentous bacterium (SFB) have been shown to induce Th17 cells producing IL-17 and IL-22 in the lamina propria of the gut and can even regulate pulmonary adaptive immune response by increasing Th17 responses in the lung [83, 84]. However, it is important to determine whether lung microbiome also has similar Th17-polarizing ability which can influence anti-*Aspergillus* host response.

It has also been observed that in germ-free mice, the absence of commensal gut microbiota leads to increase susceptibility to pulmonary viral infections. Hence, the gut microbiome can influence pulmonary immune responses by release of type 1 IFN [85, 86]. Intestinal colonization of microorganism is necessary for cytotoxic activity by NK-cell, $CD8^+$ T-cell clonal expansion, and production of specific antibodies [85].

Recently, innate lymphoid cells (ILCs) have emerged as an important cell population that has the capacity to synchronize microbiome-related immune regulation [87]. ILCs can express functional TLR2 which on stimulation induces IL-2 production, subsequently increasing the expression of IL-22, enhancing the allergic airway responses induced by *Aspergillus* spp [88]. It has also been observed that commensal bacterial limit the production of serum IgE levels which directly influences bone marrow - basophil precursors, leading to increased allergic airway responses [89].

The treatment of diseases like COPD with steroids and bronchodilators, may also alter the microbiome [90] which can subsequently increase the risk of colonization and infection by *Aspergillus* spp. In patients with Influenza, significant changes in the lung microbiome have been observed with a relative abundance of *Firmicutes* and *Proteobacteria* more specifically, *Pseudomonas* spp., which contributes to secondary invasive infections by *Aspergillus* spp. [91, 92]. Other factors like antibiotic exposure can also influence the micro-environment of the microbiome, which can affect the pulmonary immune responses to *Aspergillus* causing allergic airway diseases [93]. In patients with CF, interaction between fungal and bacterial pathogens and their biofilms may influence pathogenicity which can be observed by significant decrease in *Aspergillus* in the sputum on treatment with anti-pseudomonal antibiotics [94, 95].

9. Genetic susceptibility to aspergillosis

The genetic polymorphisms within pattern recognition receptors PRRs (*TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, Dectin-1, Dectin-2, DC-SIGN, MASP, MBL, PTX-3* surfactant protein-A2 and plaminogen) cytokines (*IL1, IL10,* IFN- γ, *CXCL10, ARNT2,)* and their receptors (*CX3CR1* and IL-4Rα) is depicted in –**Table 2**.

10. Conclusion

The clinical spectrum of *Aspergillus* related infections depends on the host immune status ranging from allergic manifestations in immunocompetent atopic individuals to invasive disease in immunosuppressed individuals. Various components of the innate and adaptive immune system form an intricate network modulating host response to *Aspergillus* exposure. Many future studies are required to study

Gene	Function	SNP position	Disease condition	Reference
Pattern Recognition receptors (PRRs)				
TLR1	TLR1 forms heterodimer with TLR2 and facilitate the fungicidal activity by various oxidative pathways	239 C/G [80 R/T] 743 A/G [248 S/N] 1063 A/G	IA	[96]
TLR 2	TLR-2 act as PRR for *Aspergillus* spp. Antigens and activate innate immune cells. Further downstream signaling via TLR2 promote the fungicidal activity by various oxidative pathways which lead to proinflammatory cytokines release.	Arg753Gln (G + 2258A) polymorphism affects the TIR domain of TLR-2 and impairs its functional activity.	IA	[97]
TLR4	TLR4 promotes fungicidal activity	[299 D/G] 1363 C/T [399 I/T] 1063 A/G [299 D/G]	IA after HSCT [EORTC] CCPA	[38, 98, 99]
TLR5	TLR-5 induction causes increase in expression of pro-inflammatory cytokines	1174C T (STOP codon)	IA	[100]
TLR6	It promotes IL-23 release and a subsequent Th17 response.	745 C/T [249 S/P]	IA after HSCT [EORTC]	[96]
TLR9	It recognizes unmethylated CpG DNA and induces innate immune responses.	1237 C/T [Promotor]	ABPA	[98] [101]
Dectin-1	Dectin-1 is act as a PRR, which is present on myeloid cells surface and expressed by DCs and macrophages. It is specialized for recognition of β-1,3-glucan of fungal species. It leads to production of chemokines and cytokines and causes recruitment of neutrophil recruitment and ROS production.	Y238X polymorphism [Stop Codon Polymorphism]	IA	[102] [103] [104]
Dectin-2	Dectin-1 is act as a PRR, which is present on plasmacytoid dendritic cells (pDCs). It is specialized for recognition of α-mannans of fungal species. It leads to cytokine production, extracellular trap (pET) formation and ROS production.	(CLEC6A – A/G) [Intron] (CLEC6A - C/T) [Intron]	IPA	[104]
DC-SIGN	DC-SIGN is a CLR. It recognizes galactomannans.	336 A/G [promoter] c.898 A/G [3′-UTR] c.74928 C/T [3′-UTR] IVS2 + 11 G/C [Intron]	IPA	[104]

Gene	Function	SNP position	Disease condition	Reference
pentraxin (*PTX3)*	PTX3 is a soluble opsonin. It is produced by phagocytes that facilitates microbial recognition and phagocytosis of conidia.	+281A/G[Intron 1] +734A/C (D48A) [Exon 2] +1449A/G [Intron 2]	IA	[105] [106]
Mannose-binding lectin-associated serine protease (MASP2)	MASP binds directly to *Aspergillus fumigatus* and promote complement activation and phagocytosis	380 A/C [D120G]	IA	[107]
MBL	MBL is a soluble PRR. It opsonizes the carbohydrate moieties of fungus and activates the lectin complement pathway using the MASPs and induces the release of proinflammatory cytokines.	868 C/T [52 C/R] 1011 A/G [Intron] 868 C/T [52 C/R]	CCPA ABPA CNPA	[108–113]
Plg	Plasminogen is produced by phagocytes that facilitates microbial recognition.	28904 A/G[a] [472 N/D]	IA	[114–116]
SFTPA2 surfactant protein-A2		1660 A/G [94 R/R] 1649 C/G [91 A/P] 1492 C/T [Intron]	ABPA	[117, 118]
Cytokines				
CXCL10	It is an 'inflammatory' chemokine. It binds to CXCR3 and mediate leukocytes recruitment such as eosinophils, T cells, NK cells and monocytes.	11101 C/Ta [Downstream] 1642 C/Ga [3′ UTR] 1101 A/Ga [Promotor]	IA	[119] [120] [121]
ARNT2	It regulates the activity and differentiation of phagocytic cells like macrophages and lymphocytes.	80732053 [Intron]	IA	[122]
IFN-ɤ	It promotes differentiation of Th1 response	1616 C/T[a] [Promotor] 1082 A/G [Promotor]	IA	[123]
IL-10	IL-10 plays a significant role in the development of atopy. It inhibits the activity of Th1 cells, NK cells, and macrophages which are essential for clearance of fungus.	2068 C/G[a] [Intron] 1082 A/G [Promotor] 1082 A/G – 819 C/T – 592 A/C [Promotor] 1082 A/G [Promotor]	IA ABPA	[124] [125] [126]
IL-4R alpha	IL-4 released by T cells binds to the IL-4 receptor (IL-4R) on B cells resulting in B cell proliferation and IgE isotype switching.	4679 A/C/G/T [75 I/L/F/V]	ABPA	[127]
Cytokine's receptors				
TNFR2 TNF receptor type 2	TNFR2 (p75) receptor is expressed by T regulatory cells for survival during clonal expansion.	322 [Promotor]	IPA	[107]

Gene	Function	SNP position	Disease condition	Reference
Interferon regulatory factor - 4 (*IRF4*)	It regulates the NFκB pathway and cell proliferation and modulates the differentiation of different DC and Th17-mediated immune responses against *Aspergillus fumigatus.*	rs12203592	IA	[128]
CX3CR1	Modulates the interaction of fungal pathogens with immune phagocytes.	39286825 [Intron] 39293757 [Intron]	IA	[122]

TLR-Toll-like receptor, IL – Interleukin, PRR – Pathogen Recognition Receptor, *Th – T helper cells, DC-SIGN - Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin, PTX3- Pentraxin,* MASP2 - Mannose-binding lectin-associated serine protease, MBL - Mannose-binding lectin, *CXCL - chemokine (C-X-C motif) ligand,* ARNT2 - *Aryl hydrocarbon receptor nuclear translocator 2, IL-4R alpha - Interleukin 4 receptor alpha, TNFR2 - TNF receptor type 2,* IRF4 *Interferon regulatory factor - 4,* CX3CR1 - *CX3C chemokine receptor 1, IA- invasive aspergillosis, IPA- invasive pulmonary aspergillosis, CCPA- Chronic cavitary pulmonary aspergillosis, ABPA – Allergic bronchopulmonary aspergillosis, CNPA – Chronic necrotizing pulmonary aspergillosis, HSCT-Hematopoietic stem cell transplantation, EORTC- European Organization for Research and Treatment of Cancer.*

Table 2.
Summary of immune system related genes mediating susceptibility to aspergillosis.

the association and impact of the complex interactions between the gut/pulmonary microbiome and the immune system in *Aspergillus*-related diseases. An understanding of the immune pathogenesis of aspergillosis can help in the development of strategies targeting *Aspergillus* itself as well as pulmonary or systemic immunity by influencing the host immune system, the microbiome and/or its metabolites.

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Section 2

Mycotoxin Production and Industrial Application

Chapter 4

The Role of Aflatoxins in *Aspergillus flavus* Resistance to Stress

Massimo Reverberi, Marzia Beccaccioli and Marco Zaccaria

Abstract

Aspergillus section Flavi produce the aflatoxins, secondary metabolites toxic to humans and animals. Why do these fungi produce aflatoxins? They do not have a clear role in pathogenicity or in niche competition. *Aspergillus* employs a considerable amount of energy to synthesize them: more than 20 enzymatic catalyzes are needed. Within the *A. flavus* species, all opportunistic pathogens of maize, more than half of the natural population are atoxigenic, indicating that aflatoxins are not so obviously linked to an enhancement of population fitness. The perspective changes in *A. parasiticus*, pathogen to peanuts, where more than 90% of the natural population produce the four aflatoxins. In this chapter, we aim to discuss our recent hypothesis that aflatoxins act as antioxidants providing more time to *Aspergillus* to "escape" an exploited substrate, that in the meanwhile is "fully charged" with reactive oxygen species and oxylipins.

Keywords: antioxidants, oxylipins, resilience to stress, lifespan, host adaptation

1. Introduction

The species belonging to the section Flavi of the genus *Aspergillus* can produce the carcinogenic aflatoxins (AF), secondary metabolites synthesized as the final product of a very complex pathway including 25 different enzymatic activities so far [1]. Aflatoxins are detrimental for animals (humans included) since, after oxidation by cytochrome P450, their 8,9-epoxide causes DNA depurination leading possibly to carcinogenesis [2]. Notwithstanding these fungi can invade the lungs of cystic fibrosis patients, the role of AF in worsening the clinical frame remains to be demonstrated. *De facto Aspergillus flavus* (and *A. parasiticus* as well) are opportunistic pathogens for animals as well as for plants and a competitive soil saprophyte [3], for which the synthesis of AF appears "luxury" or not necessary. Our idea is that AF are too expensive – in terms of biosynthesis and energy devoted – to be "non necessary". If so, why AF are produced? More than 40 years of research told us that AF are synthesized following different inputs: nutritional [4, 5], pH, light, [6], host defenses [1] and finally, oxidative stress [7, 8]. The general impression is that AF are synthesized in response to a stressful condition. In light of this, can we consider them as a part of the complex reaction set that *Aspergillus* uses for facing challenging conditions? This chapter focus on how oxidative stress can modulate (how and why modulate) AF synthesis, beginning with the description of some of the actors that switch the AF synthesis on; the following paragraph regard the important role of oxidized fatty acids in controlling several aspects of the life of these fungi and,

notably, AF synthesis; it concludes with an evolutionary point of view on the meaning of AF synthesis for the *Aspergillus* section Flavi lifestyle.

2. The role of oxidative stress in modulating aflatoxin synthesis

Oxidative stress is a condition which organisms must cope with since the process used for producing energy (namely ATP) involves a very oxidizing molecule: the oxygen [9]. Thus, aerobic organisms have evolved means for facing this stress by building up a complex antioxidant system composed of structures, proteins (enzymes) and small metabolites. The ability to control this system enables organisms to face oxidative stress and, indeed, using it to "boost" some pathways (e.g., the defense in the plants) [10]. Aflatoxins are among these: they are synthesized in response to oxidative stress conditions [7] and, as we aimed to clarify within this chapter, can act as antioxidants to enhance the survival ability of these fungi.

2.1 Reactive oxygen species (ROS)

Free radicals are, by definition, very reactive chemical species, due to their presence of one or more unpaired electrons in valence orbitals. This condition makes them highly reactive molecules, energized and unstable; free radicals will try to to give up or, as more commonly happens, to acquire an electron at the expense of another to obtain a stable configuration.

In living systems, spontaneously forming radicals are numerous, and those of greater biological interest, the so-called ROS, are those molecules in which the unpaired electron is found on O_2, such as, for example, superoxide ($\cdot O_2$-), hydroxyl ($^{\cdot}OH$), hydroperoxyl ($\cdot OOH$), peroxyl ($\cdot OOR$) and alkoxy ($^{\cdot}RO$) radicals. Oxygen is found in nature in the form of diatomic molecules that have two unpaired electrons of parallel spins arranged on two different orbitals (triplet), and therefore possessing characteristics paramagnetic. The fact of having uncoupled electrons makes O_2 particularly prone to forming covalent bonds but, in the case of incomplete reduction, ROS may be generated. These react quickly with other compounds to acquire the electrons necessary for their chemical stability, losing, in turn, their electrons and becoming radicals themselves, thus triggering a chain reaction. Once that process starts, it is determined in the cell a cascade of reactions that often begins with the peroxidation of lipids membrane (oxidation of the hydrocarbon chain), resulting in its destabilization, and which proceeds with the oxidation of other cellular components (such as proteins and DNA), to the point of causing the deconstruction of the entire cell.

The reactions in which the radical molecules can take part are many and vary significantly, for example, depending on: (i) the compartment or organelle cell in which they originate, (ii) of the antioxidant systems present, (iii) of the molecules that they attack, (iv) the water and nutritional conditions of the cell. Also, non-radical molecules, such as hydrogen peroxide (H_2O_2), can trigger responses that lead to the formation of ROS: the Haber-Weiss reaction, for example, produces hydroxyl radicals starting from H_2O_2 and O_2-. The cells of photosynthetic organisms are more subject to oxidative damage since they have concentrations of very high O_2 since, not only do they use it during breathing, but they also generate it with photosynthesis. In fact, they have membrane thylakoids composed mainly of polyunsaturated lipids (molecules subject to reactions of peroxidation) and, by means of photosynthetic pigments, absorb light energy, the excess of which favors the production of ROS. In its ground state, O_2 is relatively not very dangerous because, although it can give rise to excited states reactive

and free radicals (during photosynthesis, for example), its utilization proceeds expeditiously by means of a route in stages, in which a reduction to H_2O involving four electrons and during which intermediates can be generated partially reduced reactive species. In fungi as well as in other organisms, ROS can be produced in a tightly regulated way by the NADPH oxidase complex (NOX in fungi; [11]). This complex controls, upon stimuli, the formation of anion superoxide and controls several processes in hyphal growth and development [11, 12] and in mycotoxin synthesis too [13].

2.2 Antioxidant responses

If, as just described, the formation of free radicals can cause serious damage at the cellular level, which can sometimes lead the cell to death, it is equally true that the aerobic cells have evolved and developed efficient ROS control and detoxification systems. The latter are known as antioxidant systems and can be enzymatic and non-enzymatic in nature. The systems non-enzymatic include molecules such as: α-tocopherol, β-carotene, compounds phenolics, ascorbate, glutathione; the enzymatic ones involve: superoxide dismutases (SOD; EC 1.15.1.1), which catalyzes the dismutation of O_2.- in H_2O_2, together to others which eliminate the H_2O_2 such as catalases (CAT; EC 1.11.1.6), peroxidases, glutathione peroxidases (GP; EC 1.11.1.9), (which uses glutathione as an electron donor - GSH, reduced form, and GSSG, oxidized form) and ascorbate peroxidases (APX; EC 1.11.1.11; ASA, reduced form of ascorbic acid, and DHA, oxidized form). All the enzymes described are found in multiple forms (isoforms) that can be classified, for example, based on their metallic cofactor. The latter can also be found in different cellular compartments (such as cytosol and apoplast) or organelles [mitochondrion, chloroplast (in plants), peroxisome and vacuole]. Some of them catalyze the same reaction and can use different substrates as electron donors. In fungi, these antioxidant capacities are tightly controlled by transcriptional regulators. Main transcription factors that in fungi "react" to ROS are msn2–4 [14], skn7 [15] and Yap-1 [16]. Notably, Yap-1 orthologue ApyapA can control aflatoxin biosynthesis [17].

2.3 Oxidative stress, antioxidant system and aflatoxin synthesis

Oxidants are continuously produced within and outside fungal cells. In some way they can fuel cells to switch metabolic pathways [18] or differentiation patterns [19]. Inter alia, in *A. flavus* and *A. parasiticus* ROS boost aflatoxin formation [19–21]. In the past we showed that several oxidants amended to culture as well as increase of cell ROS were able to trigger aflatoxin synthesis [20]. Intriguingly, even external oxidants augment the titer of cell oxidants. How can these oxidants turn into "aflatoxins"? which is the "mediator" of the opening of the complex aflatoxin pathway? Our group and John Linz group demonstrated that ApYapA can orchestrate their synthesis [20]. Notably, ApYapA, similarly to its orthologue Yap-1 of *Saccharomyces cerevisiae*, is indirectly oxidized by ROS through a peroxideroxin (TSA1, [17, 22]. Once oxidized, ApYapA migrates into the nucleus and, during the exponential phase of growth it recognizes and binds to specific responsive elements present into the promoter of genes encoding antioxidant enzymes such as catalases, superoxide dismutases inter alia. During the stationary phase, indeed, it binds even to the promoter of AflR, i.e., the gene whose product controls (together with AflJ) the whole AF pathway and consequently, their biosynthesis (**Figure 1**). As suggested below (paragraph 4), aflatoxins are a subsidiary antioxidant response that fungal cells operate to "staying alive" as long as possible to differentiate conidia and "escape" from the spent - stressing, oxidizing – substrate [23].

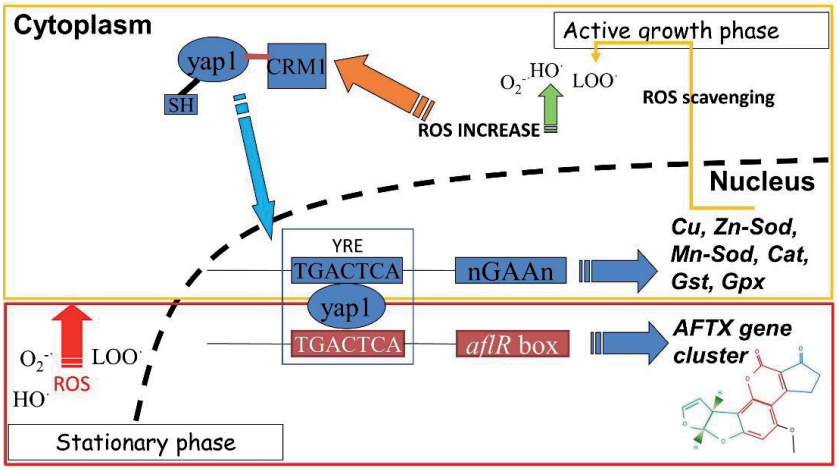

Figure 1.
Aspergillus parasiticus *produces ROS during the normal course of its lifestyle. During the exponential – Active- phase of growth their production is constant but kept low by a very efficient antioxidant system that is modulated by* ApyapA inter alia*. In this phase aflatoxin synthesis is normally shut off or very low. Indeed, during the stationary phase (or even in consequence of external stressors – E.g., herbicides), ROS scavenging though normal detoxification system (e.g., glutathione, superoxide dismutases etc) is not efficient anymore and the oxidative stress-controlled transcription factor ApYapA recognize and bound to YRE (Yap1 responsive elements) present in the promoter of* AflR*, the global transcriptional regulator of aflatoxin synthesis. In this phase, AF synthesis is switched on and contribute to scavenge oxidants present in the matrix.*

3. The role of oxylipins in the *Aspergillus* sect. Flavi lifestyle

3.1 Discovery of oxylipins

Understanding the evolution of fungal pathogenesis requires the treatment of some lipid molecules that mediate the fungus-host interaction. *A. flavus* preferentially infects maize seeds, which are rich in unsaturated fatty acids (UFAs). Furthermore, also Aspergillus species contain high levels of UFAs, including oleic ([18]: 1), linoleic ([18]: 2) and linolenic ([18]: 3) acid, which are substrates for oxygenation that converts the UFAs in oxylipins. Oxylipins are involved in the interaction-signaling of fungi, bacteria, plants and animals.

First evidence on the existence of oxylipins dates to 1987, when Champe et al., demonstrate the role of precocious sexual inducer (psi), later called oxylipins, in Aspergillus nidulans. Psi factors inhibit asexual sporulation and stimulate premature sexual sporulation, acting as hormone-like molecule [24].

Oxylipins derive from free fatty acids or from fatty acids present into membrane phospholipids. Fatty acids included in membranes, during the plant-pathogen interaction, are released by lipase action. Lipases are considered as virulence factors in plant pathogenic fungi [25].

Oxylipin oxidation may happen by two routes: the radical and the enzymatic. In fact, during the first steps of infection the production of radical species favors the accumulation of Reactive Oxygen Species (ROS). Superoxide anion (·O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) can spontaneously oxidize the free fatty acids. The second oxylipins synthesis route, i.e., enzymatic, in fungi involves the action of dioxygenases (DOX), lipoxygenases (LOX) and cyclooxygenases (COX) that convert free fatty acids in oxylipins [26].

The crosstalk established during a plant-pathogenic fungus interaction, therefore, involves a lipases-LOX concerted activity, that carries towards the oxylipin biogenesis [27].

In *A. flavus* enzymatic set for oxylipin formation is composed of four dioxygenases, PpoA, PpoB, PpoC, and PpoD, and one lipoxygenase, LOX. PpoA encodes a 5,8-linoleate diol synthase, whereas ppoC encodes for a linoleate (10R)-dioxygenase [28–30].

3.2 Oxylipins in host-pathogen interaction

Oxylipins act as modulator of many signal transduction pathways, both in plant and fungi because the chemical structure as well as the main synthesis routes of oxylipins are common between the two kingdoms. For that reason, several authors defined the oxylipins as the common language between hosts and pathogens [31]. But why do hosts and pathogens produce oxylipins?

In *A. flavus*, oxylipins regulate dissemination, affecting the production of sclerotia and conidia, and influence secondary metabolism. In addition to having an autocrine action, oxylipins are also distinguished by their paracrine action, when *A. flavus* interacts with other organisms.

When *A. flavus* produces oxylipins, the plant recognized them and alters the expression of oxylipin synthesis genes, as the LOX, but the fungus senses the plant oxylipin gradient that promotes sporulation and mycotoxin production, this signaling exchanging define the cross-talk.

Plant can release oxylipins as 9S-HPODE (9S-hydroperoxyoctadecadienoic acid) and 13S-HPODE (13S-hydroperoxyoctadecadienoic acid) able to influence the development in the Aspergilli, in addition, but at the same time they act in the regulation of plant defense and development.

The analysis of the phenotypes derived from the mutant of ppo-genes shown that in *A. flavus* that ppoA and ppoC deletion generates strains with less conidia and more sclerotia, whereas the deletion of ppoD shown the inverted situation, or rather more conidia and less sclerotia. It was considered also the deletion mutant for all four oxylipin-biosynthesis genes (dioxygenases and lox), which shows both high levels of aflatoxin production and high levels of sclerotia production. These results shown the closely link between the oxylipin production and the asexual or sexual reproduction, underlining the role of the oxylipins in the fungal regeneration.

As previously introduced, the oxylipins produced by the plant may influence the fungal lifestyle and being chemically similar to the fungal oxylipins they can substitute them. That was demonstrated in one study, where the maize lipoxygenase Zm-LOX3 cloned in *A. nidulans* mutant strain, deficient for the two genes ppoA and ppoC, restored them functions. Oxylipins for the plant assume a protective function, in fact the inactivation of Zm-LOX3 makes the plant more susceptible to *A. flavus*, that during the infection grows more and produces more aflatoxins [32]. The increase of susceptibility is linked to the accumulation of oxylipin substrates, the fatty acids, and the decrease of the jasmonic acid, in whose biosynthesis pathway Zm-LOX3 is involved. Oxylipins acting as hormones, that means that a small concentration of oxylipins can regulate physiological processes as growth and development, but several studies show that oxylipin-mediated responses are strongly influenced by the type and the nature of interaction with the host.

The development of the fungus depends on cell densities when the cell density is high *A. flavus* produces more conidia. The cell densities also influence the secondary metabolites production, in fact the aflatoxin synthesis decreases at high cell densities. The quorum sensing, or rather the phenomenon in which the set of signaling molecules enable the single cell to sense the other cells, may be associated with the oxylipins release. The deletion of ppo and lox genes inhibits the development of the oxylipins [33, 34]. The exposure to exogenous seed oxylipins, as 9-HpODE and 13-HpODE stimulate the sporulation and the aflatoxin synthesis. The 13-HpODE

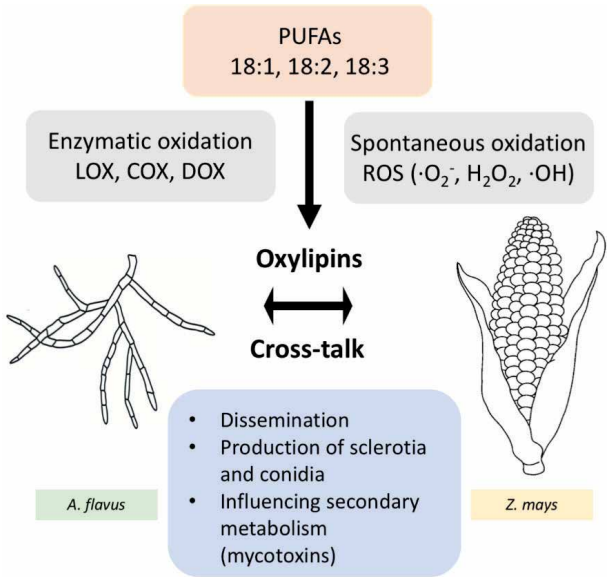

Figure 2.
Signaling oxylipin-mediated in A. flavus. *Polyunsaturated fatty acids (PUFAs) may be converted by enzymatic and spontaneous oxidation in oxylipins, that mediate the interaction with the host (Z. mays).*

seems to inhibit the sclerotia formation, suggesting the role of this oxylipin in the sexual/asexual reproduction in *Aspergillus* species [35]. These results proposed the oxylipins as quorum sensing mediator in *Aspergillus* species [36] (**Figure 2**).

Although the role of oxylipins in development and host-pathogen communication is recognized, little is known about their perception. Mammal oxylipins are sensing by G protein-coupled receptors (GPCRs), but in fungi GPCRs are not actually identified also if in *A. flavus* have been found some genes homologous to mammal GPCRs required for the high-density growth [37].

GPCR-mediated signaling seems to be linked to pathogenesis, therefore it is hypothesized that they could be potential targets for disease control [38].

4. Evolving a strategy for enhancing the resilience to stress: the role of aflatoxins

A. flavus is highly infectant, fast growing, efficient and versatile bio factory to its core [39]. Among the wide inventory of secondary metabolites it produces, aflatoxins are not the least puzzling, even after decades of dedicated research. Their physiological role has proven hard to frame unequivocally, having been linked over the years to several different purposes, including messengers for quorum sensing, facilitators of dispersal and resistance factors to UV stress [40], inhibitors of environmental competitors [41], mutagenic agents employed to compensate the limited intraspecific variability derived by a conidiogenesis-centred reproduction strategy and, finally, antioxidants.

Aflatoxins have been linked to oxidative stress since the 1980s [42], their synthesis being convincingly linked to increase in the presence of oxidants, *in vitro* and *in vivo* [43, 44]. However, the mechanism behind aflatoxins' role as direct or indirect reducing agents has also been elusive. In a general view, secondary metabolites such as mycotoxins are described by several experts as the by-product of housekeeping processes whose production is an indirect consequence of the very activity of

primary metabolism [45]. In this framework, it is possible that aflatoxins could fit the picture as the by-products of a secondary pathway aimed at channeling and exhausting environmental oxidative stress through a dedicated metabolic pathway, which only culminates in aflatoxin synthesis; aflatoxins would therefore achieve their alleged biological purpose through an indirect role. This eventuality is intriguing but, also, does not preclude the avenue of a more direct role as a ROS scavenger. As all secondary metabolites, aflatoxin production is mainly triggered after the biological switch from trophophase to idiophase, when *Aspergillus* transitions from a growth-centred lifestyle to differentiation and dispersal. At such a stage of mycelial development, aflatoxin must provide a benefit to growth/survivability to an appreciable, but not substantial extent. Given that even within *A. flavus* strains only half are aflatoxigenic, it would be sensible to hypothesize that aflatoxins in no way define or drive *A. flavus* ecology, but also in no way do they burden it beyond redemption, otherwise they would be excised from secondary metabolism altogether through selection, if nothing else, in the context of evolution.

A. parasiticus is an aflatoxin producer, and close relative to *A. flavus,* with a well-documented tolerance to intense oxidative stress, both *in vitro* and *in vivo.* Hong et al. [46] have provided precious insight into *A. parasiticus* aflatoxigenic biological triggers. In their work, antioxidant enzymes are upregulated during growth stage and into early stationary phase; it is only once stably into stationary phase that the oxidative stress-related transcription factory AP-1 like triggers aflatoxin synthesis. In this context, it is easy to see how strongly an intrinsic, direct antioxidant potential of the aflatoxin molecule would constitute a substantial clue to the proof of mechanism researchers have wondered about.

A recent research by Finotti et al. [47] aims at elucidating this aspect. Finotti explored the intrinsic potential (**Figure 3**) of the four main AFs congeners (B1, B2, G1, G2) as scavengers of reactive oxygen species (ROS). In this work, 2,2′-Azobis,

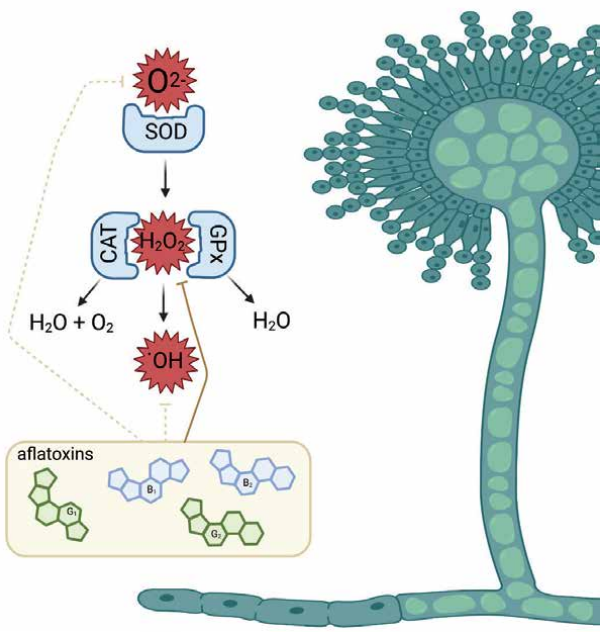

Figure 3.
ROS are highlighted with a red star. Antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are adjuvated by aflatoxins' scavenging activity on H_2O_2 (observed), and on O^{2-} and ·OH (putative). Created with BioRender.com.

2-amidinopropane (APAB) was used to generate oxidants *in vitro* in hydrophilic and lipophilic environments. In the former case, all aflatoxin variants proved capable at inhibiting the oxidant-induced bleaching of crocin, each with different degrees of efficacy, namely: G1 > B2 > G2 > B1, ranked from most to least effective. Notably, AFG1 presented an antioxidant value (Ka/Kc = 2.49) comparable to that of the hydrophilic fraction of select polyphenols known for their remarkable antioxidant activity. A second *in vitro* test was run by Finotti and collaborators to assess survivability of *E. coli* K12 cells, when faced with hydrogen peroxide-induced oxidative stress, in the presence of aflatoxin B1. *E. coli* was selected for the test because it is non-susceptible to aflatoxin toxicity, most likely due to its lack of cytochrome p450, the enzyme whose interaction is necessary to incur into the toxic effect of AFs. 20 ug/mL of AFB1 provided increased carrying capacity to populations of *E.coli* K12 when challenged with hydrogen peroxide concentrations within 0 and 0.6 mM, and no difference with the control beyond such interval. It is of note how Finotti's data substantiate the hypothesis of AFs as active scavengers of ROS. We do not think, however, that this evidence disproves in any way the considerations on AFs' putative, ulterior biological roles. It is indeed a complicated endeavor to frame the purpose of a molecule whose ecological role is likely residual, and whose benefit to the producing organism arguably pertains more than one aspect of life, but possibly none decisively.

5. Conclusions

Fungal species belonging to the *Aspergillus* sect. Flavi synthesize the animal health-hazardous aflatoxins, the most potent natural carcinogenic on earth. Why do these fungi produce them? Our suggestion, recently supported by our findings and by other scientists, is that aflatoxins are a way for resisting an oxidizing environment; namely, they are produced to provide fungi "more time" to induce conidiogenesis and - literally - escape from the stressing environment. Considering this, oxidant stressors produced in different contexts - ranging from herbicide treated soil to host tissues - trigger aflatoxin biosynthesis that - in turn - enhance the antioxidant capacity of *Aspergillus* sect. Flavi and provide a chance to face challenging environments and exploit them.

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Several young and "less" young scientists have a minor but important role in this play call "why Aspergillus produces aflatoxins": I reserve a special thanks to Corrado Fanelli, Anna Adele Fabbri, Enrico Finotti, Alessandra Ricelli, Marzia Scarpari, Marta Punelli and Valeria Scala *inter alia.*

Conflict of interest

"The authors declare no conflict of interest."

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Chapter 5

Mycovirus Containing *Aspergillus flavus* and Acute Lymphoblastic Leukemia: Carcinogenesis beyond Mycotoxin Production

Cameron K. Tebbi, Ioly Kotta-Loizou and Robert H.A. Coutts

Abstract

Carcinogenic effects of *Aspergillus* spp. have been well established and generally attributed to a variety of mycotoxin productions, particularly aflatoxins. It is known that most carcinogenic mycotoxins, with the exception of fumonisins, are genotoxic and mutagenic, causing chromosomal aberrations, micronuclei, DNA single-strand breaks, sister chromatid exchange, unscheduled DNA synthesis *etc*. Some *Aspergillus* spp. are infected with mycoviruses which can result in loss of aflatoxin production. The effects of mycovirus containing *Aspergillus* on human health have not been fully evaluated. Recent studies in patients with acute lymphoblastic leukemia, in full remission, have revealed the existence of antibody to the products of a certain *Aspergillus flavus* isolate which harbored an unknown mycovirus. Exposure of blood mononuclear cells from these patients, but not controls, to the products of this organism had reproduced cell surface phenotypes and genetic markers, characteristic of acute lymphoblastic leukemia. Carcinogenic effects of *Aspergillus* spp. may not always be mycotoxin related and this requires further investigation.

Keywords: Acute lymphoblastic leukemia, Mycovirus, Aspergillus, Cancer, Etiology, Leukemogenesis, Carcinogenesis, Virus, Mycotoxin

1. Introduction

With a worldwide distribution and a significant level of genetic diversity, fungi are of importance in both medical and agricultural fields and represent major health and commercial concerns. Medically, fungal organisms can be a part of the normal flora of humans and animals. However, these also have the potential to cause mild to severe life-threatening invasive infections or toxicities. The immune response to fungal agents is variable and complex, ranging from lack of recognition to severe inflammatory reactions resulting in significant morbidity and mortality [1–6].

There is a broad and diverse spectrum of human and animal diseases attributed to fungi. Major effects of fungal agents in human health include, but are not limited to, organ-specific and systemic infections, especially in immunocompromised individuals, toxicity emanating from fungal products, carcinogenicity, mutagenicity, growth impairment and stimulation of allergic reactions. Common and usually

non-life-threatening infections caused by fungal agents affecting humans are well recognized and often localized on nails, skin, oral cavity, throat and vagina. Severe and fatal infections, however, can be caused by a variety of fungi including *Aspergillus, Blastomyces, Candida, Coccidioides, Cryptococcus, Histoplasma, Mucoromycetes, Pneumocystis, Talaromyces, etc*. Despite the significance of fungal infections an understanding of their pathophysiology has lagged behind other human pathogens. While the immune system of healthy individuals, in general, can effectively prevent some fungal infections, this is not the case in immunosuppressed patients [7, 8].

In addition to causing direct infections, the products of some fungal organisms can be toxic to animals and humans. Also, the mycobiome has been implicated in the pathogenesis of various types of cancers. An example is the link between *Malassezia spp.* and development of pancreatic ductal adenocarcinoma (PDA) [9]. Based on a reported murine experiment, fungal migration from the intestinal lumen to the pancreas initiates the pathogenesis of PDA by driving the complement cascade through the activation of mannose-binding lectin (MBL) [10]. Another example is the carcinogenic potential of *Candida spp*. Some findings indicate that *Candida albicans* is capable of promoting cancer by several mechanisms, including production of carcinogenic byproducts, inflammation, induction of T helper type 17 (Th17) cell response and molecular imitations [10–12]. As will be discussed later in this article, possible relationships between fungal agents and hematological malignancies have been explored.

In light of the above, here the well-established significance of mycotoxins in carcinogenesis is discussed and novel findings illustrating that mycovirus infections may also play a role in human diseases is highlighted. In particular, focus is placed on a mycovirus containing *Aspergillus flavus* and its effects on leukemogenesis.

2. Mycotoxins

The toxicity, mutagenic and carcinogenic effects of some fungi is often attributed to their production of mycotoxins. Mycotoxins are low molecular weight metabolites produced by yeasts and filamentous fungi. These metabolites are heterogeneous chemicals, toxic to vertebrates, including humans. Several mycotoxins also have toxicities to invertebrates, plants, and other microorganisms [13, 14].

Currently, there are over 450 known mycotoxins, which along with their secondary metabolites, can produce varying degrees of toxicity ranging from mild gastrointestinal symptoms to cancer. A large number of common mycotoxins have been identified that are of major concern to human health, among which are aflatoxins, fumonisins, ochratoxins, patulin, zearalenone and nivalenol/deoxynivalenol. Some organisms can produce several different mycotoxins, and many different species may produce the same mycotoxins. Mycotoxin producing fungi are usually found in improperly saved edibles and agricultural commodities. They can enter and contaminate human and animal food supplies. Animals fed contaminated foods can pass aflatoxins through their eggs, milk, and meats, thus indirectly transmitting aflatoxins to humans [15, 16]. While toxicity in humans is often due to ingestion of large doses of mycotoxins, these can also permeate through the skin [17].

Many mycotoxins are cytotoxic and suppress the functions of lymphocytes, granulocytes, and monocytes. Exposure to some mycotoxins inhibits interferon gamma producing Th1 cells and results in decreased number of these cells. Mycotoxins may lead to T cell polarization toward the Th2 phenotype and is a risk factor for the development of allergies [18–23]. The principal function of Th1 cells is cell-mediated immunity and inflammation. In normal conditions, there is

a balance between Th1 and Th2 cells. A shift of such a balance results in various disorders. Th1 cells play an important role in the functions of immunity related cells such as macrophages, B cells, and cytotoxic $CD8^+$ T lymphocytes (CTLs). The latter stimulate cellular immune response, participate in the inhibition of the activation of macrophages and invigorate B cells to produce IgM and IgG1. For instance, it is found that T cells of children exposed to *Aspergillus* have significantly lower Th1 cytokines, including tumor necrosis factors (TNFs), interferon-γ, interleukin-2 and -10. These cytokines are involved in the development of CTLs and natural killer (NK) cells which are responsible for the cell-mediated immune response against viruses and detection and removal of tumor cells. Thus, exposure to fungal agents may significantly change cellular composition and cytokine production and immune function [24, 25].

Exposure to aflatoxins can lead to life threatening acute poisoning (aflatoxicosis) [26]. In turn, acute aflatoxicosis can result in acute hepatic necrosis often manifested by symptoms of liver failure [27]. This eventually may cause development of cirrhosis in the liver and hepatic carcinoma. Chronic low-level exposure to mycotoxins, particularly aflatoxins and especially aflatoxin B1, is known to be associated with increased risk of hepatic damage, liver and gallbladder cancer and impaired immune activity [27–29]. Several studies have documented liver and gallbladder toxicity and carcinogenicity related to mycotoxins. Other organs, including bones, kidneys, pancreas, bladder, viscera and central nervous system, can be subject to carcinogenesis [30].

A variety of mycotoxins have carcinogenic potential in animals and humans [16, 17, 26, 28, 31–35]. Certain mycotoxins, especially aflatoxins, produced by genetically diverse *Aspergillus* spp. including *A. fumigatus, A. parasiticus* and *A. flavus* can be genotoxic with damage to DNA, which is attributed to the development of cancer in animals and humans. The effects of aflatoxins B1, B2, G1 and G2 and their metabolites such as aflatoxins M1, M2a, P1, Q1, Q2a, R0, H1; B2a, M2; GM1, GM2a, parasiticol (B3) and GM2, produced by the *Aspergillus* spp., are well recognized [35].

The carcinogenesis of mycotoxins is reported to be due to the intercalation of aflatoxin metabolites into DNA which alkylate the bases through epoxide moiety. This can be as a result of the mutations in the *p53* gene or signaling apoptosis. The third base of codon 249 of the *p53* gene is reported to be more susceptible to aflatoxin-mediated mutations. For example, in hepatocellular carcinoma, upon exposure to aflatoxin, mutation of *p53* gene is fixed at codon 249 third base and take the form of G to T transversion [36, 37].

In one report, using a mammalian cell line, the mutagenicity of various mycotoxins and the efficiency of mutagenic mycotoxins in producing DNA single strand breaks and chromosome aberrations were investigated. These experiments revealed that aflatoxin B1, mycophenolic acid, patulin, penicillic acid, and sterigmatocystin induce 8-azaguanine-resistant mutations. At higher concentrations, aflatoxin B1, mycophenolic acid, and sterigmatocystin were found to have minimal effects on single-stranded DNA. In contrast, treatment with patulin and penicillic acid at higher concentrations had resulted in severe breaks. Chaetoglobosin B, fusarenon X, luteoskyrin, and ochratoxin A had not induced 8-azaguanine-resistant mutations [38].

Overall, the mutagenicity of mycotoxins varies significantly and depends on their efficiency in causing DNA single-strand breaks, resulting in chromosomal aberrations. Adults are believed to have a higher tolerance to mycotoxins but exposure of children, while controversial and not uniformly accepted, can lead to delayed development and stunted growth [16, 31–33].

In addition to laboratory-based experiments, reports regarding isolation of mycotoxin producing strains of fungi, including that of *A. flavus*, from the

residences of leukemia patients are available [39–42]. In many reports, except for recent publications, fungal carcinogenesis is attributed to mycotoxins and their immunosuppressive effects. One report describes examination of sera from 36 cancer patients against an aflatoxin producing *A. flavus* which was isolated from the home of a patient with leukemia. A modified microimmunodiffiusion technique was used for this immunological evaluation. This study had found that 30% of cancer patients, 15 of whom had leukemia or lymphoid malignancy, and 6% of controls had shown a precipitation band indicating positive results [39]. Another published article reports four leukemic patients, from three families, in a residence where a mycotoxin producing fungus was isolated. The leukemogenesis was attributed to the immune depressive effects of mycotoxins [41]. In a house where a husband and wife had developed acute myelomonocytic and undifferentiated leukemia, respectively, fungal surveyance of the residence had been performed. Three fungal isolates were found, an extract of which had shown a depressive effect on a phytohemagglutinin skin test in guinea pigs as compared to negative findings using extracts isolated from a control residence [40]. As described below, a significant amount of data regarding the correlation of a mycovirus containing *A. flavus*, isolated from the home of a patient with acute lymphoblastic leukemia, has been recently published.

3. Viruses and human cancer

A vast amount of data on several viruses and their possible association with cancer development has been published [43–52]. While not the focus of this article, a brief review of the subject reveals the importance of the study of viral agents and their relation to occurrence of malignant disorders. Both DNA and RNA viruses are capable of causing cancer in humans. Some of the known DNA viruses that are capable of causing human cancers are Epstein-Barr (EB) virus, human papilloma virus, hepatitis B virus, and human herpes virus 8. The relationship of EB virus to the development of Burkitt's lymphoma and nasopharyngeal carcinoma is well established [53–59]. Likewise, the relation of human papilloma virus and the development of cervical cancer and retention of HPV viral oncoproteins E6 and E7 for their continued expression and proliferation has been demonstrated [60–63]. Human T lymphotropic virus type 1, human immunodeficiency virus (HIV) and hepatitis C viruse are some of the RNA viruses that contribute to human cancers. It appears that viruses have diverse biological pathways to malignant disorders. The presence of viral gene products in cancer and precancerous cells are known. Despite the well-known carcinogenic role of viruses, little data regarding any possible health effects of mycoviruses alone, or in conjunction with their host, are available. This area needs to be further explored.

4. Mycoviruses

Viruses that infect fungi, also known as mycoviruses (*myco* = 'fungus' in Greek), are widespread geographically and are expected to infect all fungal taxa, from early divergent lineages to the most well-studied ascomycetes (sac fungi) and basidiomycetes (mushrooms). Mycovirus infection is persistent but does not result in disease or death of the host fungus, and often does not lead to obvious alterations in its phenotype under controlled laboratory conditions; therefore, mycovirology is an underappreciated and understudied field, similar to all non-disease associated virology [64].

Mycoviruses are currently classified in 22 taxa (21 families and one genus) by the International Committee on Taxonomy of Viruses (ICTV; https://talk.ictvonline.org/) (**Figure 1**). Some of these taxa exclusively accommodate viruses infecting fungi, such as the families *Hypoviridae* and *Polymycoviridae*. Other taxa also accommodate viruses infecting protozoa, plants, insects and mammals, such as the families *Botourmiaviridae*, *Chrysoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae*. Members of the DNA-containing *Genomoviridae* family have been discovered in sequencing data from a variety of samples, including plant and insect tissue, animal blood, serum and feces, human blood, plasma, cerebrospinal fluid, cervical biopsies, and feces, and sewage [65]. Mycoviruses may be closely related to viruses pathogenic for humans. For instance, family *Mymonaviridae* belongs to the order *Mononegavirales*, as are viruses that cause Ebola, measles, mumps, rabies and respiratory diseases. Families *Metaviridae* and *Pseudoviridae* belong to order *Ortervirales*, together with human immunodeficiency virus (HIV), cause of acquired immunodeficiency syndrome (AIDS), and other retroviruses.

Classification of exemplar mycoviruses known to infect *Aspergillus* spp is shown in **Figure 2**.

Almost all known mycoviruses have double stranded (ds) RNA genomes or single stranded (ss) RNA genomes, either positive sense or negative sense, with one family of mycoviruses having circular ssDNA genomes. Virions are often proteinaceous in nature, composed of virus capsid proteins and their structure may range from spherical, to bacilliform in the case of barnaviruses, to filamentous in the case of flexiviruses and mymonaviruses. The absence of true virions is also common: narnaviruses and mitoviruses exist as naked RNA molecules respectively in the cytoplasm and mitochondria, hypoviruses are encapsulated in host derived lipid vesicles, polymycoviruses are non-conventionally encapsidated by a viral protein [66, 67]. Mycoviruses move intracellularly within the infected fungus and spread in mycelia during cell division and growth. Almost all known mycoviruses lack an extracellular phase in their replication cycle; they are transmitted vertically during asexual and/or sexual spore production and horizontally between fungal strains following cell fusion. The absence of an extracellular phase explains the general lack of lipid envelopes in virions.

Early reports focused on the mycovirus-mediated alterations on fungal phenotype, including morphology, pigmentation, asexual and sexual sporulation, and growth. Production of viral toxins conferring a competitive advantage to the fungal host [68], clearly illustrate that viral infection can be beneficial to the host and viruses are undeserving of their name, derived from the Latin word for 'poison' or 'venom'. These killer yeast systems have been primarily studied in the eukaryotic model organism *Saccharomyces cerevisiae* [69], extensively used in biotechnological applications such as baking, brewing and winemaking. However, interest in mycoviruses stems mainly from their effects on the interaction between their host fungus and the plant, insect or mammalian/human host of the fungus.

An increasing number of studies clearly illustrate the importance of mycoviruses in host-microbe interactions. The discovery of 'transmissible hypovirulence', i.e., mycovirus-mediated decrease in fungal pathogenicity represents a major advance in the field and the first mycovirus-based biological control application to combat chestnut blight caused by the plant pathogen *Cryphonectria parasitica* [70, 71]. The opposite phenomenon called hypervirulence, i.e., mycovirus-mediated increase in fungal pathogenicity, has also been noted. For instance, two variants of Aspergillus fumigatus polymycovirus 1 (AfuPmV-1), the first virus demonstrated to be infectious as dsRNA [66], respectively cause hypovirulence in an immunosuppressed mouse infection model [72] and hypervirulence in the greater wax moth *G. mellonella* infection model [73]. Additionally, AfuPmV-1 renders its fungal host more

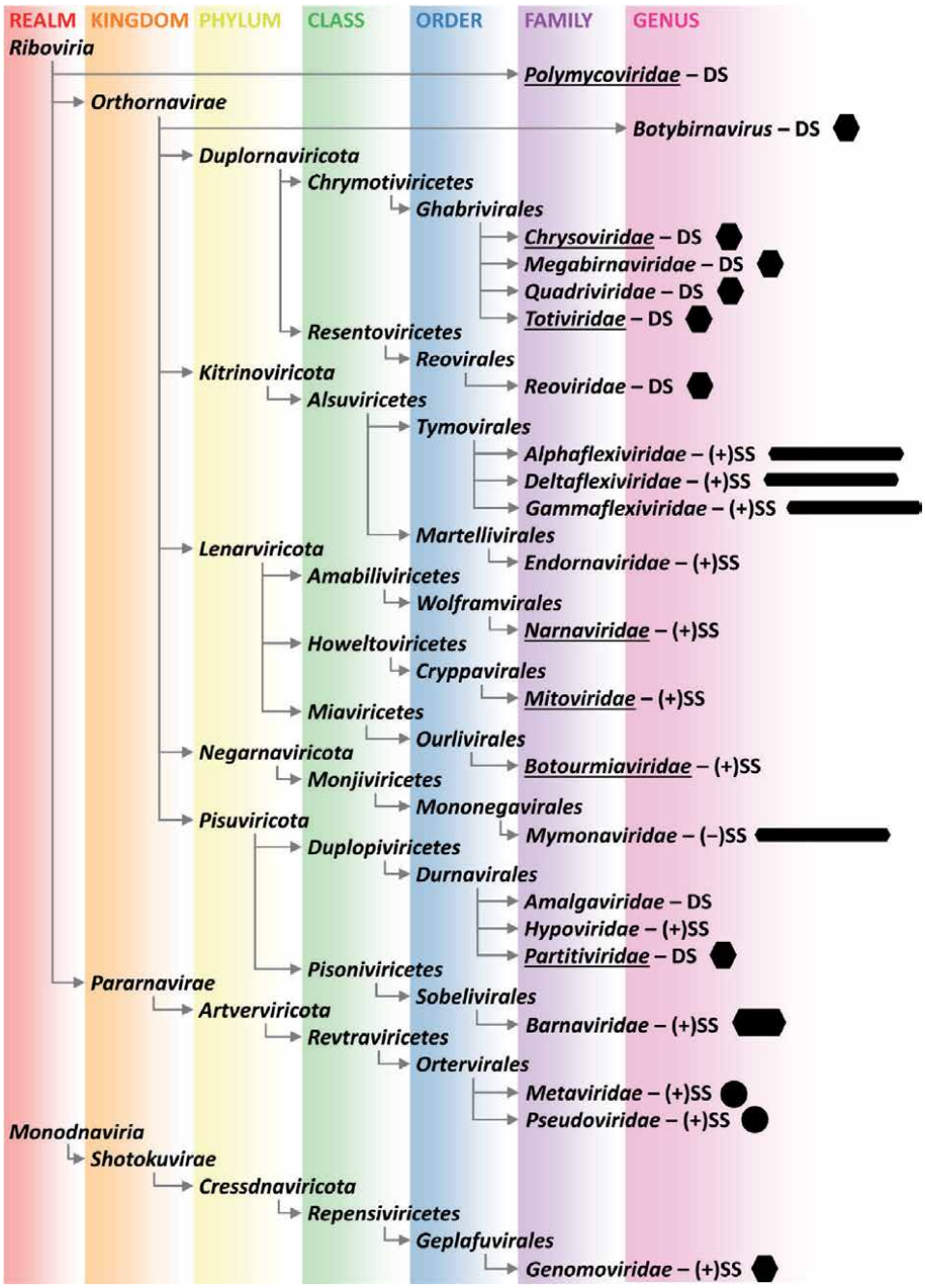

Figure 1.
Current classification of mycoviruses according to the International Committee on Taxonomy of Viruses. The realms Riboviria *and* Monodnaviria *accommodate viruses with respectively RNA and DNA genomes. Underlying family names accommodate mycoviruses known to infect* Aspergillus *spp. Next to family/genus names, (+)SS, (–)SS and DS indicate respectively, positive-sense single-stranded, negative-sense single-stranded and double-stranded genomes; hexagons indicate the presence of true virions, either isometric, bacilliform of filamentous.*

sensitive to the bacterium *Pseudomonas aeruginosa* [74]. Furthermore, partitivirus infection of *Talaromyces marneffei* leads to hypervirulence in a BALB/c mouse model [75]. Mycoviruses dsRNA genomes or replication intermediates are recognized by Toll-like receptor 3 (TLR-3) [76] and may induce an interferon immune response

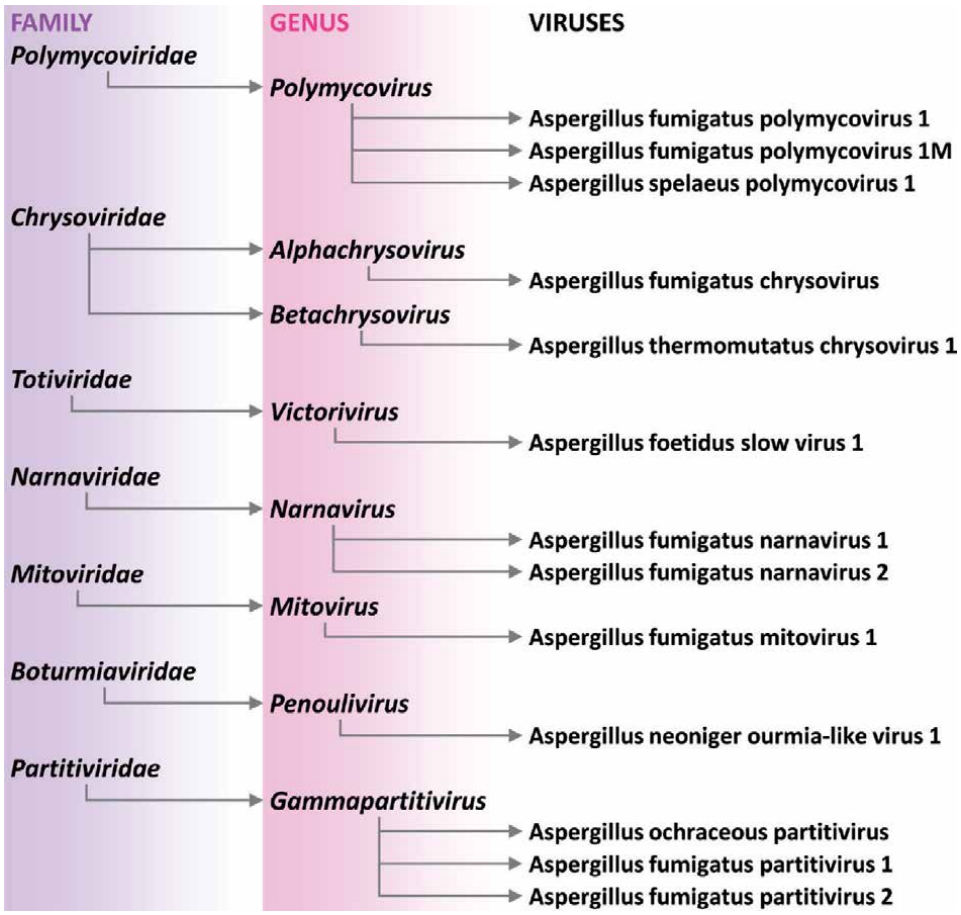

Figure 2.
Classification of exemplar mycoviruses known to infect Aspergillus *spp. Not all known mycoviruses found in* Aspergillus *spp. are officially assigned to recognized taxa. The phenotypes and effects of the majority of these mycoviruses on their* Aspergillus *host is unknown.*

in a TLR-3 dependent or independent manner, as illustrated with totivirus infected *Malassezia* [77, 78]. A link between azole resistance and mycovirus infection has been noted in *Penicillium digitatum* [79]. Finally, mycovirus infection is known to be responsible for modulation of fungal toxins and this phenomenon has been studied mainly in *Aspergillus* spp [80]. Carcinogenic aflatoxin production may be repressed by the presence of a mycovirus in *A. flavus* [81–84], while ochratoxin A is enhanced by the presence of a partitivirus in *A. ochraceus* [85].

Currently most mycovirus studies are focused on economically important phytopathogenic fungi, while scant data regarding fungi containing mycoviruses and human disorders are available. Since mycoviruses do exist in fungi, and humans are exposed to them, further research on these organisms may expand our knowledge of their possible role and effects of their interaction with humans.

5. Studies of mycovirus containing *Aspergillus flavus*

A report describing plasma of patients with acute lymphoblastic leukemia (ALL) having a positive reaction to an *A. flavus* isolate containing an unknown mycovirus is available [86]. Exposure of the peripheral blood mononuclear cells

(PBMCs) obtained from a group of ALL patients who were in a complete remission to the culture of this organism was reported to reproduce genetic and cell surface phenotypes, characteristic of active ALL [87]. Conversely, this was not observed in the control group of patients [87]. To describe these findings (which are patented) in more detail, in a series of experiments, a mycovirus infected *A. flavus* separated from the home of a patient with B-cell ALL was found to contain unknown mycovirus particles. These mycovirus particles were found within the body of the organism and culture supernatant. Chemical analysis of the isolated mycovirus containing *A. flavus* had revealed a lack of aflatoxin production [86]. The latter may be due to the influence of the unknown mycovirus which may have caused suppression of the production of aflatoxin as described previously [80–84]. Utilizing fast protein liquid chromatography (FPLC) for the analysis of the supernatant of the culture of this isolate, three separate peaks were identified. As noted above, in controlled experiments using plasma of patients with ALL in complete remission, with no evidence of the disease, using crude supernatant of the culture of the mycovirus containing *A. flavus* and enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies, plasma of patients with ALL had reacted positively. The plasma obtained from three separate groups of controls, including normal individuals, patients with sickle cell disease and individuals with various solid tumors, had been negative. In a separate study evaluating peaks obtained by fractionation using FPLC, of the three peaks which were found, peak 1 had the strongest positive effect [86]. The authors suggest that this technique can be used for screening for ALL or a test to identify patients who have had this disease [86].

As noted before, in a related publication, exposure of PBMCs obtained from ALL patients in complete remission, and long-term survivors of this disease, to the supernatant of the culture of the mycovirus containing *A. flavus* resulted in the re-development of the genetic and cell surface phenotypes, characteristic of ALL. The cell surface phenotypes examined were CD10/CD19, CD19/CD34 and CD34/CD117. The redevelopment of the ALL cell surface phenotypes was reported to be gradual, completed in 24 hours, and remained stable thereafter. Following exposure to the supernatant of the mycovirus containing *A. flavus*, alterations in gene expression were evaluated using microarray technique. Some of these alterations were reported to be upregulation of JAK1 (12.87-fold), JAK2 (1.5-fold), JAK3 (2.73-fold), IKZF1 (10.12-fold), MCL1 (59.37-fold), MYC (14.19-fold), HDAC1 (26.39-fold) and downregulation of PAX5 (3.05-fold). Following incubation, a significant and robust activation of transcription factor NF-κB p65 was reported by immunoblotting in ALL patients without any changes in the controls. The supernatant of the culture of *Mycocladus corymbifer*, which was used as a negative control, was reported to have no effects on PBMCs either from the ALL or control patients [87]. The above studies suggest a possible role for the mycovirus containing *A. flavus* in the process of leukemogenesis and opens a venue for vaccination and prevention of this disease.

6. Conclusion

It is apparent that fungal *spp*. are important in human and animal health. The mechanism of the effects of fungal agents in the development of human diseases appears to be multifaceted. Fungi are widespread in nature and inevitably, humans encounter these organisms. Many fungi contain mycoviruses. Although a significant amount of data regarding the carcinogenic effects of mycotoxins in the development of malignant disorders are available, possible pathogenicity and role of the mycoviruses in fungi, if any, in human and animal health, including malignant disorders, are not known. Recent reports describing *in vitro* effects of a mycovirus

containing *A. flavus* isolate in redeveloping characteristic ALL cell surface and genetic phenotypes in the PMBCs of acute lymphoblastic leukemia patients in complete remission is of interest. The existence of antibody to this organism in plasma of these patients is intriguing and further indicates its possible role in leukemogenesis. This area needs to be further investigated.

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Chapter 6

Industrial Applications of Nanomaterials Produced from *Aspergillus* Species

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Abstract

There is a great demand for green methods of synthesis of nanoparticles. Fungi play an important role in the synthesis of nanoparticles, of which *Aspergillus* spp. are known to secrete different enzymes responsible for the synthesis of nanoparticles. The process of biosynthesis of nanoparticles is simple, rapid, cost-effective, eco-friendly, and easy to synthesize at ambient temperature and pressure. Mostly, the metal nanoparticles such as silver, gold, lead and the oxides of titanium, zinc, and copper are synthesized from *Aspergillus* spp. These include mainly *Aspergillus fumigatus, A. flavus, A. niger*, *A. terreus*, and *A. clavatus*. The fabrication of different nanoparticles is extracellular. In the present chapter, we have discussed the role of different species of *Aspergillus*, mechanism of biogenic synthesis particularly enzymes involved in the reduction of metal ions into nanoparticles. The biogenically synthesized nanoparticles have demonstrated several biomedicals, agricultural, and engineering applications. The biogenic nanoparticles are mostly used as antimicrobial and cytotoxic agents. Their use as fungicidal agents is important for sustainable agriculture.

Keywords: *aspergillus* spp., nanomaterials, biogenic synthesis, industrial, biomedical, agriculture

1. Introduction

Nanomaterials (NMs) are the structures fabricated in the nanoscale, i.e. 1 to 100 nm and having at least one dimension in the nanoscale. The fabrication, study, and application of nanostructures are known as nanotechnology. The exhibition of novel physicochemical properties by the nanoscale materials has provided a unique opportunity for researchers to design and develop materials with applications in the diverse fields of science and technology. This has attracted attention towards nanoparticles (NPs) and their fabrication as compared to other sectors of NMs. Some of the nanomaterial productions have reached to the industrial scale due to the high demand for NMs in consumer products and their number is increasing at the moment with their developing applications. Ever-increasing demand for different NPs has generated the need for easy, safe, efficient, rapid, and eco-friendly procedures for their large-scale production.

Nanomaterials can be produced by two general approaches, i.e. top-down approach and bottom-up approach. Another classification includes different methods like physical, chemical, biological, and hybrid methods of nanoparticle production. The physical method requires an expensive setup, is high energy-consuming, and hazardous to health and the environment. Whereas chemical methods are highly efficient as compared to physical methods, but involve a toxic reducing agent, solvent, and stabilizing/capping agents. Recently, the biological method of nanoparticle production has attracted attention because of its ease, eco-friendly nature, high efficiency, and high yield. In this method, a biological agent or a biomolecule plays a significant role in the production of NMs [1]. Production of NMs by a biological method is a promising alternative for physical and chemical methods [2].

Among the different biological systems like bacteria, actinomycetes, fungi, plants, protozoa, and animals, fungi have shown great potential for the production of NPs on large scale. Bacteria normally produced NPs intracellularly, where large-scale production and purification of NPs is complicated and expensive. Unlike bacteria, fungi produce NPs extracellularly and are easy to use and purify NPs for large-scale production [3]. Fungi are easy to handle, versatile, tolerant, and economical biological systems for industrial production of biotechnology products and have been used extensively in large-scale production of different metabolites. The tremendous ability of fungi in the secretion of proteins up to 100 g/L, metabolic diversity, and high production capacity have made them a unique option for industrial biotechnology for decades. Hence, filamentous fungi are the first choice, since they are capable of secreting a large amount of proteins and other metabolites extracellularly. Moreover, the fabrication of NPs by a fungal system is a green process [4]. Among the fungal sources, *Aspergillus* is a very promising candidate for the production of NPs, this is because there are more than 350 species of this genus with enormous biochemical versatility in addition to the secretion of a large quantity of proteins [5]. Different *Aspergillus* species produce NPs of diverse sizes and shapes with interesting physicochemical properties like enhanced thermostability, stability over a wide pH range, greater solubility, and biocompatibility. Moreover, the compounds produced by *Aspergillus* are classified as generally regarded as safe (GRAS) status, which can be safely used in the industry [6]. NPs fabricated by fungi have been used for different applications such as in medicine, as an anti-cancer drug, antibiotic, antifungal, antimicrobial, and antiviral agents [7], in diagnostic, bioimaging, biosensor, agricultural, and other industrial applications [8]. A new term "Myconanotechnology", was proposed by Rai and co-workers [9] to highlight the research on fungi in the production of NPs and their role in the nanotechnology research.

Industrial biotechnology processes demonstrate a significant reduction of greenhouse gas emissions using renewable resources. The process is environment friendly and do not result in the accumulation of toxic compounds in the ecosystem. In industrial biotechnology, biomass input is used under the process of biological agents like metabolites and biomolecules to create a wide spectrum of products. There is a worldwide interest to enable the production of different NPs on biotechnological lines because of their eco-friendly nature, less energy-intensive, ease of execution, and ability to modify biological agents, and products [10].

In the present chapter, we are going to focus on the need for large-scale productions of NPs by biological methods in general and by *Aspergillus* spp. in particular. Different NPs fabricated by the *Aspergillus* spp., their advantages over the other methods, details of the mechanistic aspects of nanoparticle production, and various applications of fabricated NPs. Toxicity concerns of the large-scale production of NPs will also be discussed.

2. Diversity of *aspergillus* spp. for the synthesis of different nanomaterials

More than 6400 different biologically active substances have been reported from filamentous fungi which have potential bioactivities and different applications [11]. As these fungi have greater tolerance to high metal ion concentration and have the ability to internalize and bio accumulate metal ions they can be used for metal ion reduction and stabilization in nanomaterial synthesis [12–16]. A huge range of fungi is shown to have the ability to synthesize NPs. Out of which *Aspergillus* is one of the major contributors in the mycosynthesized (fungus mediated synthesis) NMs with various biological activities. A huge range of *Aspergillus* spp. have been reported to synthesize different NMs including metal and metal oxide NPs. The cell-free extracts, as well as supernatant of fermented medium, can also be used for the synthesis of NPs [14, 15, 17]. The following **Table 1**, summarizes the *Aspergillus* spp. and the respective NMs synthesized by them.

***Aspergillus* spp.**	**Nanomaterial synthesized**	**Reference**
Aspergillus tubingiensis	Silver	[18]
Aspergillus niger IPT856	Silver	[19]
Aspergillus oryzae	Silver	[20]
Aspergillus flavus	Silver	[21]
Aspergillus versicolor	Silver	[22]
Aspergillus terreus	Silver	[23]
Aspergillus versicolor	Silver	[24]
Aspergillus oryzae (MTCC no. 1846)	Silver	[25, 26]
Aspergillus fumigatus BTCB10	Silver	[27, 28]
Aspergillus niger	Silver	[14, 15]
Aspergillus tamari *Aspergillus niger*	Silver	[29]
Aspergillus flavus	Silver	[30]
Aspergillus terreus	Silver	[31, 32]
Aspergillus flavus	Silver	[33]
Aspergillus fumigates	Silver	[34]
Aspergillus oryzae var. *wiridis*	Silver	[35]
Aspergillus flavus	Silver	[36]
Aspergillus fumigatus DSM819	Silver	[37]
Aspergillus niger	Silver	[17]
Aspergillus niger	Silver	[38]
Aspergillus niger	Silver	[39]
Aspergillus fumigatus	Silver	[40]
Aspergillus flavus	Silver	[41]
Aspergillus clavatus	Silver	[42]
Aspergillus flavus NJP08	Silver	[43]
Aspergillus terreus CZR-1	Silver	[44]
Aspergillus terreus	Silver and Gold	[45]
Aspergillus sydowii	Gold	[46]
Aspergillus niger	Gold	[47]

***Aspergillus* spp.**	**Nanomaterial synthesized**	**Reference**
Aspergillus niger	Gold	[48]
Aspergillus terreus IF0	Gold	[49]
Aspergillus niger	Gold	[50]
Aspergillus clavatus	Gold	[51]
Aspergillus flavus	TiO_2	[52]
Aspergillus flavus TFR7	TiO_2	[53]
Aspergillus niger	ZnO	[54]
Aspergillus fumigatus	ZnO	[55, 56]
Aspergillus oryzae	$FeCl_3$	[57]
Aspergillus tubingensis	$Ca_3P_2O_8$	[58]
Aspergillus versicolor mycelia	Hg	[59]
Aspergillus aureoterreus Samson et al. AUMC 13006	CuO	[60]
Aspergillus carneus Blochwitz AUMC 13007	CuO	[60]
Aspergillus flavus var. *columnaris* Raper and Fennell AUMC 13012	CuO	[60]
Aspergillus fumigatus Fresenius AUMC 13024	CuO	[60]
*Aspergillus sydowii (*Bainier and Sartory) Thom and Church	CuO	[60]
Aspergillus terreus Thom *AUMC 13019*	CuO	[60]

Table 1.
Various aspergillus *spp. and respective nanomaterials synthesized by them.*

The cell-free extracts of *Aspergillus* spp. are challenged against the precursor salt for direct synthesis of NPs. But *Aspergillus* extract fermented lupin was reported by Mosallam et al., [61], for the biological synthesis of selenium NPs in the presence of gamma radiation. Balakumaran et al. [45] reported the various strains of *Aspergillus* isolated from Kolli hills and Yercaud hills, South India, and identified their ability to synthesize extracellular gold and silver NPs. Out of all the screened isolates, *A. terreus* showed the most stable nanoparticle synthesis. Vala [46] reported the synthesis of gold NPs by marine-derived fungus *Aspergillus sydowii* [62]. The intracellular synthesis of gold NPs by *Ammophilus fumigatus* has been reported by Bathrinarayan et al., [63, 64]. In one of the studies on the experimental rat model have demonstrated the wound healing ability of *A. niger* mediated silver NPs [65]. Ghareib et al., [60] isolated *Aspergillus* strains from Egyptian soil and reported their biomass and culture supernatant mediated synthesis of copper oxide (CuO) NPs. Biogenic zinc oxide NPs are synthesized from the cell-free fungal filtrate of *A. niger,* which has antimicrobial and dye degradation ability [54].

All these various types of NPs synthesized using different isolates and strains of *Aspergillus* are shown to have distinguishing bioactivities, numerous functions, and applications in various fields [14, 15, 66]. These fungus-mediated metal and metal oxide NPs are synthesized intra or extracellularly and are reported to appear in various shapes and sizes [67].

3. Advantages of nanomaterial production by *aspergillus* spp

The green chemistry approach highlights the usage of microorganisms which offers a cheaper, lighter, reliable, nontoxic, and eco-friendly process [68, 69]. Fungi

secrete a higher amount of proteins owing to significantly higher productivity of NPs [70] which effectively proved a potential source for the extracellular synthesis of different NPs without using harmful toxic chemicals. The advantages made fungi more suitable for large-scale production and easy downstream processing, also economic [70, 71]. Besides, enzyme nitrate reductase is found to be responsible for the synthesis of NPs in fungi [68, 69]. Biofabrication of NPs using fungi (eukaryotic organism) has several advantages over the prokaryotic mediated approach for reproducibility of nanosized materials. Also include ease to multiplication, grow, handling, and rest of downstream process for this top-down approach of nanobiosynthesis through nano factories [72, 73]. Tarafdar et al., [74] observed rapid, low cost, and eco-friendly iron nanoparticle fabrication by using the fungi *Aspergillus oryzae* TFR9. This study evaluated the morphological and elemental characterization of the biosynthesized iron NPs [74].

Zielonka et al., [75] demonstrated fungi are almost ideal biocatalysts for NPs biosynthesis. In contrast to bacteria, as they are well-known for producing greater amounts of biologically active substances that make the fungus more appropriate for large-scale production [31, 32]. Moreover, fungal biomass can resist flow pressure, agitation, and harsh conditions in chambers such as bioreactors. Also, they exude extracellular reductive proteins which can be used in subsequent process steps. However, the fungal cell is deprived of unessential cellular components since NPs are accelerated outside the cell and can be immediately used in manifold ways without pre-treatment [76]. There are a large number of fungi, which can efficiently synthesize silver NPs, such as *Aspergillus clavatus,* and have many biomedical applications [77].

Here we highlighted the advantages of NMs produced by using *Aspergillus* spp. The high-scale production of NPs from fungi has wide applications in protein engineering, synthetic biology, and downstream processing (**Figure 1**). For large-scale production, fungi can be effectively employed. Gade et al., [14, 15] studied extracellular biosynthesis of AgNPs by *Aspergillus niger* isolated from soil. The nitrate-dependent reductase enzyme reduced the silver ions and a shuttle quinone extracellular process. The reduction of silver ions was an extracellular process.

AgNPs released silver ions in the fungal cell, which increased its antifungal function. AgNPs synthesized by using *A. terreus* HA1N sp. There is a large number of fungi, which can efficiently synthesize silver NPs, such as *Aspergillus clavatus* (*A. clavatus*) or ZnO NPs are produced by *Aspergillus terrus* [57]. The effect of prepared zinc oxide NPs on the growth and mycotoxin production by mycotoxigenic molds was evaluated which was concentration-dependent. The levels of produced mycotoxins were decreased when the concentration of ZnONPs increased [78]. Bathrinarayanan et al., [63, 64] produced gold NPs by *Aspergillus fumigatus*. It was found to be stable, spherical, and had irregular morphologies which were confirmed by SEM analysis.

El-Desouky et al., [79] demonstrated the synthesis AgNPs by an eco-friendly and low-cost method using the fungi *Aspergillus terreus* HA1N. It is an alternative to chemical procedures which require drastic experimental conditions emitting toxic chemical byproducts. The AgNPs are widely used as a novel therapeutic agent as antibacterial, antifungal, antiviral, anti-inflammatory, and anti-cancer agents [80, 81]. The AgNPs synthesized by *Aspergillus* ssp. present potential advantages such as fast growth rate, the rapid capacity of metallic ion reduction, nanoparticle stabilization, and facile and economical biomass handling. Moreover, these fungi have significantly higher productivity when used in nanoparticle biosynthesis due to their higher protein secretion [82]. Husain et al., [83] demonstrated the immobilization of *Aspergillus oryzae* β-galactosidase on native ZnO and zinc oxide NPs (ZnO-NP) by using a simple adsorption mechanism. The ZnO has wide

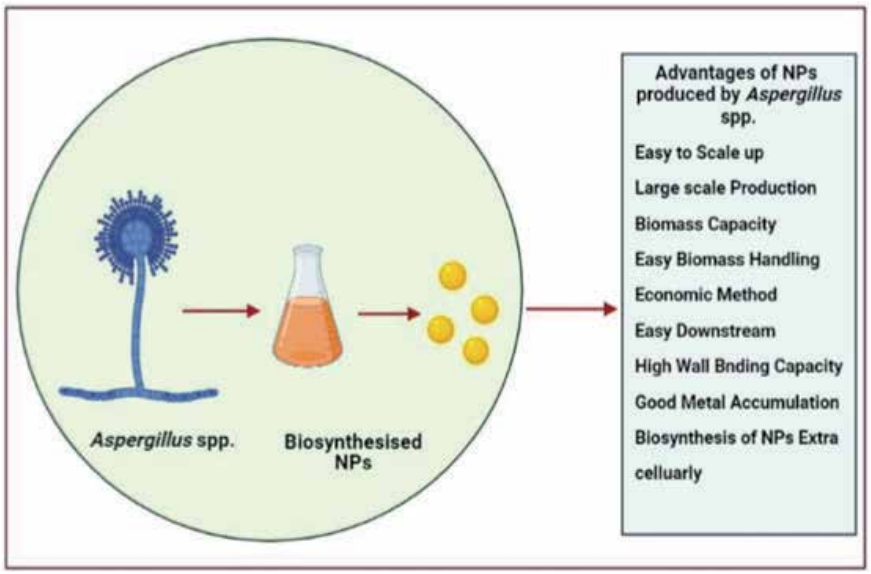

Figure 1.
Advantages of nanoparticles produced by aspergillus *spp.*

applications. In addition to this easy production, improved stability against various denaturants, and excellent reusability, ZnO-NP bound β galactosidase. There are many applications in constructing enzyme-based analytical devices for clinical, environmental, and food technology. *Aspergillus niger* showed effective fabrication of AgNPs [84]. The mycotoxin produced by mycotoxigenic fungi such as *Aspergillus* sp. Food toxin can be detected by nano-based biosensors. The functionalized NMs are used as catalytic tools, immobilization platforms, or optical or electroactive labels to improve the biosensing performance to obtain higher sensitivity, stability, and selectivity. Recently, these nano biosystems are also bringing advantages in terms of the design of novel food toxin detection strategies [85].

4. Mechanistic aspect of nanomaterial synthesis by *aspergillus* spp

It is well-identified that biological systems can fabricate the number of metallic and non-metallic nanoparticles. Synthesis of nanoparticles can be achieved at low cost by biological system especially from the fungal system at low pH, temperature, and salt concentration. Various studies have been proved that fungus-like *Fusarium* [7], *Phoma* [4], *Aspergillus* [86], and many more were found to be excellent factory for synthesis different types of nanoparticles. Every fungal species has unique biomolecule contents, which play a crucial role in the synthesis of nanoparticles. Due to this convolution still, an exact mechanism for nanoparticles synthesis from specific fungal species is yet to be revealed.

Even though, various studies have been initiated to understand the mechanism for the synthesis of nanoparticles from *Aspergillus* spp. Jain and co-workers [43] proposed two-step mechanism for silver nanoparticles synthesis from *Aspergillus flavus* NJP08. In the first step, 32 kDa reductase protein secreted by fungus might be responsible for the synthesis, and in the next step 35 kDa protein is responsible to provide stability to silver nanoparticles. In one of the study by Phanjom and Ahemad [86] proposed that the nitrate reductase enzyme secreted by *Aspergillus oryzae* (MTCC No. 1846) is responsible for the conversion of Ag + to Ag0. Selenium

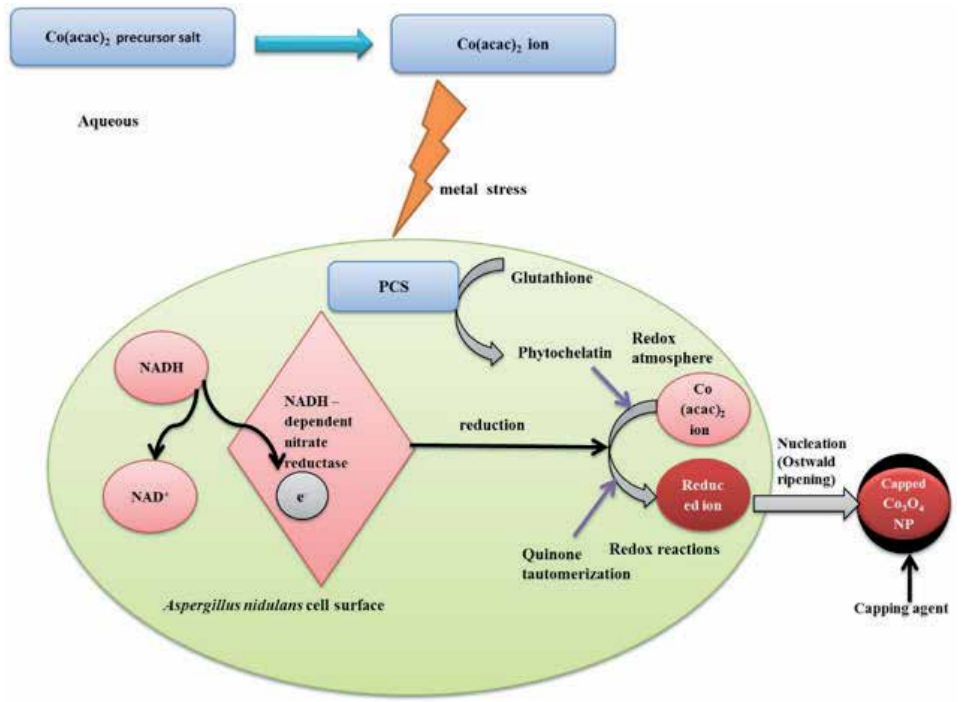

Figure 2.
Possible mechanism for the biosynthesis of Co_3O_4 nanoparticles in A. nidulans *[87].*

nanoparticles are also proved to be synthesized by the aqueous extract of fermented Lupin using *Aspergillus oryzae* and nucleation by gamma-ray (30.0 kGy) [61]. The authors confirmed that due to unique characteristics and novel biosynthesis method, selenium nanoparticles could be a good green antimicrobial candidate in biomedicine, cosmetics, and pharmaceutics. Endophytic fungi *Aspergillus nidulans* also produced cobalt oxide nanoparticles through the detoxification mechanism. In the synthesis, nitrate reductase along with electron shuttling compounds and other peptides are responsible for the reduction and synthesis [87]. Pavani et al. [88] reported the possible reductase or cytochrome base synthesis of lead nanoparticles from *Aspergillus* species. In the first step, the mechanism stated the trapping of lead ions on the fungal cell wall through electrostatic attraction. In the next step, these ions get entered into the cell and might get reduced by enzymes existing in the cell wall and inside the cell wall. In one of the study conducted by Li et al., [31, 32] reported the stabilized nanoparticles synthesis through reducing agent nicotinamide adenine dinucleotide (NADH) present in the *Aspergillus terreus* (**Figure 2**).

5. Applications of nanomaterials synthesized using *aspergillus* spp

The numerous NMs have been synthesized by *Aspergillus* spp. and applied in various fields, for instance, Ag, Cu, Fe, Fe_3O_4, and ZnO NMs are some of them [55, 56, 72, 89–95]. Nanotechnology platform finds application of NMs in almost in each and every field such as agriculture, environmental science, health sciences, portable water treatment large/small scale plants, industrial separation, catalyst, electronics, energy storage, and energy regeneration [96–99]. The applications of NMs synthesized using *Aspergillus* spp. in various fields are shown in the graphical representation of **Figure 3**.

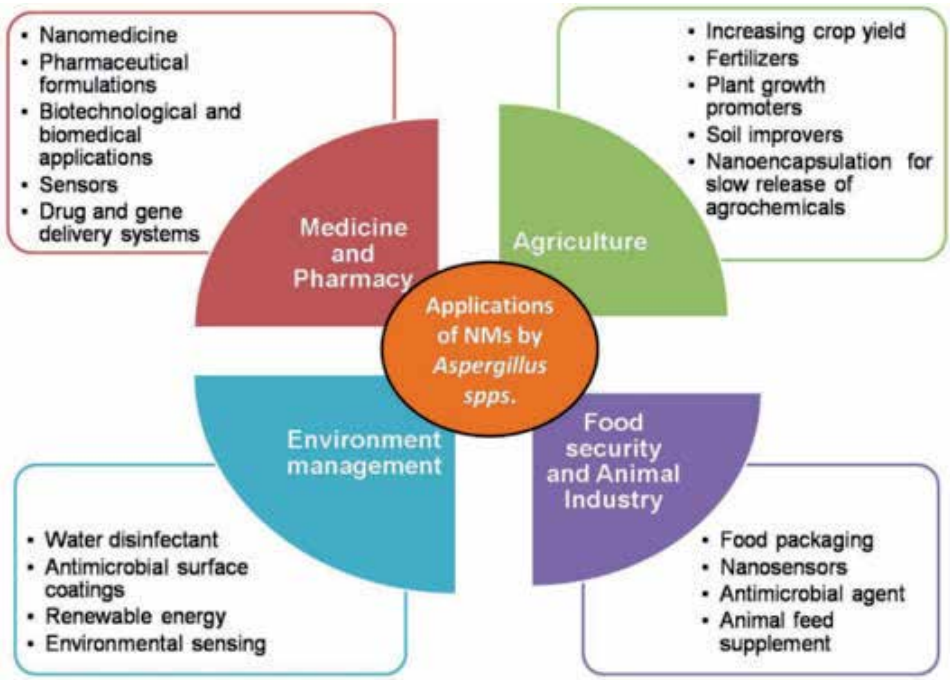

Figure 3.
Graphical representation of different applications of NMs synthesized using aspergillus *spp.*

NMs synthesized by *Aspergillus* species have tremendous applications broadly in the areas like agriculture, food security and animal industry, environmental management, and medicine and pharmacy are some of them. The detailed description is given in the sections below.

5.1 Applications in agriculture

In recent years, the nanotechnological advances in the field of agriculture have been increasing as the application of various NMs in the development of nano-based products like nanofertilizers for increasing crop yield and soil improvement, for plant growth promotion, nanopesticides, nanofungicides, nanoencapsulation for slow release of agrochemicals, and more in which NMs plays a vital role. The application of NPs as agrochemicals has become more common as technological advances make their production more economical for employment in the agriculture sector. For the potential application of NPs in plant disease control primarily included the information about the antimicrobial activity of different nano-size compounds against phytopathogens and the development of better application strategies to enhance the efficacy of disease suppression [100]. The antimicrobial activity of *Aspergillus* spp. synthesized NMs have been reported by many researchers. Silver NPs have been reported as most effective against phytopathogenic fungi, *Magnaporthe grisea* and *Bipolaris sorokiniana*, *in-vitro* [101], *Alternaria solani* and *Erwinia cartovora* pv. *cartovora* [102], and *Alternaria* blight as well as *Phytophthora* blight [103]. Elgorban et al., [24] demonstrated the silver NPs synthesis using fungus *Aspergillus versicolor* and evaluate its antifungal activity against *S. sclerotiorum* and *Botrytis cinerea* in strawberry plants. The silver NPs showed the concentration-dependent activity towards both the tested organisms but showed the greatest effect against *B. cinerea*. In another study, Ismail et al., [104] evaluated the combined effect of silver and selenium NPs against fungus *A. solani* that causing

early blight disease of potato. The fungus isolated from leaf spot was identified by microscopy and treated with NPs suspension, which showed the formation of pits and pores. Therefore, the authors concluded that the myco-synthesized AgNPs were able to penetrate and distribute throughout the fungal cell area and interact with the components, and cause cell death. Silver/chitosan nanoformulations (NFs) were applied against various seed-borne plant pathogens, particularly seed-borne disease-causing fungi, isolated from chickpea seeds [105]. These studies reveal the possibilities of NPs application as an antifungal agent, alternative to the fungicide for controlling plant pathogens.

Nanoformulations of copper-chitosan (Cu/Ch) has been prepared as an antifungal agent against *A. solani* that causing early blight disease of tomato (*Solanum lypersicum* Mill). These NPs caused mycelia growth inhibition and spore germination in *A. solani* and *F. oxysporum*, respectively, *in-vitro* model [106]. Recently, Shende et al., [94] synthesized the CuNPs using *Aspergillus flavus* and tested its activity against selected fungal plant pathogens namely *Aspergillus niger*, *Fusarium oxysporum,* and *Alternaria alternata*, which reveals significant antifungal activity. The study suggested the application of CuNPs as an effective fungicide for sustainable agriculture. ZnO NPs have also been investigated as an effective fungicidal agent against plant pathogens. ZnO NPs have many advantages over silver NPs for fungal pathogen control efforts [107]. He et al., [108] evaluated the antifungal effect of ZnO NPs and their mode of action against two post-harvest pathogenic fungi *viz*. *B. cinerea* and *Penicillium expansum*. Different concentrations of NPs, when applied to fungal hyphae demonstrated cell wall damage and collapse fungal hyphae. Raliya and Tarafdar [55, 56] reported the synthesis of ZnO NPs by *Aspergillus fumigatus* TFR-8, with the size range between 1.2 ~ 6.8 nm and Oblate spherical and hexagonal shape and evaluated its effect on phosphorous-mobilizing enzyme secretion and gum contents in cluster bean (*Cyamopsis tetragonoloba* L.). The antibacterial potential of photocatalytic nanoscale titanium dioxide (TiO_2), nanoscale TiO_2 doped with zinc (TiO_2/Zn; Agri-Titan), and nanoscale TiO_2 doped (incorporation of other materials into the structure of TiO_2) with a silver (TiO_2/Ag) has been evaluated against *Xanthomonas perforans*, bacteria causing bacterial spot disease in tomato [109]. Shenashen et al., [110] synthesized and characterized the mesoporous alumina sphere (MAS) NPs and evaluated their biological activity against *F. oxysporum*, that causing root rot disease in tomatoes, in comparison with the recommended fungicide tolclofomethyl, under laboratory and green house conditions. The authors reported cell death because of the entry of NPs in fungal cells due to disruption of the cell membrane and malformation of hyphae.

5.2 Applications in food security and animal industry

The application of NMs in the food security and animal industry is attending the great interest of the scientific community in recent years. Food security is usually the preparation, treatment, and storage of food products in which the food-borne pathogens or illness will not going to cause any damage or spoilage to the product [96, 97, 111]. Food insecurity, like illegal additives, pathogens, pesticide residues, allergens, and other unsafe factors, those are not only seriously affects human health, but also limit the rapid development of food industries to a certain extent [112–114]. The identification and quantitative analysis of bacteria is a very important and crucial issue in food safety. Conventional practices require long culture time, highly skilled operators, or specific recognition elements of each type of bacteria [113]. For this purpose, the analytical methods or equipments that meet the requirement of modern detection of various hazardous substances present in the foods for example packaging materials, sensors, and food containers coated with

NPs are develop using NMs. The novel nano-based food packaging materials have the unique characteristics involving oxygen scavengers, antimicrobial potential, and barriers to gas or moisture, and many other. In view of these multiple benefits of nanopackaging, its application in the pathogens detection, antimicrobials, allergens and contaminants, UV-protecting activity, high gas barrier plastics, etc. are some important areas of research [115]. The use of such NMs in food packaging enhances the shelf life of food devoid of undesirable alteration in its quality.

The application of smart packaging systems has increased tremendously in animal industries the muscle-based food products such as meat, chicken, etc. that are prone to contamination. The packaging of meat and muscle products suppress the spoilage, enhance the tenderness by allowing enzymatic activity, avoid contamination, retain the cherry red color in red meats and reduce the loss in its weight [116]. Plastic food packaging is one of the most important areas of research that employ nanotechnology to make stronger and lighter packaging materials and also enhances its performance. Besides this, NMs with strong antimicrobial properties such as Ag and TiO_2 NPs could be used in the packaging of foods to prevent spoilage [117]. Additionally, the application of NPs of clay in food packaging helps to control the entry of carbon dioxide, oxygen, and moisture towards food materials, thus preventing food spoilage.

Nowadays, more researchers have been paying attention to the development of nanosensors, which are being added in plastic packaging to spot the gases released from spoiled food. In the food spoilage or contamination condition, the packaging material will alert the consumer by detecting toxins, microbial contamination, and pesticides in food products, based on flavor production and color changing [118]. Moreover, plastic films entrenched with silicate NPs are being developed to maintain food fresh for a longer period. In this case, NPs play a vital role in dropping the oxygen flow and also facilitate to impede the moisture seeping out from the package. In animal industries, *Aspergillus* spp. synthesized NMs such as ZnO NPs are used as antimicrobial agents. For instance, *Aspergillus fumigatus* JCF and *Aspergillus niger* synthesized ZnO NPs with 60 ~ 80 nm and 61 ± 0.65 nm size, respectively and Spherical shape demonstrated the antimicrobial potential [54, 93]. The antifungal activity was observed in the ZnO NPs, which were synthesized by *Aspergillus terreus* [90]. Recently, Mausa et al., [119] reported the mycosynthesis of various NMs *viz.* Co_3O_4, CuO, Fe_3O_4, NiO, and ZnO NPs by endophytic fungus *Aspergillus terreus*, and studied its antimicrobial and antioxidant activities, which leads to their application in different fields.

5.3 Applications in healthcare, medicine, and pharmacy

In medicine and pharmacy, NMs have been successfully applied due to their high surface area that is able to adsorbed or conjugate with an extensive variety of therapeutic and diagnostic agents such as drugs, vaccines, genes, antibodies, and biosensors. In recent years, antibiotic resistance is an emerging major global health problem and novel antimicrobial formulations are essentially needed to fight against these drug-resistant microbes, therefore nano-based medicine as antimicrobial agents have gained considerable attention in the field of microbial drug resistance [119, 120]. Hence, the NPs synthesized by Mousa et al., [119] using the endophytic fungus *Aspergillus terreus* were studied to discover their efficacy against different multi-drug-resistant bacterial strains as well as some human and plant pathogenic fungi. The authors have reported the broad-spectrum antimicrobial action of all the mycosynthesized NPs, where the bacterial and fungal strains were inhibited. Furthermore, Co_3O_4 NPs among the five types of mycosynthesized NPs exhibited the strongest antimicrobial potential against the tested pathogens. There

are very few reports on the antimicrobial activity of Co_3O_4 NPs and only very less reported their antibacterial potential only [121]. Meanwhile, previous reports have observed the antimicrobial activity of CuO, Fe_3O_4, NiO, and ZnO NPs [122–125].

There are several reports on the synthesis and antimicrobial applications of *Aspergillus* spp. synthesized NMs. Netala et al., [22] demonstrated the antibacterial activity of Ag NPs synthesized by *Aspergillus versicolor* against *Staphylococcus aureus*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* at concentration 1 mg/mL. In another study, *Aspergillus terreus*-mediated synthesis of Ag NPs, showed antibacterial activity against *Salmonella typhi*, *S. aureus*, and *Escherichia coli* [23]. The synergistic effect with Ag NPs synthesized by *Aspergillus flavus* and conventional antibiotics against multi-drug-resistant bacteria such as *Bacillus spp*., *Micrococus luteus*, *S. aureus*, *Enterococcus faecalis*, *E. coli*, *P. aeruginosa*, *Acinetobacter baumanii*, and *K. pneumoniae* at concentration 100 ppm [21]. Rodrigues et al., [18] reported the synthesis of Ag NPs using *Aspergillus tubingiensis* and demonstrated the antimicrobial activity against *Candida* sp. and *P. aeruginosa* at concentrations 0.11-1.75 μg/mL and 0.28 μg/mL respectively. Ag NPs synthesized by *Aspergillus oryzae* revealed the antifungal effect against *Trichophyton rubrum* at concentration > 7.5 μg/mL [20]. Another strain of *Aspergillus oryzae* (MTCC no. 1846) synthesized Ag NPs using 1 mM $AgNO_3$, produced 7-27 nm-sized spherical particles, which showed the antibacterial effects [25, 26]. Ottoni et al., [19] reported the synthesis of Ag NPs by *Aspergillus niger* IPT856 and its antibacterial activity against *E. coli*, *S. aureus*, and *P. aeruginosa*. In another study, *Aspergillus fumigatus* BTCB10 synthesized Ag NPs with a spherical shape, which demonstrated the antibacterial and cytotoxic effects [27, 28]. The ZnO NPs as a dietary supplement in the animals gives health benefits, which improves the quality of egg in poultry, help in wound healing, act as an antioxidant, improve growth performance, hormone production, bone formation, immune system, a cofactor for enzymatic process, and reproduction system [126]. The antibacterial and antifungal potential improves the health of the livestock. In another study, Farrag et al., [92] reported the synthesis of Ag NPs by *Aspergillus niger* isolated from soil by treatment with silver nitrate. AgNPs exhibited significant inhibition of *Allovahlkampfia spelaea* viability and growth of both trophozoites and cysts, with a reduction of amoebic cytotoxic activity in host cells that suggested the Ag NPs possibly will give a promising future for the treatment of *Allovahlkampfia spelaea* infections in humans.

5.4 Applications in environmental management

NMs offer a unique platform for the purification of water contaminated with pollutants namely organics, metal ions, biological contaminants, and arsenic from the water because of the high surface area of nanosorbents and their ability of chemical modification as well as easier regeneration [127–130]. Chatterjee et al., [91] reported the synthesis of superparamagnetic iron oxide NPs (IONPs) (Fe_3O_4) of 20-40 nm size by manglicolous (mangrove) fungus *Aspergillus niger* BSC-1 and employed for the removal of hexavalent chromium from aqueous solution. Therefore, suggested the utilization of mycosynthesized IONPs could be employed for the heavy metal remediation from contaminated wastewater. The enzymatic bioremediation of textile industry wastewater containing direct green or reactive red azo dye by utilization of enzymes immobilized onto magnetic NPs for the improvement of industrial and environmental applications have been reported by Darwesh et al., [131]. Different types of magnetic NPs have been used to remove heavy metal ions from industrial wastewater [132].

In another study, the Au NPs was synthesized by *A. niger* that was found to be very effective against the mosquito larvae. The AuNPs were tested using the larvae

of three mosquito species viz. *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti*. Among them, it has been observed that the effect of Au NPs was found to be significant against *C. quinquefasciatus* larvae than the *A. stephensi* and *A. aegypti* larvae. All larval instars of *C. quinquefasciatus* showed 100% mortality after 48 hours of exposure to the Au NPs synthesized by *A. niger* [50]. In conclusion, the authors suggest that the application of mycosynthesized Au NPs by *A. niger* could be the fast and environmentally friendly approach towards the control of mosquitoes than the currently available approaches. This may possibly lead to a novel potential strategy for vector control [50].

Other than this, nowadays NMs could be applied in antimicrobial surface coatings, environmental sensing, renewable energy, and many other environmental applications.

6. Toxicity aspects of nanomaterials

Assessment of toxicity of synthesized NPs is the critical step for ensuring their safe and sustainable applications. Hence, toxicity evaluation of all the newly synthesized nanoparticle must be considered before their industrial applications. As far as the comparison of biosynthesized NPs with NMs synthesized by other methods especially the chemical method is concerned, the biosynthesized NPs seems to be biocompatible [133]. For instance, the green synthesized NPs were found to enhance the plant seedling growth, yield and quality, suggesting the biocompatibility of biosynthesized NPs as compared to the chemical synthesis NPs [134]. In contrast, few studies have shown the toxicity of biosynthesized or green synthesized NPs. Sulaiman et al., [135] have synthesized silver NPs (AgNPs) by using *Aspergillus flavus* and investigated its cytotoxic effect on HL-60, a human promyeloid leukemia cells. The study reported the dose-dependent toxicity of AgNPs at concentration of 5 and 10 μg/ml. The study claimed that although AgNPs have a toxic effect on normal cells but they also have the potential to act as a potential anticancer agent. However, the toxicity of AgNPs was found to be higher than silver nitrate solution. The said toxic effect was claimed to be due to the physicochemical interaction of silver atoms of AgNPs with the functional groups of intracellular proteins, nitrogen bases, and phosphate groups of DNA. The AgNPs may induce the accumulation of reactive oxygen species (ROS) leading to cellular apoptosis. Such effect will be helpful for the anti-cancer, antiproliferative, and antiangiogenic effects *in vitro*. Othman et al., [136] have synthesized AgNPs by using *A. terreus* and studied its antitumor activity against Human Caucasian breast adenocarcinoma (MCF7). It was found to inhibit the growth in dose-dependent manner with IC_{50} value of 46.7 μg/ml. Similarly, ZnO NPs synthesized by using the culture filtrate of *A. terreus* have been reported to be cytotoxic to HeLa cells. It was shown to induce apoptosis by inhibiting the production of cellular superoxide dismutase, catalase, glutathione peroxidase levels and inducing the accumulation of ROS, and reduction in mitochondrial membrane potential. Moreover, further investigation found it to induce oxidative damage via down-regulating expression of p53, Bax, Caspase-3, Caspase-9, and up-regulating Cytochrome-C expression [137]. The nanoparticle when come in contact with the target cell membrane, gets accumulates at the cell surface and induces pore formation causing the leakage of cytoplasmic material outside the cell. The NPs entered in the cell can interact with intracellular protein and DNA and thus disturbing the cell regulation [138]. The discussed mechanism of nanoparticle toxicity, in general, is represented in **Figure 4**. In general, toxicity studies are performed on human and animal cell line and plants. But there is also a need to give attention to the toxicity studies on microbes that are directly

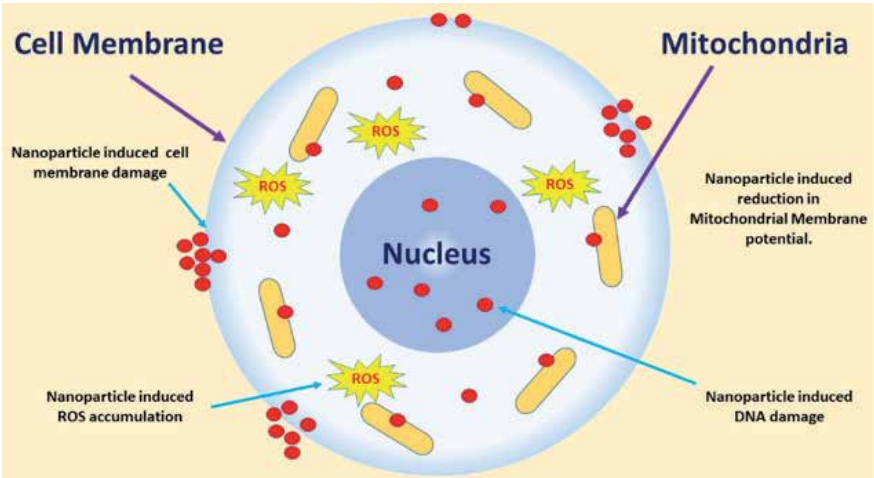

Figure 4.
Mechanism of cytotoxicity of mycosynthesized nanoparticles.

or indirectly beneficial to humans. Gupta et al., [139] have explored the toxicity of AgNPs synthesized by different fungi. The study found the mycosynthesized AgNPs to show toxicity to soil beneficial bacteria *Pseudomonas putida* KT2440 at the concentration of 0.4 μg/ml. Mycosynthesized Selenium nanoparticles (Se-NPs) were found to alter Wi-38, a normal lung fibroblast cells. At the IC50 value of 461 ppm, it exerted the loss of typical cell shape, granulation, loss of monolayer, and shrinking or rounding of cells [140].

Considering all of these observations from various studies it is suggested that before the actual application of any biosynthesized nanoparticle there is a need to undertake the toxicity studies and then make their use at biocompatible dose. For example, *A. terreus* strain AF-1 was exploited for the synthesis of CuO NPs which were integrated in cotton fabric. The said nanoparticle was used at a safe dose making it applicable for desired purpose [141]. Although mycosynthesized NPs are quite biocompatible as compared to NPs synthesized by other methods, it is recommended to verify the toxic effects of each type of NPs and choose their safe dose so as to make them safer for any kind of application.

7. Conclusion

Nanomaterials as the structures fabricated in the nanoscale have gained increasing attention for diagnostic and therapeutic purposes especially for those produced in a green safe approach by using fungi and other microorganisms. Among fungal species successfully used for this purpose, members of the genus Aspergillus are in the first line of investigation because of their huge diversity and capability to grow in abundance in laboratory conditions. Although there are many reports on the synthesis and biological activities of nanomaterials of different origins by fungi, little has been documented about important disciplines such as their mode of action and applications in medicine and industry. This chapter has highlighted the diversity of *Aspergillus* species and their advantages for nanomaterial production, mechanistic aspects of nanomaterial synthesis by selected Aspergilli, applications in healthcare, medicine, and pharmacy, role in environmental management as a unique platform for water decontamination, and finally, the cytotoxicity of introduced nanomaterials as a critical step for ensuring their safety and sustainability. Overall, these

results further substantiate the importance and priority of *Aspergillus* species for nanomaterial production at an industrial scale in a safe and cost-effective manner which enables researchers to use them for diagnostic, detoxifying, and therapeutic purposes in industry, medicine, and agriculture.

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This book highlights recent advances in the pathogenicity, mycotoxin-producing ability, and industrial application of members belonging to the genus *Aspergillus*. It is divided into two sections and six chapters that address different aspects and the importance of Aspergilli in relation to *Aspergillus*–human interactions, immunopathogenesis of invasive aspergillosis, the role of aflatoxin in *Aspergillus flavus* resilience to stress, mycovirus-containing *A. flavus* and carcinogenesis beyond mycotoxin production, and industrial application of *Aspergillus* species in conjunction to nanoparticle synthesis. This book brings readers several cutting-edge aspects of *Aspergillus* research with useful information for mycologists, microbiologists, toxicologists, plant pathologists, and pharmacologists, who may be interested in understanding the impact, significance, and recent advances within the genus *Aspergillus* that have not been critically noticed elsewhere.

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