UNIVERSITY OF CRETE – FACULTY OF MEDICINE

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DISSECTING THE ECHINOCANDIN PARADOXICAL ACTIVITY AGAINST *ASPERGILLUS FUMIGATUS*

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ABSTRACT

Echinocandins comprise a novel class of antifungal compounds with potent *in vivo* activity against *Candida* and *Aspergillus*. However, despite causing morphologic changes in germinating hyphae of *Aspergillus,* echinocandins fail to inhibit fungal growth *in vitro*. This paradoxical lag in *in vitro* activity of echinocandins against *Aspergillus* implies the presence of additional mechanisms of action of these antifungal agents *in vivo*. Echinocandin-mediated β-1,3 glucan exposure in *Aspergillus* hyphae triggers activation of potent immune responses and could represent an alternative mechanism of action of these compounds. However, the precise mechanisms of echinocandin induced augmention of *in vivo* activity have not been characterized.

In this study, we attempted to decode this echinocandin paradox. By performing *in vitro* experiments in conditions of high (5%) CO2, we attempted to simulate *in vivo* growth of *Aspergillus fumigatus* in tissue, and noticed a significant increase in echinocandin activity. Additionally, we found that *Aspergillus fumigatus* hyphae pre-treated with caspofungin showed a remarkably enhanced exposure of β-1,3 glucan. Surprisingly, caspofungin treated hyphae presented significantly more damage after osmotic shock compared to the untreated controls. Of interest, we did not find a significant enhancement in neutrophil activity against *Aspergillus* upon pretreatment with echinocandins. Collectively, these data could partially explain the echinocandin paradoxical activity against *Aspergillus*, and provide a simple method for a more accurate and reliable estimation of *in vitro* activity of these compounds against *Aspergillus fumigatus*.

ΠΕΡΙΛΗΨΗ

Οι εχινοκανδίνες αποτελούν μια νέα κατηγορία αντιμυκητιασικών φαρμάκων, με ενεργότητα εναντίον μυκήτων των γενών *Candida* και *Aspergillus*. Όμως, αν και προκαλούν δομικές μεταβολές σε αναπτυσσόμενες υφές των μυκήτων του γένους *Aspergillus*, δεν είναι ικανές να εμποδίσουν την αύξηση του μύκητα *in vitro*. Αυτή η παράδοξη αδυναμία των εχινοκανδινών εναντίων των μυκήτων του γένους *Aspergillus* υπονοεί την ύπαρξη επιπλέον μηχανισμών δράσης τους μέσα στους οργανισμούς. Η αποκάλυψη στο τοίχωμα των υφών των μυκήτων του γένους *Aspergillus* της β-1,3 γλουκάνης οδηγεί σε ενεργοποίηση του ανοσοποιητικού, και θα μπορούσε να αποτελεί έναν εναλλακτικό τρόπο δράσης αυτών των φαρμάκων. Παρόλα αυτά, οι ακριβείς ανοσοενισχυντικοί μηχανισμοί δράσης των εχινοκανδινών και η βελτιωμένη *in vivo* δραστηριότητά τους, δεν έχουν επαρκώς χαρακτηριστεί.

Πραγματοποιώντας *in vitro* πειράματα σε συνθήκες υψηλού διοξειδίου του άνθρακα (5%), προσπαθήσαμε να μιμηθούμε το περιβάλλον ανάπτυξης του *Aspergillus fumigatus* στους ιστούς και παρατηρήσαμε μια σημαντική βελτίωση της δράσης των εχινοκανδινών. Επιπλέον, βρήκαμε πως οι υφές του *Aspergillus fumigatus* που είχαν εκτεθεί σε κασποφουγκίνη εκφράζουν στην επιφάνεια του μύκητα σημαντικά περισσότερη β-1,3 γλουκάνη. Περιέργως, οι υφές που είχαν εκτεθεί σε κασποφουγκίνη παρουσίασαν περισσότερη βλάβη μετά από οσμωτικό σοκ. Παρόλα αυτά, δεν παρατηρήσαμε βελτίωση της δράσης των ουδετεροφίλων εναντίον υφών του *Aspergillus fumigatus* ύστερα από έκθεση σε εχινοκανδίνες. Συνολικά, αυτά τα δεδομένα θα μπορούσαν να ερμηνεύσουν, έστω εν μέρει, το παράδοξο των εχινοκανδινών, αλλά και να παράσχουν ένα καλύτερο μέσο για τα τεστ αντοχής στις εχινοκανδίνες έναντι του *Aspergillus fumigatus*, από τα ήδη υπάρχοντα.

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ABBREVIATIONS

Allergic Bronchopulmonary Aspergillosis (ABPA)
C-type Lectin Receptors (CLR)
Chronic Granulomatous Disease (CGD)
Cluster of Differentiation (CD)
Complement Receptor (CR3)
Food and Drug Administation (FDA)
Human Immunodeficiency Virus (HIV)
Invasive Aspergillosis (IA)
Minimum Effective Concentration (MEC)
Minimum Inhibitory Concentration (MIC)
Neutrophil Extracellular Traps (NETs)
Optical Density (OD)
Optical Density at 450nm (OD450)
Optical Density at 650nm (OD650)
Pathogen Associated Molecular Patterns (PAMPs)
Pattern Recognition Receptors (PRRs)
Polymorphonuclear cells / neutrophils (PMNs)
Reactive Oxygen Species (ROS)
Toll Like Receptors (TLRs)

A. INTRODUCTION

A1: *Aspergillus fumigatus*

A1.1: The *Aspergillus* genus

The fungal gerera *Aspergillus* were initially described and named by Micheli in 1729 after 'aspergillum', a device used in some liturgies of the Roman Catholic Church by the clergy to sprinkle holy water, because of its similarity with the microscopic structure of the fungus [1]. Fungi, including *Aspergillus*, are eukaryotic organisms developed early in evolution, with hundreds of members, few of which are pathogenic to humans. *Aspergilli* are saprophytic organisms that can grow in many different substrates of decaying vegetation, like leaf litter, or mouldy hay, but also in animal manure and even in human tissues [1]. Fungi of the genus *Aspergillus* are commonly used in the industry for the production of food and drinks (e.g. the Japanese alcoholic beverage 'sake' by *Aspergillus oryzae*), enzymes (e.g. glucose oxidase), chemicals (e.g. citric acid, gluconic acid) and drugs (e.g. statins) [1].

Spores of the genus *Aspergillus* have small size and weight and physical characteristics, like a hydrophobic exterior, that allow them to travel long distances in aerosols via air currents. When spores reach a solid or liquid surface, and if moisture and nutrient supply are sufficient, they germinate [1]. Their ability to be drifted by air currents and their relatively small requirements for nutrients allow global distribution of *Aspergillus* spores.

Aspergilli like other fungi and animals are heterotrophic, which means that they utilize organic carbon for their growth. However, unlike heterotrophic animals, instead of up-taking food and then digesting it, *Aspergilli* secrete enzymes and acids into their environment to break down polymeric molecules into smaller molecules and utilize them as carbon and nitrogen sources. The ability of germinating *Aspergilli* to grow towards and into their food substrate while they also degrade it makes them important from an ecological point of

view, since they contribute to the recycling of carbon dioxide and other inorganic compounds [1, 2].

Growth and reproduction of *Aspergilli* is quite different from the stereotypical binary fission that is used by most unicellular microorganisms. When seen under the microscope, colonies of *Aspergilli* are made of long, branched and interconnected structures, termed hyphae, justifying their characterization as filamentous fungi. In the appropriate environment, fungal spores, the monocellular form of the fungus named conidium, can germinate,

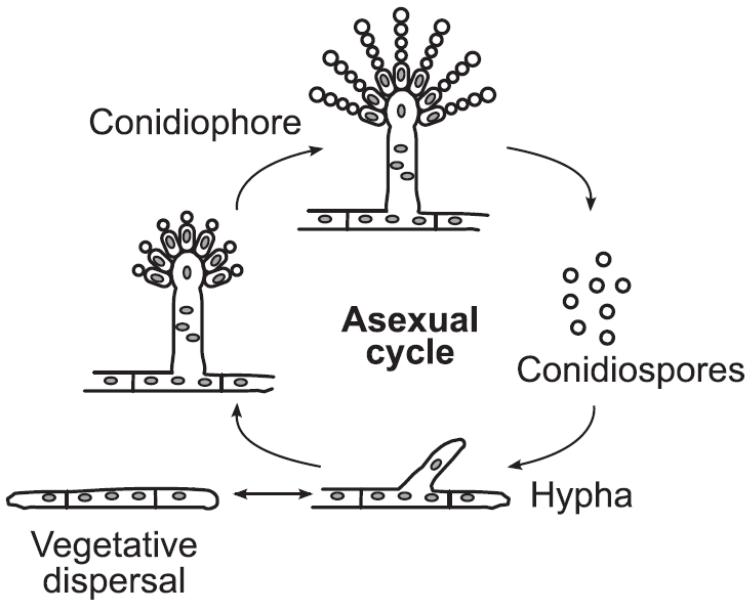

Figure A.1: Asexual reproduction of *Aspergillus* species. Conidia released from a conidiophore can germinate into hyphae that can disperse vegetatively and also produce conidiophores as vertical projections of the hyphae. Adapted from [3].

by expanding their cell wall toward a direction leading to the production of germinating mycelia and the development of branched hyphae. Vertical development of hyphal branches gives rise to the conidiophores, leading to the production and release of the fungal spores, termed conidia. This asexual form of reproduction leads to the formation of conidia that can be dispersed in the environment. However, some species of the genus *Aspergillus*, like *Aspergillus fumigatus* are also able to reproduce sexually by producing cleistothecia, which are closed and most often round structures that contain the meiotic ascospores, the sexual spores of the fungus [1, 4].

A1.2: *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophytic eukaryotic microorganism that belongs to the fungal genus *Aspergillus*. Similar to other *Aspergillus spp*, it is ubiquitously present in soil, in decaying organic material, able to grow with minimum nutrient requirements if the moisture content is higher than 15%. *Aspergillus fumigatus* is a thermotolerant fungus, able to grow at a wide range of temperatures (from 12oC to 52oC), and survive at temperatures as high as 70oC [5]. Its ability to grow rapidly at mammalian physiologic temperatures, 37oC, in contrast to most other environmental fungi, contributes to the ability of *Aspergillus fumigatus* to infect and cause disease in mammals.

Over the past few decades *Aspergillus fumigatus* has emerged as the most important airborne fungal pathogen in humans. It is the most frequent species of the *Aspergillus* genus that causes life threatening lung infections, in immunocompromised individuals, known as invasive aspergillosis (IA) [6]. Invasive aspergillosis is a life threatening infection of the lungs, with a propensity for angioinvasion and dissemination to other organs in severely immunocompromised individuals. Over the past two decades there is a rise in incidence of invasive aspergillosis, due to the increasing number of immunocompromised individuals who are at high risk for developing this potentially lethal infection. Patients with hematologic malignancies and recipients of hematopoietic stem cell and solid organ transplantation are at highest risk for invasive aspergillosis, with an incidence rate of 1% to 26%, and mortality rate approaching 90% in case of disseminate infection [6, 7, 8]. However, *Aspergillus fumigatus* can also cause a spectrum of non-invasive diseases in humans, including aspergilloma, which is a ‘fungal ball’ formed in pre-existing lung cavities, caused by overgrowth of *Aspergillus fumigatus* mycelia [6]. Furthermore, *Aspergillus fumigatus* can also cause hypersensitivity disorders, such as allergic bronchopulmonary aspergillosis (ABPA), an inflammatory lung disease in patients with severe asthma, manifesting with pulmonary infiltrates and bronchiectasis [9, 10].

A1.3: Pathogenicity of *Aspergillus fumigatus*

Conidia of *Aspergillus fumigatus* enter the human body via inhalation, leading to their deposition on the mucosa of the nasal cavity, the larger airways, or, due to their small size, at the distant lung alveoli. Alveolar epithelial cells can phagocytose an amount of inhaled conidia of *Aspergillus fumigatus* [11, 12, 13]. Following their uptake by epithelial cells, *Aspergillus fumigatus* can escape killing and germinate to hyphae.

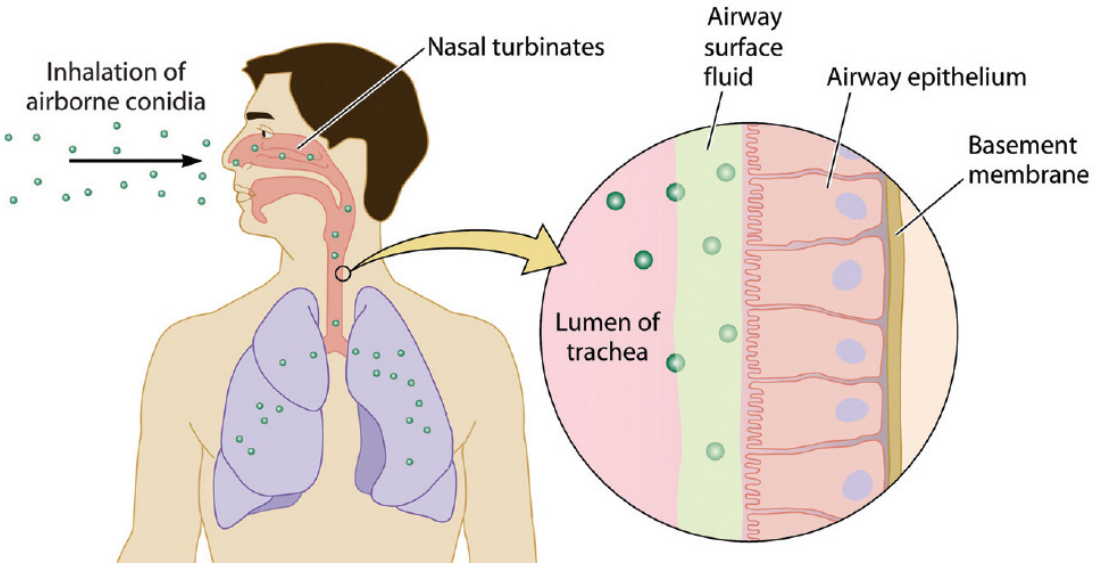

Figure A.2: Inhalation of *Aspergillus fumigatus* conidia. Airborne conidia are able to reach the alveoli of the lungs due to their small size. Adapted from [14].

Germination of intracellular and extracellular fungal spores into hyphae facilitates tissue invasion [15]. Breach of the first line of host innate immunity, that includes the alveolar epithelial barrier, by *Aspergillus* hyphae, allows the fungus to invade tissue, facilitates endothelial cell damage and results in angioinvasion and systemic dissemination via bloodstream [15].

A2: Innate immunity

The immune system consists of cellular and humoral host defence mechanisms that are responsible for the elimination of disease-causing

organisms, which are named pathogens. When a pathogen enters the host, it

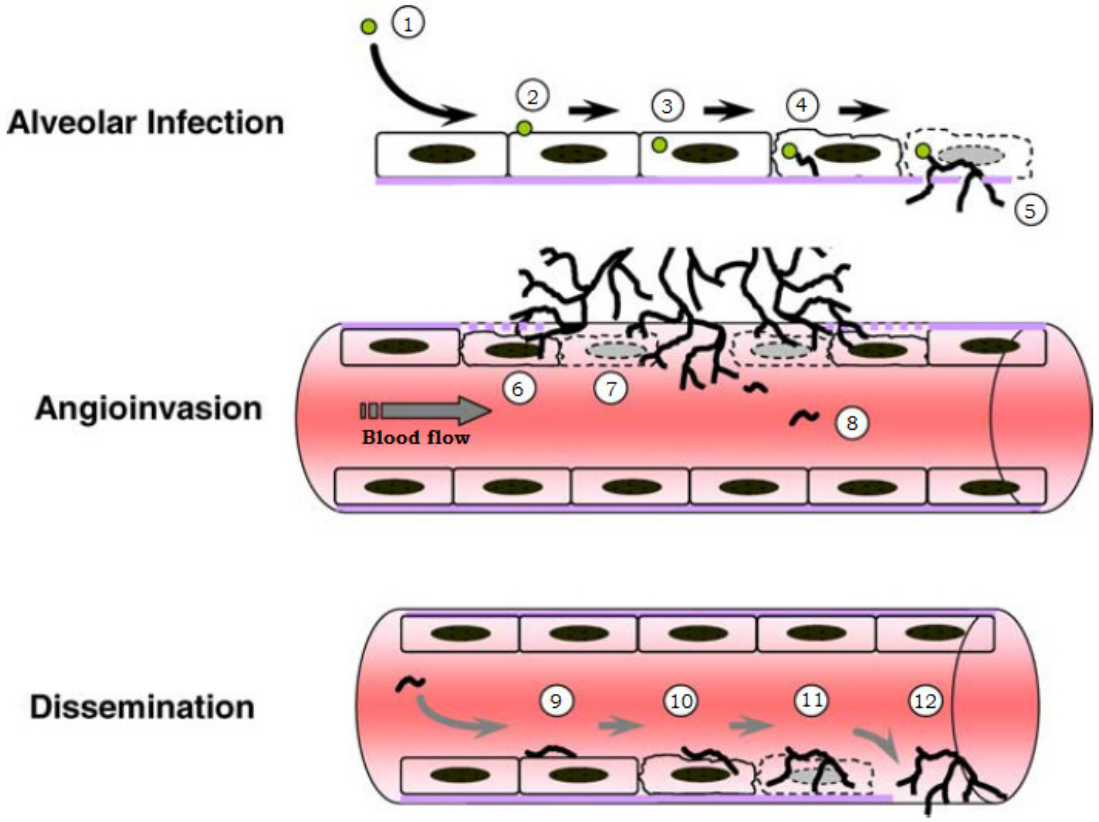

Figure A.3: Schematic representation of alveolar infection, angioinvasion and fungal dissemination in invasive aspergillosis. Invasive aspergillosis starts with the deposition of the conidia on the alveoli (1), and proceeds with the interaction with the epithelial cells of the alveoli (2), the endocytosis of conidia by the epithelial cells (3), the germination of conidia inside the endosomes (4) that leads to the escape from the epithelial cells (5), the penetration of the endothelial cells that cover the lung blood vessels (6), that eventually leads to endothelial cell damage and the access of hyphae to the bloodstream (7), the dispersal of hyphal fragments in the vasculature (8), that can reach distal vessels (9), penetrate the luminal surface of the endothelial cells (10), damage the endothelial cells and gain access to their abluminal surface (11) and then penetrate the distal organs (12). Adapted from [15].

triggers a systemic response in two waves. Initially, a rapid, multifaceted, but less specialized arm of host defence, named ‘innate immunity’ is activated upon sensing of host damage in response to an infectious or non-infectious threat; this acute phase response is followed by a second line of specialized immune response, termed ‘adaptive immunity’. Adaptive immunity utilizes

clonal expansion of immune cells and production of antibodies that recognize particular epitopes on the invading pathogen and also result in long term immunological memory.

A2.1: Overview of the innate immunity

Epithelial barriers represent a physical barrier for tissue invasion by bacteria, viruses and parasites. An additional, underappreciated epithelial defence strategy includes the inducible release of antimicrobial molecules upon microbial sensing. If a pathogen breaches these barriers, a second line of innate immune response is activated, starting with tissue resident macrophages, which are cells that recognise the pathogens via cell surface receptors and then, by phagocytosis, engulf them in phagosomes and kill them after the fusion of the phagosomes containing the pathogen, with lysosomes [16].

Macrophage activation, leads to the production of a battery of inflammatory molecules, including chemokines and cytokines, and results in the initiation of a complex inflammatory response, leading to recruitment and activation of other immune cell subsets, changes in endothelia and activation of complement and other components of humoral immune response. Important cells of the innate immune system which are recruited upon inflammation are neutrophils and monocytes, which differentiate to inflammatory macrophages and dendritic cells upon arrival at the site of infection [16].

Neutrophils, the dominant fraction of blood leucocytes (~60%), are professional phagocytic cells that arrive first in the inflamed tissue. They are able to kill microorganisms by multiple effectors mechanisms, including the engulfment and intracellular degradation of pathogens, but also via the production and release of reactive oxygen species (ROS) and antimicrobial molecules. Another recently discovered effector function of neutrophils upon their activation includes the process of release of DNA decorated with

antimicrobial proteins, called NETosis, which results in the entrapment and killing of microbes [17].

Both macrophages and dendritic cells originate from monocytes that circulate in the blood, so these two populations share many common functions. However, there are several differences between macrophages and the dendritic cells. Macrophages are terminally differentiated cells that mature continuously from circulating monocytes and after their maturation, they reside in the tissues throughout the body, even without inflammation, and in especially high numbers in the spleen, the liver, the lung, the gastrointestinal tract and the connective tissue.

Dendritic cells are professional antigen presenting cells that link the innate with the adaptive immune system, by up-taking pathogens, processing them and efficiently presenting molecules of the pathogen able to initiate an adaptive immune response, termed antigens, bound on proteins of the major histocompatibility complex (MHC), to T cells upon their migration to lymphoid regions of the draining lymph nodes. Although other cells are also capable of presenting antigens to T cells, dendritic cells are highly specialized and unique among all other cell types in their ability to present antigens and expand naïve T-cells, which are T lymphocytes that have not encountered an antigen before) [16].

A2.2: Innate immunity against *Aspergillus fumigatus*

The interaction between the human host and *Aspergillus fumigatus* leads to a variety of phenotypes, ranging from asymptomatic interaction, to hypersensitivity reactions leading to allergic bronchopulmonary aspergillosis in immunocompetent hosts or even to fatal invasive disease in severely immunocompromised individuals.

In the alveoli, *Aspergillus fumigatus* conidia are entrapped by the respiratory epithelium and are also exposed to the activity of soluble pattern

recognition receptors and antimicrobial peptides on the epithelium of the alveoli [14].

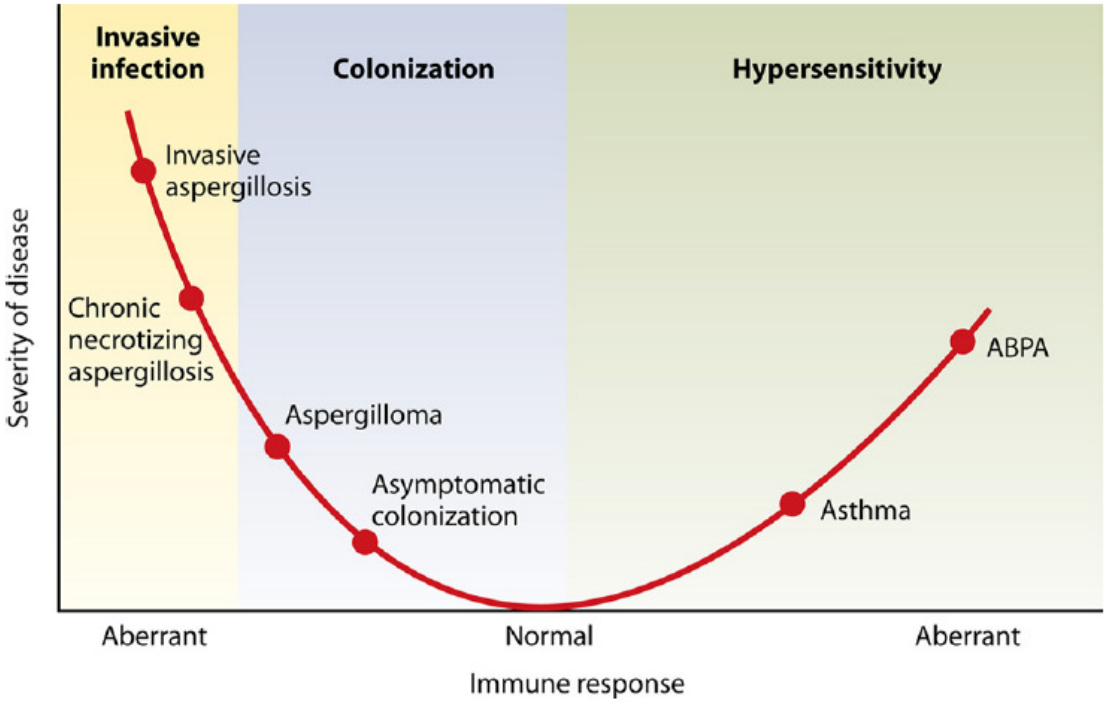

Figure A.4: Diagrammatic representation of the range of phenotypes resulting from the interaction of *Aspergillus fumigatus* with the immune system. Adapted from [14].

The innate immune response begins with the activation of the resident alveolar macrophages that phagocytose the conidia as well as with the dendritc cells that can be found in the infected tissues and also manage to phagocytose the conidia. Furthermore, since the resident cells are activated, they release cytokines and chemokines that lead to the recruitment of other leucocytes from adjacent vessels. Meanwhile, a few hours after the conidia reach the alveoli, they swell, which is the first stage of the germination process. Generally, resident macrophages are capable of eliminating a high load of conidia that are typically inhaled on a daily basis, but even if this is not enough, the recruited neutrophils and monocytes are capable of killing excessive numbers of inhaled conidia [14, 18, 19]. Dendritic cells are able to phagocytose conidia and hyphae and then process them in order to expose fungal antigens and present them to the draining lymph nodes of the lung in

order to trigger adaptive immune responses [14, 18]. Attenuated immune responses, in the case of severe immunosupression, allows germination of *Aspergillus fumigatus* conidia, tissue penetration by germinating hyphae and

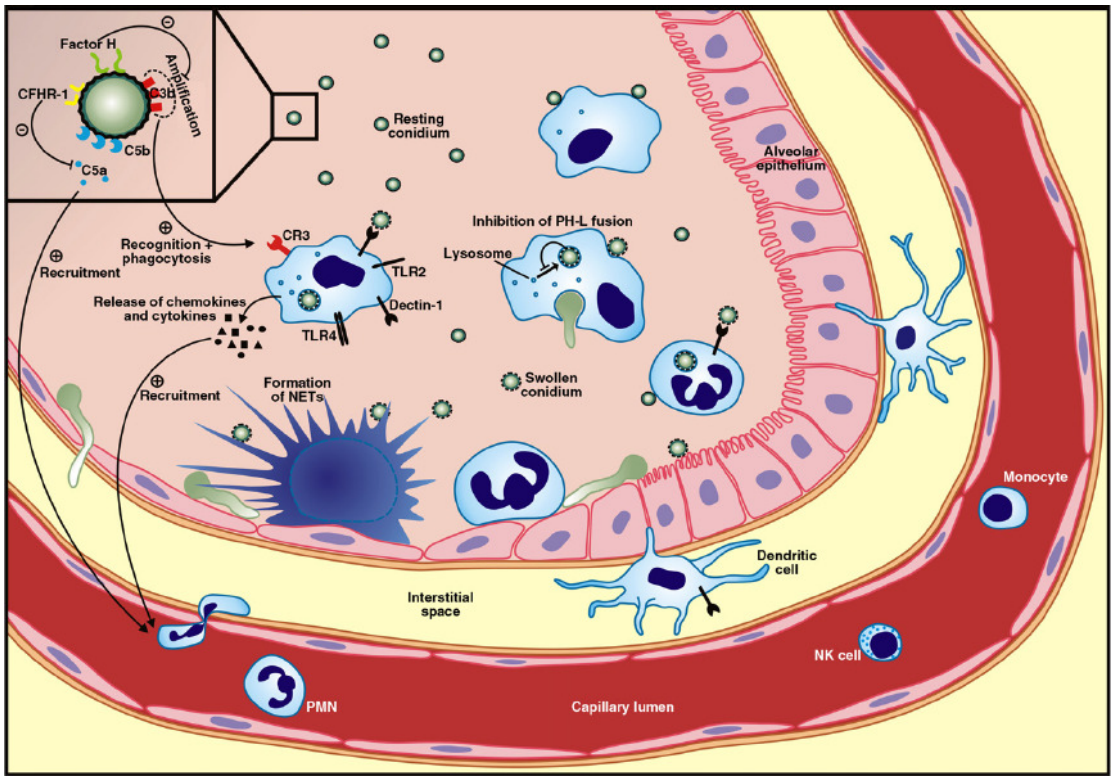

Figure A.5: The innate immune response against *Aspergillus fumigatus* in the lungs. Resting conidia are inhaled and deposited in the lungs where resident macrophages recognize the conidia with cell surface receptors and phagocytose them, while neutrophils and monocytes are recruited from the blood and also target conidia by phagocytosis, soluble antimicrobial peptides and neutrophil extracellular traps (NETs) by neutrophils, which entrap conidia and hyphae. If the innate immune response fails to eliminate resting and swollen conidia, and germination proceeds to hyphae, invasive disease of the lungs could occur. Adapted from [18].

development of invasive fungal disease. Specifically, patients with prolonged neutropenia or qualitative defects in phagocytes, as a result of prolonged administration of corticosteroids or because of genetic defects in NADPH dependent ROS production (Chronic granulomatous disease – CGD), are uniquely predisposed to develop invasive disease from *Aspergillus fumigatus* [20, 21, 22, 23].

Conidia and hyphae of *Aspergillus fumigatus* are recognised by soluble and cell surface pattern recognition receptors (PRRs) that bind to microbial molecules called pathogen associated molecular patterns (PAMPs). However, the resting conidia of *Aspergillus fumigatus* are generally protected from PAMPs, since they are covered with a hydrophobic protein layer that protects them from recognition by the immune system [24, 25]. Apart from that, the cell wall of *Aspergillus fumigatus* is composed mainly of polysaccharides, and specifically by β-1,3 glucan and β-1,6 glucan, chitin (a polymer of N-acetylglucosamine), galactomannan and mannans (chains of hundreds of mannose molecules added to fungal proteins via O- or N- linkages) [26], which have immunogenic properties, since they can bind to PRRs and trigger immune responses [27].

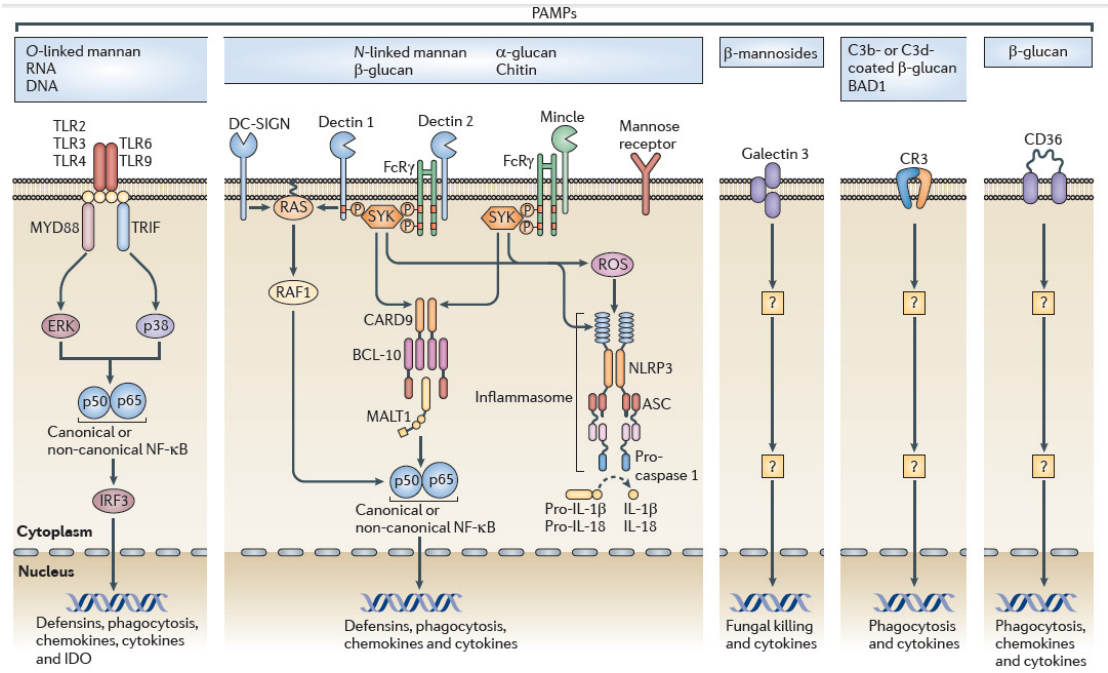

Figure A.6: Innate immune recognition of fungal PAMPs and downstream signalling pathways. The major PRRs implicated in the innate immune recognition of fungi are Toll Like Receptors (TLRs), C-type Lectin Receptors (CLRs: DC-SIGN, Dectin-1, Dectin-2, Mincle and Mannose Receptor), galectin family proteins (like galectin 3) and Complement Receptor 3 (CR3). These PRRs recognise fungal PAMPs leading to activation of downstream signalling cascades towards activation of the inflammasome, the canonical or the non-canonical NF-kB pathway, leading to leucocyte effector functions, like phagocytosis, the production of cytokines, chemokines and reactive oxygen species (ROS). Adapted from [27].

Of particular interest are β-glucans, which are a heterogeneous group of polymers of glucose found in fungi, plants and also in some bacteria. They consist of linear β-1,3 linked backbones with β-1,6 linked side chains whose length and distribution varies, and they can form tertiary structures which can be stabilized by interchain hydrogen bonds. They are of great interest in mammalian immunology, since they have a profound immunogenic role. Indeed, administration of purified β-glucans has many beneficial effects like protection against tumor development and fungal, viral, bacterial and protozoan infections [28, 29]. The immunogenic properties of β-glucans stem from their ability to activate phagocytic, cytotoxic and anti-microbial activities of phagocytes and production of pro-inflammatory mediators, chemokines and cytokines like IL-1β, IL-6 and TNF-α [30]. The recognition of fungal β-glucans in humans is mainly mediated by cell surface receptors, like Dectin-1, Complement Receptor 3 (CR3), and possibly scavenger receptors including CD36 [27].

Importantly, the morphological state of *Aspergillus fumigatus* seems to be critical for its recognition from the immune system, since resting conidia seem to be unable to trigger strong innate immune responses and initiate adaptive immune responses, even though they are phagocytosed [24, 31, 32, 33, 34]. This seems to be related to the fact that immunogenic molecules in the fungal cell wall, like β-glucans, are masked at resting conidia, so innate immune receptors cannot detect them and trigger adequate activation of the innate immune cells. Several lines of evidence support this idea, including the fact that removal of the hydrophobic layer of *Aspergillus fumigatus* by genetic deletion of the proteins that form it or by chemical means leads to immune activation [24], or that exposure of immunogenic molecules like β-glucan during germination of *Aspergillus fumigatus* correlates with the activation of immune cells [32, 34]. Furthermore, treatment with antifungal drugs that target components of the fungal cell wall and alter its composition, like caspofungin (described extensively below), lead to exposure of immunogenic molecules, like β-glucan, that increase the inflammatory immune responses [34, 35].

A3: Echinocandins

A3.1: Echinocandins in general

Even though microorganisms pose a threat against humans, several drugs used currently in medicine are by-products of otherwise potentially harmful microbes. This is also the case with echinocandins, which were discovered during a systematic screening of microbial fermentation compounds with novel antifungal activity. Even though they have a short antifungal spectrum and weak solubility, they have proved to be promising antifungal agents due to their activities against *Candida* and *Aspergillus* species and their remarkable activity against azole-resistant strains [36].

From the 1970s, when the first drug of the echinocandin family was isolated, until now, more than 20 natural echinocandins have been discovered. They all have a cyclic lipo-hexapeptide structure, even though several minor differences in their structure due to differences in their chemical composition exist [36, 37, 38, 39]. As suggested by several lines of evidence, echinocandins act by obstructing the biosynthesis of the fungal cell wall by non-competitive inhibition of the catalytic subunit of the β-1,3 glucan synthase complex, which is responsible for the synthesis of the cell wall β-1,3 glucans [40, 41, 42]. Echinocandins have been shown to increase the susceptibility of baker's yeast to osmotic stress and even cause lysis in biofilms due to the inhibition of β-1,3 glucan synthase [43, 44, 45]. The cell lysis is mainly taking place to the growing tip of the daughter cell [44, 45].

Secondary effects are also taking place as a result of the echinocandin activity, with reduction in the content of membrane sterol as well as increased amount of chitin in the fungal cell wall [46, 47]. Furthermore, inhibiting the synthesis of β-1,3 glucan causes morphological alterations. After treatment with echinocandins, cells form aggregates due to the failure of separation of the daughter and mother cell [48], while the formation of hyphae is inhibited as well [49, 50]. In the case of the *Aspergillus* species, the effects of

echinocandins are not fungicidal, but rather fungistatic, and they have as a result only partial decrease of fungal growth rate, as well as the vulnerability of hyphal tips to osmotic lysis, an increase of the chitin content of the fungal cell wall and the induction of severe morphological changes, like the formation of branched hyphal tips, balloon-like cells and swollen germ tubes [51, 52, 53, 54].

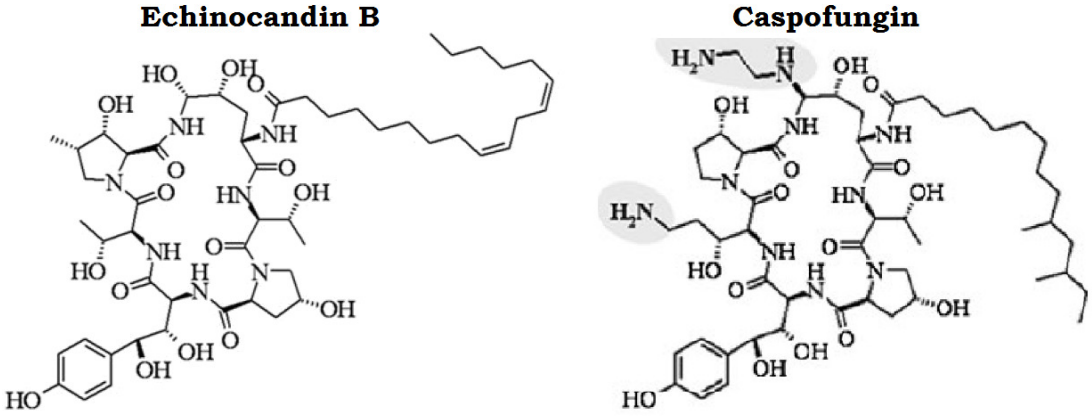

Figure A.7: Chemical structure of echinocandins. Echinocandin B is the first echinocandin discovered and caspofungin is a semi-synthetic product. Highlighted areas show the sites of modifications. Adapted from [36].

Even though there are more than 20 antifungal drugs in the family of echinocandins, only 3 semi-synthetic echinocandins have been approved by Food and Drug Administration (FDA) for clinical use, namely, caspofungin, micafungin and anidulafungin, while another one, aminocandin is under clinical evaluation [36]. Caspofungin, anidulafungin and micafungin have been approved for the treatment of oesophageal candidiasis and invasive candidiasis in adults and in children (caspofungin) older than three months. Micafungin has also been approved by FDA for antifungal prophylaxis in patients with hematopoietic cell transplantation, while caspofungin has been approved for empirical therapy of patients with febrile neutropenia and also for salvage and primary therapy of invasive aspergillosis. All three echinocandins have limited oral bioavailability, so they are administered via intravenous infusion [36]. These novel antifungal drugs show good antifungal activity

against the human pathogenic fungi *Candida* and *Aspergillus* spp *in vitro*, at clinically attainable concentrations [55, 56, 57, 58, 59].

A3.2: The echinocandin paradox with *Aspergillus fumigatus*

Echinocandins have a fungistatic effect against *Aspergillus* spp [36, 55, 60, 61, 62]. As echinocandins cannot fully inhibit the growth of these fungi, and more particularly, *Aspergillus fumigatus*, determination of the minimum inhibitory concentration (MIC; that is the concentration of an antifungal compound that is needed to inhibit the growth of the microbe) is not possible. To overcome this problem, an alternative method has been occupied. Since echinocandins induce morphological changes to the growing morphotypes of the fungus, a determination of the minimum effective concentration (MEC) was introduced to describe the effect and activity of echinocandins against *Aspergillus* species. MEC is defined as the minimum concentration of the antifungal that causes severe morphological changes, like the formation of branched hyphal tips, balloon-like cells and swollen germ tubes [54, 63]. The measured MEC values for the human pathogenic *Aspergillus* species are between 0,015-0,25 μg/ml [64].

However, the clinical and biologic significance of MEC is controversial, in view of the potent activity of echinocandins against *Aspergillus fumigatus in vivo* [59, 65, 66, 67, 68, 69, 70, 71]. So, it is unclear why echinocandins display a mediocre *in vitro* activity against *Aspergillus fumigatus*, and whether there are any other mechanisms of action that account for their *in vivo* potent antifungal efficacy [67, 72, 73]. To this end, there is evidence of immunomodulatory action of echinocandins at concentrations well below the MIC (or the MEC for *Aspergillus fumigatus*), mediated by increased unmasking of the immunogenic fungal cell wall polysaccharide β-1,3 glucan [35, 74, 75, 76]. Indeed, as previously described, increased exposure of β-1,3 glucan due to treatment with caspofungin leads to immune recognition of *Aspergillus fumigatus* and activation of macrophages and neutrophils towards

inflammatory response and increased neutrophil activity against hyphae of *Aspergillus fumigatus* [35, 74].

Thus, it would be interesting to test whether this paradoxical difference between the *in vitro* and *in vivo* activity of echinocandins could be related to different environmental conditions of fungal growth in tissue (e.g., increased CO2 concentration) than in culture. Also, it would be important to explore whether the immunomodulatory properties of echinocandins could partially explain their improved *in vivo* activity, by focusing on whether there is indeed increased β-1,3 glucan exposure in the *in vitro* environment and the environment that simulates the *in vivo* conditions of *Aspergillus fumigatus* growth. Furthermore, it would be of particular interest to see if exposure of germinating conidia to echinocandins leads to increased damage of *Aspergillus fumigatus* hyphae.

A4: Objectives of this study

Aim 1: Explore the *in vitro* activity of echinocandins against *Aspergillus fumigatus* under conditions simulating physiologic, *in vivo* growth.

Aim 2: Explore whether the immunomodulatory properties of echinocandins could partially explain their improved *in vivo* activity, with a focus on immune augmentation via exposure of immunostimulatory cell wall β-glucans.

Aim 3. Explore the effect of echinocandins in neutrophil activity against *Aspergillus fumigatus* hyphae.

B. MATERIALS AND METHODS

B1: Chemicals and reagents

RPMI-1640+L-glutamine, Fetal Calf Serum (FCS), Hepes solution 1M, Sodium pyruvate solution 100mM, 7,5% Bovine serum albumin fraction V (BSA) solution, Phosphate buffered saline (PBS) tablets and 0,4% Tryptan Blue solution were purchased by Gibco. Mowiol powder, Propidium Iodide, (2,3)-bis (2-Methoxy 4-Nitro 5-Sulphenyl) (2H) Tetrazolium Carboxanilide (XTT) powder, menadione powder and Histopaque 1119 were purchased by Sigma-Aldrich. PFA 37% stock solution was purchased by Merck. Penicillin & streptomycin were purchased in solution by Biosera. Ficoll-paque was purchased by GE Healthcare. Water for injection was administered by the Internal Medicine Department of University hospital of Crete. Caspofungin, amphotericin B and voriconazole were obtained from pharmacy formulary and were dissolved at a stock concentration of 5mg/ml. The FITC-conjugated monoclonal antibodies were: anti-CD14 (Biolegend), anti-CD15 (Invitrogen), anti-CD16 (BD pharmingen) and IgG2a isotype control (eBioscience). Murine anti-β-1,3 glucan monoclonal antibody was purchased by Biosupplies, Australia. Fluorescent Alexa^{555} conjugated secondary goat anti-murine antibody was purchased by Molecular Probes (Invitrogen).

Fetal Calf Serum (FCS) was heat inactivated for 30 minutes at 55oC. Phosphate buffered saline (PBS) was dissolved in distilled water and filter sterilized with a 0,22μm filter system (Corning).

B2: Growth media

Yeast glucose (YAG) agar plates supplemented with trace elements, vitamin mix, and MgSO4, were made by adding the ingredients in distilled water, sterilizing the solution with an autoclave and then spitting the solution in non culture treated sterile 100mm plates (Corning).

Yeast peptone dextrose (YPD) liquid medium was made by adding the ingredients in distilled water and autoclaving. For YPD agar plates, the same procedure was performed along with the addition of agar powder and then the solution was split in non culture treated sterile 100mm plates (Corning) after autoclaving.

For pre-exposure of *Aspergillus fumigatus* conidia and *Candida albicans* yeast to caspofungin, caspofungin was added to YAG agar plates or in the liquid YPD medium respectively, after autoclaving.

For antifungal susceptibility testing of *Candida albicans* strains GFP and CAF-2 and *Aspergillus fumigatus* strain 293, for germination of *Aspergillus fumigatus* strain 293 and for the co-culture of *Aspergillus fumigatus* strain 293 hyphae with neutrophils, RPMI-complete was used (RPMI-1640 supplemented with 10% heat-inactivated FCS, 10mM hepes, 1mM sodium pyruvate, 100 U/ml penicillin, 100μg/ml streptomycin).

B3: Fungal isolates

Aspergillus fumigatus strain 293 was grown on yeast extract dextrose (YAG) agar plates for 2–4 days at 35oC. Conidia were then collected at sterile conditions by gently scraping the surface of the plate with a sterile flame bent Pasteur pipette in sterile PBS. Then, the solution was collected and passed through a 40μm nylon cell strainer (BD Falcon), washed once with sterile PBS, centrifuged at 4.000rpm for 10 minutes at room temperature. Then the conidia were resuspended in sterile PBS, counted with a Neubauer hemocytometer and stored at 4oC until use.

Candida albicans GFP and CAF-2 strains were cultured overnight on yeast peptone dextrose (YPD) agar plates at 37oC, and then, single colonies were picked at sterile conditions and cultured in YPD liquid medium overnight at 37oC on a stirrer. Then, yeast cells were counted with a Neubauer hemocytometer and stored at 4oC until use.

B4: XTT and menadione

XTT powder was dissolved as previously described [77]. Briefly, XTT powder was dissolved in sterile PBS in a final concentration of 0,5mg/ml, heated at 60oC for 30 minutes and filter sterilized with a 0,2μm syringe filter. Menadione was dissolved in acetone in a final concentration of 50μM, and filter sterilized with a 0,2μm syringe filter. Both solutions were kept at 4oC until use.

B5: XTT assay standardization

Serial dilutions of conidial inocula (10^5, $5x10^4$, 10^4, $5x10^3$ and 10^3) were added in RPMI-complete medium in a 96-well plate for 18 hours at 37oC to allow germination to mature hyphae. Then, XTT and menadione were added at a final concentration of 0,25mg/ml and 25μM respectively, and the plate was put at 37oC for 1 hour. Then, 125μl from the supernatant were transferred in a fresh 96-well plate and the optical density at 450nm (OD450) was measured with a microplate reader (Bio-Rad model 680). The relationship between XTT conversion (OD450) and fungal inoculum was assessed by linear regression analysis, and the goodness of fit (r^2) was reported.

B6: Susceptibility testing

Minimum inhibitory concentrations (MICs) for caspofungin were determined for both GFP and CAF-2 *Candida* strains, while minimum effective concentrations (MECs) for caspofungin and MICs for amphotericin B and voriconazole were determined for *Aspergillus fumigatus* strain 293. The broth microdilution method of the CLSI (M38-A) [78] was followed using 96-well microtitration plates, with slight modifications. Briefly, the fungi were cultured fresh before each susceptibility test, and added at a concentration of 10^4 – $5x10^4$/ml in RPMI-complete for 48 hours at 35oC, pH 7,4 or in RPMI-complete at 37oC and 5% CO2, pH 7,4. Serial dilutions of the antifungal agents were prepared to

yield concentrations of 8ng/ml to 16μg/ml for caspofungin, 1ng/ml to 500ng/ml for voriconazole and 1ng/ml to 4μg/ml for amphotericin B at a final volume of 200 μl after inoculation. At 48 hours, the growth was studied both visually and microscopically, while the MECs of caspofungin for *Aspergillus fumigatus* strain 293 were determined microscopically by 2 different investigators.

Additionally, after the visual and microscopical determination of fungal growth and the MECs, XTT and menadione were added in the wells of the 96-well plates in a final concentration of 0,25mg/ml for XTT and 25μM for menadione and the plates were returned at 35oC or 37oC & 5% CO2 (in the same conditions they had been previously) for 1 – 2 hours, and then 125μl of the supernatant were transferred to a new flat bottom 96-well plate and the optical densities at 450nm (OD450) were measured with a microplate reader (Bio-Rad model 680). MICs were determined as the concentration of the antifungal that yielded at least 80% reduction of growth compared to the untreated control, and they were comparable with the growth reported visually and microscopically for the antifungal drugs tested.

B7: Immunostaining of β-1,3 glucan exposure for FACS analysis

Conidia of *Aspergillus fumigatus* strain 293 that had been pre-exposed, during culture, to 500ng/ml, 250ng/ml or 0ng/ml caspofungin, and yeast cells of *Candida albicans* GFP strain that had been pre-exposed, during culture, to 20ng/ml, 10ng/ml, 5ng/ml or 0ng/ml caspofungin, were stained for surface exposure of β-1,3 glucan. Briefly, $5x10^6$ – 10^7 spores were blocked with PBS/2% BSA in 1,5ml Eppendorf tubes for 15 minutes at 4oC on a rotator, incubated for 2 hours at 4oC with a murine anti-β-1,3 glucan primary antibody (diluted 1/200) on a rotator, washed once with PBS, incubated for 30 minutes at 4oC with a goat anti-murine secondary fluorescent $Alexa^{555}$ conjugated antibody (diluted 1/500) on a rotator, washed twice with PBS, and resuspended in 350μl of PBS. Single cells were acquired with a FACSCalibur (BD biosciences) and analyzed using the FlowJo software (Tree Star).

B8: Immunostaining of β-1,3 glucan for confocal microscopy

$5x10^4$ conidia of *Aspergillus fumigatus* strain 293 were put in a 96-well plate at 37oC for 18 hours to allow hyphal outgrowth with 125ng/ml, 63ng/ml or 0ng/ml caspofungin in RPMI-complete. Then, the samples were blocked for 15 minutes in PBS/2% BSA, incubated for 2 hours with a murine anti-β-1,3 glucan primary antibody (diluted 1/200), washed once with PBS, incubated for 30 minutes at 4oC with a goat anti-murine secondary fluorescent Alexa^{555} conjugated antibody (diluted 1/500), washed twice with PBS; all treatments were performed at 4oC rotating incubation followed by 20 min fixation with 4% PFA at room temperature. Then, hyphae were transferred on slides, mounted with mowiol, covered with coverslips and sealed.

Alternatively, $5x10^4$ conidia of *Aspergillus fumigatus* strain 293 were put on sterile coverlslips in a 24-well plate at 37oC and 5% CO2 for 18 hours to allow hyphal outgrowth with 250ng/ml, 125ng/ml, 63ng/ml, 31ng/ml or 0ng/ml caspofungin in RPMI-complete, and were then stained as before.

Images were acquired with a confocal microscope (Leica TCS SP2) and analysed with the Leica software LAS AF version 2.6.0.

To normalize the mean fluorescence intensity of the β-1,3 glucan staining to the hyphal biomass, an estimate of the fungal biomass was needed. So, resting conidia of *Aspergillus fumigatus* strain 293 were fixed with a PBS solution containing 4% PFA for 20 minutes, washed with PBS, stained with the DNA binding dye propidium iodide for 20 minutes, washed twice, and transferred on slides. Images were acquired with a confocal microscope (Leica TCS SP2) and analysed with the Leica software LAS AF version 2.6.0. Areas containing 100%, 98%, 96%, 94%... 6%, 4%, 2%, 0% of propidium iodide stained conidia, were chosen and the mean fluorescence intensity of the signal of the transparent light channel for these areas was compared to their mean fluorescence intensity of the signal of the propidium iodide channel. Regression analysis showed a linear correlation with r^2 = 0,9763 and

p<0,0001, allowing the use of the mean fluorescence intensity of the signal of the transparent light channel as an indicator of the fungal biomass.

B9: Isolation of neutrophils

Blood from healthy blood donors was obtained from the Blood Transfusion Unit of University Hospital of Crete in EDTA coated tubes. The blood was diluted 1/2 – 1/2,5 with sterile PBS. Then, 3ml of sterile cold 1119 Histopaque were put at the bottom of 15ml Falcon tubes, and 3ml of sterile cold Ficoll-Paque were carefully layered on top of them. After that, the diluted blood was carefully layered on top of the Ficoll-Paque layer, and the tubes were centrifuged at 650g for 30 minutes at 16-20oC without brakes. After the centrifugation, the zone between ficoll-paque and histopaque was collected, put in a fresh 50ml Falcon tube and washed with sterile PBS. After decanting the supernatant, hypotonic lysis was performed by resuspending the pellet in 2ml of sterile water for injection (WFI) and stirring gently the tube for 15 seconds. Osmolarity was restored by filling the tube with sterile PBS. Then, the hypotonic lysis step was repeated once, and the cells were resuspended in RPMI-complete, and counted with a Neubauer hemocytometer under a microscope. Dead cells were excluded by tryptan blue exclusion. The cells were typically >97% alive.

B10: FACS staining of neutrophils

$3x10^5$ of cells isolated with the neutrophil isolation protocol were used per condition. These cells were put in FACS tubes, washed once with 2ml PBS/5% FCS, centrifuged at 1.500rpm for 5 minutes at 4oC, resuspended in 100μl PBS/5% FCS with one different antibody per FACS tube (anti-CD14, anti-CD15, anti-CD16, and IgG2a isotype control) at a 1/100 dilution, incubated for 30 minutes at 4oC on a rotator, washed once with PBS/5% FCS, and resuspended in 350μl of PBS. Single cells were acquired with a FACSCalibur

(BD biosciences) and analyzed using the FlowJo software (Tree Star). The isolated cells were >90% neutrophils.

B11: Hyphal damage assay

$5x10^4$ conidia of *Aspergillus fumigatus* 293 per well of a flat bottom 96 well plate were incubated at 37oC and 5% CO2 in 200µl of RPMI-complete for 18 hours to induce mature hyphae with 250ng/ml, 125ng/ml, 63ng/ml, 31ng/ml or 0ng/ml caspofungin. After 18 hours, the medium was completely removed and fresh RPMI-complete was added, containing 10^6, $5x10^5$, $2,5x10^5$, $5x10^4$ or 0 human neutrophils, to achieve the 20/1, 10/1, 5/1, 1/1 and 0/1 PMN/hyphae ratio, which is the ratio of neutrophils to the starting conidial inoculum, and the plate was put at 37oC and 5% CO2. After 2 hours of incubation, the medium was completely removed and 200µl of sterile water for injection (WFI) were added to each well, to perform hypotonic lysis of neutrophils, and the plate was incubated at 37oC and 5% CO2 for 20 minutes. Then, the WFI was completely removed and the hypotonic lysis step was repeated once. After that, 200µl of a sterile PBS solution containing 0,25mg/ml XTT and 25µM menadione were added to each well and the plate was put at 37oC and 5% CO2 for 1-2 hours. Then, 125µl from the supernatant were transferred in a new 96-well plate and the optical density at 450nm (OD450) was measured with a microplate reader (Bio-Rad model 680), along with a background measurement at 655nm (OD655) for normalization. All conditions were performed in triplicates. The percentage of hyphal damage was calculated as follows:

$$\% \text{ Damage} = 100\% \times \frac{(OD450\text{-}OD655)_{control} - (OD450\text{-}OD655)_{experiment}}{(OD450\text{-}OD655)_{control}}$$

B12: Hyphal susceptibility to osmotic shock

$5x10^4$ conidia of *Aspergillus fumigatus* 293 per well of a flat bottom 96 well plate were incubated at 37oC, 5% CO2 in 200µl of RPMI-complete for 18 hours to induce mature hyphae with 250ng/ml, 125ng/ml, 63ng/ml, 31ng/ml or 0ng/ml caspofungin. After 18 hours, the medium was completely removed and 200µl of sterile water for injection (WFI) were added to some of the wells, to perform hypotonic lysis, and the plate was put back at 37oC and 5% CO2 for 20 minutes. Then, the WFI was completely removed and the hypotonic lysis step was repeated once. After that, 200µl of a sterile PBS solution containing 0,25mg/ml XTT and 25µM menadione were added to each well and the plate was incubated at 37oC and 5% CO2 for 1-2 hours. Then, 125µl from the supernatant were transferred in a new 96-well plate and the optical density at 450nm (OD450) was measured with a microplate reader (Bio-Rad model 680), along with a background measurement at 655nm (OD655) for normalization. All conditions were performed in triplicates.

Then, in order to acquire a measurement long after the osmotic shock, the content of the well was replaced with sterile PBS and the plate was kept at room temperature overnight. Then, the content of the wells was completely removed and 200µl of a sterile PBS solution containing 0,25mg/ml XTT and 25µM menadione were added to each well and the plate was put at 37oC and 5% CO2 for 1-2 hours. Then, 125µl from the supernatant were transferred in a new 96-well plate and the optical density at 450nm (OD450) was measured with a microplate reader (Bio-Rad model 680), along with a background measurement at 655nm (OD655) for normalization.

B13: Statistical analysis

All statistical analyses were performed using Prism (GraphPad Software). A p value < 0.05 was considered statistically significant for all variables tested.

C. RESULTS

C1: Exploring the *in vitro* activity of echinocandins against *Aspergillus fumigatus* under conditions simulating physiologic, *in vivo* growth

Paradoxically, caspofungin displays significant *in vivo* activity in preclinical studies and patients with invasive aspergillosis, but lacks meaningful *in vitro* activity against *Aspergillus fumigatus* [54, 65, 66, 67, 68, 79] So far, *in vitro* activity of echinocandins against *Aspergillus* spp is based on visual detection of morphologic changes in fungal hyphae, whereas a reliable assay for assessment of antifungal activity in fungal biomass in lacking. Thus, in order to have a more accurate estimate of the effect of echinocandins on *Aspergillus fumigatus* fungal biomass, we adapted a previously described colorimetric assay that relies on the reduction of XTT to formazan derivatives by actively metabolizing fungal cells, by spectrophotometric quantification of the product of this reaction at 450nm [77]. To apply the XTT assay, we first had to establish that it is sensitive enough to detect minimal amounts of fungal cells and that there is linear association between XTT reduction reflected by color development and the corresponding fungal metabolic activity. After preliminary experiments, we determined the optimized concentration of 0,25mg/ml XTT and 25μM of the electron acceptor menadione that was used in subsequent studies. Then, serial dilutions of resting conidia (the metabolically inactive resting spores of the fungus), at final concentrations ranging from 10^5 to 10^3, were incubated in 96-well plates at 37oC for 18 hours in complete medium to allow for germination to hyphae. After 5-6 hours of incubation, germination is evident with the swelling of the fungal cell wall, the formation of fungal germ tubes, and later on, the formation of fungal syncytia termed hyphae, which at 18 hours have already expanded across the area of the well. After 18 hours, XTT/menadione was added for 1h in the wells at the optimized concentrations of 0,25mg/ml/and 25μM, respectively. We found a linear association between XTT reduction, measured by spectrophotometry (OD 450 nm), and starting

fungal inoculum (linear regression: r^2=0,959 and p<0,0001), which allowed us to use this assay for assessment of the fungal biomass for further experiments (Figure C.1).

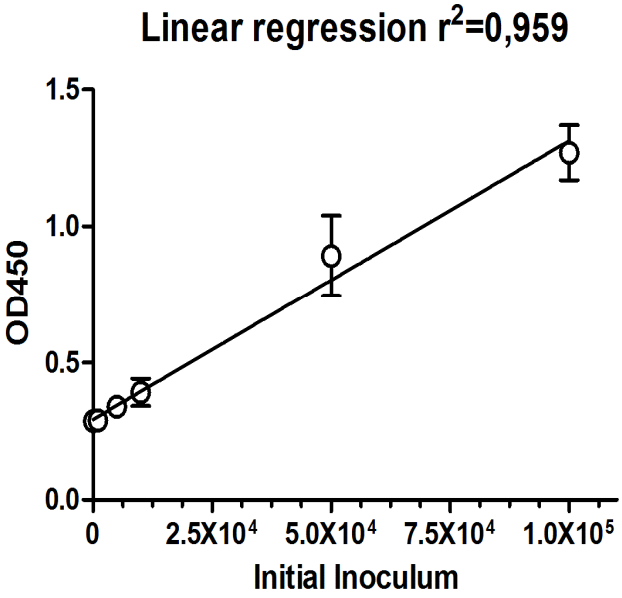

Figure C.1: Inoculum size and XTT reduction by 18 hour grown hyphae of *Aspergillus fumigatus* strain 293. X-axis shows the number of the conidia per well at the beginning of the experiment. Y-axis shows the optical density at 450nm. Data show means +/- standard deviations. Linear regression analysis revealed a linear association (r^2=0,959 and p<0,0001).

Then, in order to confirm in our experimental setting that caspofungin has minimal effect on the growth of *Aspergillus fumigatus in vitro*, we performed susceptibility tests of *Aspergillus fumigatus* using serial dilutions of caspofungin at 35oC for 48 hours using the colorimetric XTT assay. The Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) guidelines recommend using RPMI medium, pH 7 for susceptibility testing of *Aspergillus fumigatus* [78]. In our studies, we used RPMI complete medium, pH 7,4, in order to assess susceptibility of *Aspergillus fumigatus* to antifungal drugs in conditions more relevant to our *ex vivo* studies with human phagocytic cells. We noticed that the pH of the RPMI-complete medium after 2 days of incubation had increased to 9. The range of concentrations of the tested antifungal agents was based on previous susceptibility studies on *Aspergillus fumigatus* and *Candida albicans* [80, 81, 82]. The activity of caspofungin on two different strains of *Candida albicans*, is shown in Figure C.2 (a) and (b), where y-axis

shows the growth of the fungus as a percentage of the untreated control, and

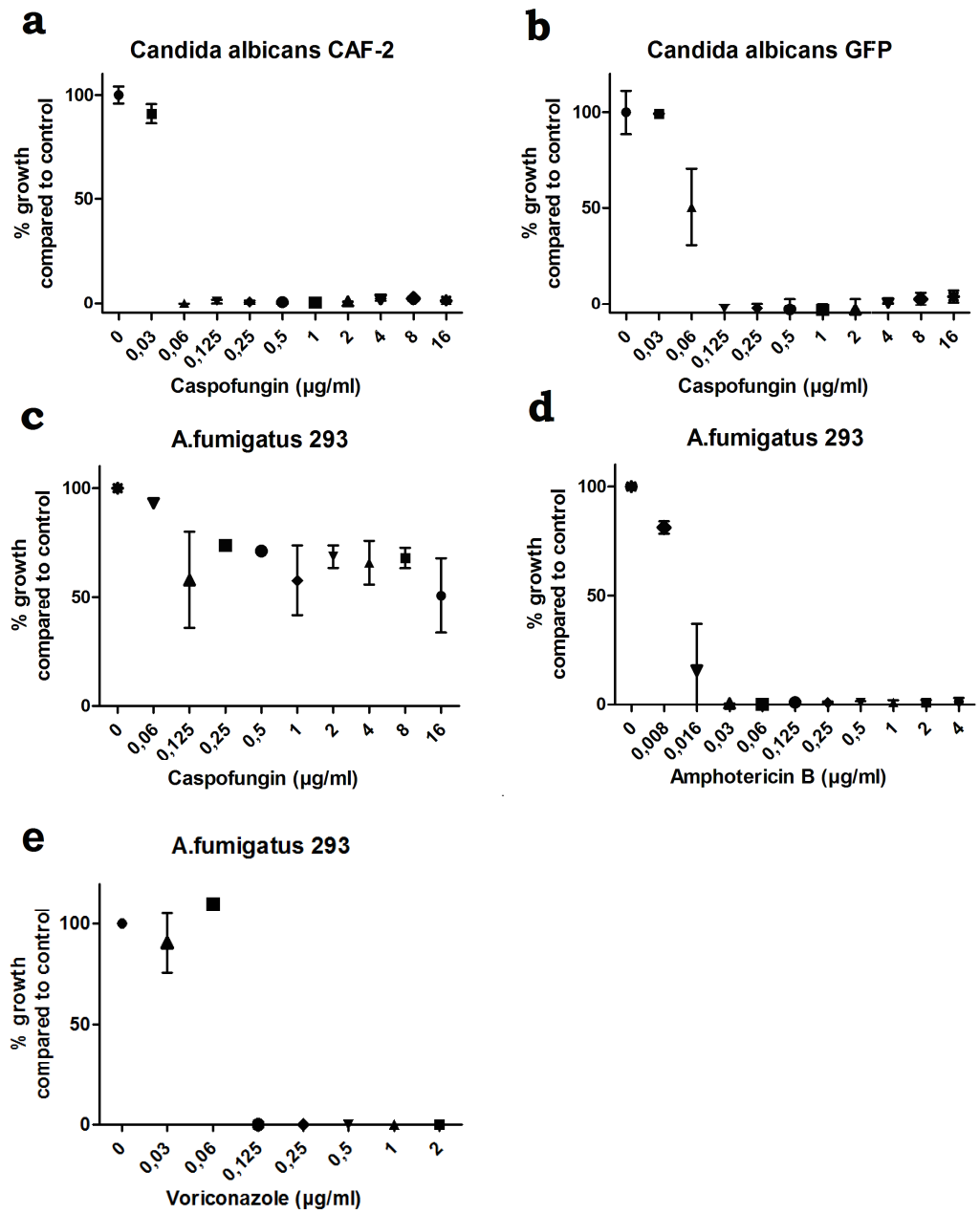

Figure C.2: XTT-based analysis of *in vitro* activity of caspofungin (a), (b), (c), amphotericin B (d) and voriconazole (e) against 2 strains of *Candida albicans* (a), (b) or *Aspergillus fumigatus* 293 (c), (d) and (e) after 48 hours in RPMI-complete at 35oC. Data show means +/- standard deviations.

the x-axis shows the concentration of the antifungal compound used in this experiment. Caspofungin resulted in complete inhibition of fungal growth both visually and by XTT reduction, which allows the determination of the MIC for

Candida albicans CAF-2 and GFP strain, at 0,06μg/ml and 0,125μg/ml respectively. The susceptibility of *Aspergillus fumigatus* strain 293 to caspofungin, amphotericin B and voriconazole after 48 hours of incubation at 35oC in complete medium is shown in Figure C.2 (c), (d) and (e) respectively. In agreement with previous studies, we found that caspofungin was not able to completely inhibit *Aspergillus fumigatus* strain 293 growth even at relatively high concentrations. In sharp contrast, amphotericin B and voriconazole, two antifungal agents targeting the fungal cell membrane, were able to completely inhibit the growth of *Aspergillus fumigatus* strain 293 at a concentration as low as 0,016μg/ml for amphotericin B and 0,125μg/ml for voriconazole, which are defined as the Minimum Inhibitory Concentrations (or MICs) for these drugs. We also confirmed that even though the activity of caspofungin on *Aspergillus fumigatus* metabolic activity reduction was minimal, there was an obvious effect in the morphology of hyphae under the microscope, with the hyphae being shorter and with many short stumpy branches, at concentrations as low as 0,5μg/ml, as determined by 2 independent investigators. The minimum concentration of caspofungin that leads to these morphologic changes, is used instead of the MIC for caspofungin in the case of *Aspergillus fumigatus*, and is termed Minimum Effective Concentration (MEC) [54]. Notably MEC does not correlate with the reduction in fungal growth, as evidenced by the rate of XTT conversion.

To address this paradoxical difference between the *in vitro* and the *in vivo* activity of caspofungin against *Aspergillus fumigatus*, we used an *in vitro* setting, that has not been described before for susceptibility testing of *Aspergillus fumigatus*, and resembles the physiologic fungal growth upon tissue infection, by performing the susceptibility test with the use of the colorimetric XTT assay in the incubator at 37oC with 5% CO2, with all the other variables remaining the same. Interestingly, as shown in Figure C.3 (a), at 48 hours of incubation, there was evidence of a significant reduction in *Aspergillus fumigatus* strain 293 growth starting from 31ng/ml caspofungin, which resulted in almost 80% decrease in fungal metabolic activity. In addition,

there was evidence of morphologic changes in caspofungin treated hyphae, as can also be seen in Figure C.4 (e) at the transmitted light (DIC) channel, with the hyphae having more sharp and stumpy branches. Thus, in addition to the effect of caspofungin in fungal metabolic activity reduction there was a significant decline in MEC, based on morphologic criteria. As opposite to the dramatic increase in echinocandin antifungal activity against *Aspergillus* under physiologically relevant conditions

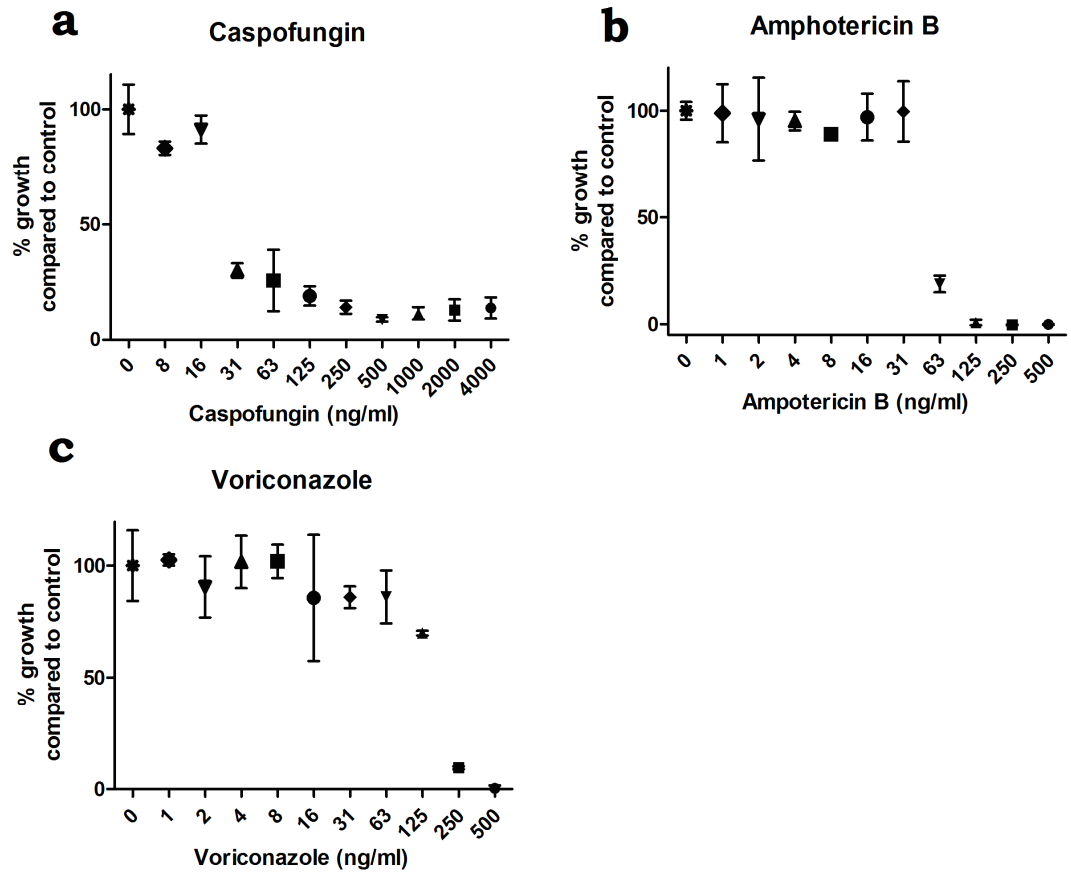

Figure C.3: XTT-based analysis of *in vitro* activity of caspofungin (a), amphotericin B (b) and voriconazole (c) against *Aspergillus fumigatus* 293 after 48 hours in RPMI-complete in an environment simulating *in vivo* growth (37oC & 5% CO2). Data show means +/- standard deviations.

(5% CO2), as shown in Figure C.3 (b) and (c), there was no evidence of significant effect of CO2 in MIC of either amphotericin B or voriconazole, which are antifungal drugs that also target the fungal cell membrane, since the growth of *Aspergillus fumigatus* strain 293 after 48 hours in RPMI-complete

medium at 37oC and 5% CO2 is completely inhibited in the presence of 63ng/ml amphotericin B, or 250ng/ml voriconazole respectively, which are the MICs for these antifungal drugs.

C2: Exploring whether the immunomodulatory properties of echinocandins could partially explain their improved *in vivo* activity: focus on immune augmentation via exposure of immunostimulatory cell wall β-glucans

We performed immunostaining with an antibody against β-1,3 glucan in order to check for β-1,3 glucan exposure of *Aspergillus fumigatus* resting conidia and hyphae and *Candida albicans* yeast. As shown in the FACS analysis in Figure C.4 (a), resting conidia of *Aspergillus fumigatus* strain 293 showed minimal amount of β-1,3 glucan exposure. Importantly, there was no evidence of enhanced β-1,3 glucan exposure following pre-exposure of resting conidia with different echinocandin concentrations close to the MEC range, since 6,73% of the untreated exposed β-1,3 glucan, while 5,49% and 5,81% of the conidia treated with 250ng/ml (1/2 MEC) and 500ng/ml (1/1 MEC) of caspofungin respectively, exposed β-1,3 glucan. As expected, the isotype control (resting conidia incubated only with the secondary fluorescent antibody) were less than 0,5% positive for β-1,3 glucan, while the positive control, that is swollen conidia that are known to expose high levels of β-1,3 glucan on their surface during the germination process [34], expose β-1,3 glucan at 46,1%. On the other hand, immunostaining of *Candida albicans* GFP strain with the anti-β-1,3 glucan primary antibody, assessed by FACS analysis, revealed that *Candida albicans* exposes β-1,3 glucan after caspofungin treatment. Specifically, untreated yeast spores had less than 0,5% β-1,3 glucan exposure similarly to the isotype control, as shown in Figure C.4 (b). Importantly, yeast spores of *Candida albicans* GFP strain treated with caspofungin at concentrations even below 1/6 MEC, displayed a higher amount of β-1,3 glucan exposure in up to 16,8% of total cells, as shown in

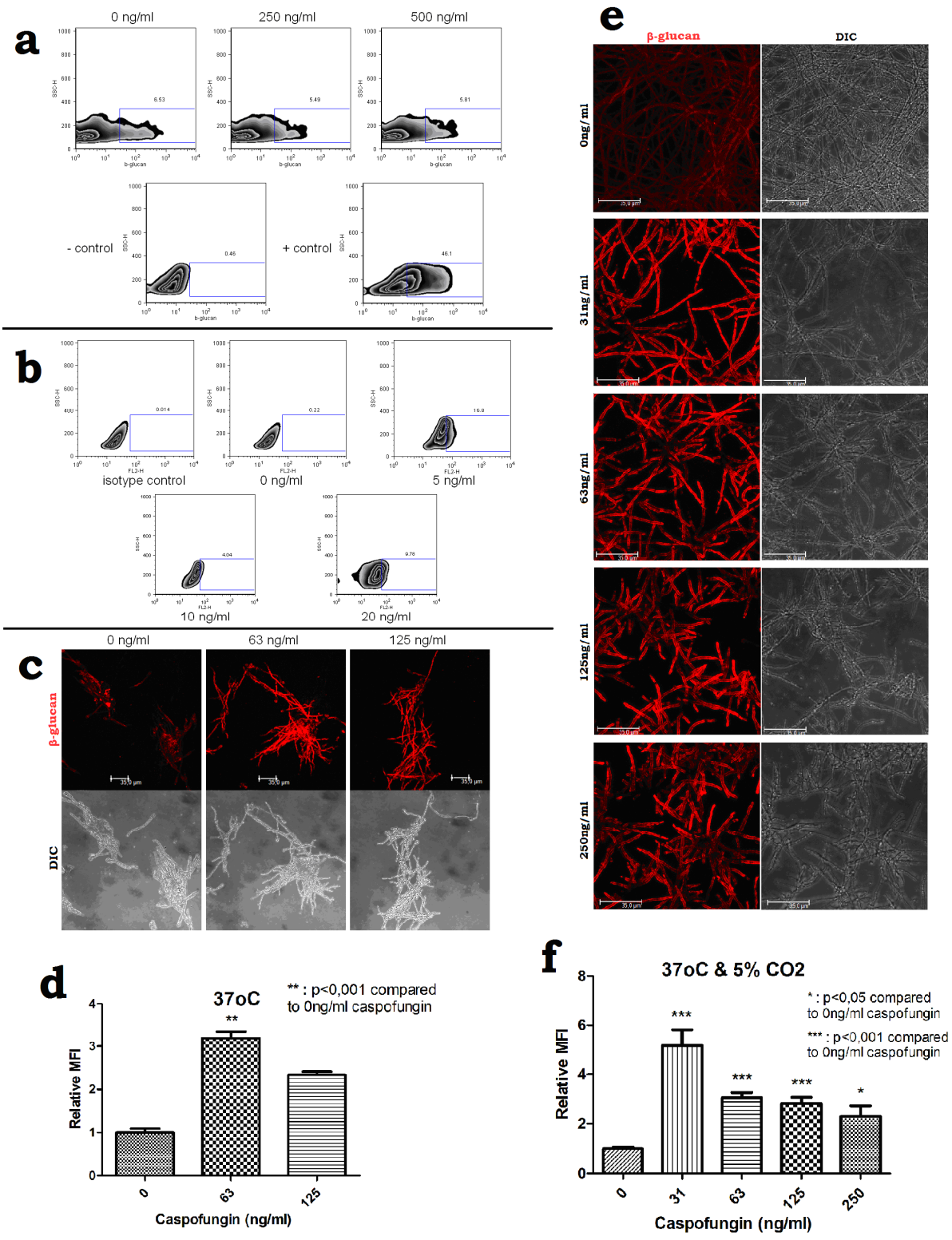

Figure C.4: Caspofungin treated *Aspergillus fumigatus* and exposure of β-1,3 glucan. Conidia of *Aspergillus fumigatus* 293 grown in YAG plates with different concentrations of caspofungin (a), or yeasts of *Candida albicans*-GFP grown overnight in liquid YPD medium with different concentrations of caspofungin (b) were stained with anti-β-glucan antibody and secondary antibody and studied with FACS. Hyphae of *Aspergillus fumigatus* 293 grown at

Figure C.4 continued: 37oC (c) and (d), or 37oC & 5% (e) and (f) in RPMI-complete with different concentrations of caspofungin were stained with anti-β-glucan antibody and secondary antibody and visualized with confocal microscopy. Software assisted quantification of β-glucan exposure shown normalized to fungal biomass shown in (d) and (f). Data show means +/- standard errors of the means. Statistical analysis in (d) was performed with the non-parametric Kruskal Wallis test with Dunn's post test, and in (f) with the one way ANOVA with Dunett's post test.

Figure C.4 (b). Collectively treatment with caspofungin had no significant β-1,3 glucan unmasking effect in *Aspergillus* spores.

Next, in order to evaluate whether caspofungin treatment affected β-1,3 glucan surface exposure in hyphae of *Aspergillus fumigatus*, we cultured *Aspergillus fumigatus* strain 293 at 37oC with or without CO2, alone or in the presence of different concentrations of caspofungin for 18 hours in order allow germination and formation of mature hyphae. After immunostaining for β-1,3 glucan, we analyzed the effect of caspofungin on β-1,3 glucan surface exposure in hyphae by confocal microscopy as shown in Figure C.4 (c) and (e). When we stained *Aspergillus fumigatus* hyphae pretreated with caspofungin for β-1,3 glucan, as shown in Figure C.4 (c), there was a significant increase in exposure of β-1,3 glucan, when compared to untreated hyphae, at concentrations 1/4 MEC and 1/8 MEC 125ng/ml and 63ng/ml respectively). In order to quantify the amount of β-1,3 glucan exposure, we quantified the volume (biomass) of the hyphae based on the fluorescent signal of hyphae at the transmitted light (DIC) channel (data not shown). Next we calculated the amount of β-1,3 glucan exposure in hyphae by normalizing the fluorescence intensity of β-1,3 glucan antibody staining to the calculated fungal biomass. Results on β-1,3 glucan exposure following caspofungin treatment are shown in Figure C.4 (d). As shown, exposure of *Aspergillus fumigatus* strain 293 to concentrations of caspofungin 1/8 – 1/4 of MEC (63ng/ml – 125ng/ml) resulted in 2 – 3 fold increase in the amount of surface β-1,3 glucan.

Next, we examined whether β-1,3 glucan exposure of hyphae occurred at the *in vitro* conditions that best simulated the *in vivo* environment. To that

end, we cultured conidia of *Aspergillus fumigatus* strain 293 on coverslips at 37oC and 5% CO2 for 18 hours to allow germination to mature hyphae with different concentrations of caspofungin around and below the MEC, stained with an antibody against β-1,3 glucan, and studied the hyphae by confocal microscopy. As shown in Figure C.4 (e), there was a significant difference in the exposure of β-1,3 glucan between the hyphae produced by conidia treated with caspofungin and the hyphae produced by conidia that were not treated with caspofungin, while the exposure of β-1,3 glucan increased from 250ng/ml to 31ng/ml. In order to quantify the amount of β-1,3 glucan exposure of these hyphae, we again calculated the relative exposure of β-1,3 glucan, that is the exposure of β-1,3 glucan normalized to the fungal biomass, and depicted it in Figure C.4 (f). Conidia that germinated at 37oC and 5% CO2 with caspofungin around and below the MEC for 18 hours, produced hyphae that had 2,5 – 5 fold increased β-1,3 glucan exposure compared to the ones that were not treated with caspofungin, while, the β-1,3 glucan exposure was less with the higher concentrations of caspofungin, and maximum with 31ng/ml which was the lowest concentration of caspofungin in this experiment, and also below the MEC.

C3: Effect of echinocandins in neutrophil activity against *Aspergillus fumigatus* hyphae

To examine the effect of caspofungin in neutrophil activity against *Aspergillus fumigatus* hyphae, we established an assay using human neutrophils isolated from healthy volunteers and mature hyphae of *Aspergillus fumigatus* strain 293. Using blood from healthy donors, and a previously described isolation method [83], we isolated neutrophils and verified their purity by immunostaining with fluorescent antibodies against CD15 and CD16 which are typical surface markers of neutrophils, and CD14 which is a surface marker mainly expressed by circulating monocytes [84]. The results of the FACS analysis are shown in Figure C.5 (a). The cells studied comprise the

predominant population as evidenced by Forward and Side Scatter at the dot plot named 'Gating', and were negative for CD14, as only 1,75% of those cells were positive for CD14, and were also positive for CD15 and CD16, since 93,2% and 91,5 of those cells were positive for CD15 and CD16 respectively, as shown in the corresponding dot plots, where y-axis shows the side scatter, and the x-axis shows the fluorescence intensity. Thus, we found that the isolated leucocytes had the necessary purity (> 90%) to be characterized as neutrophils.

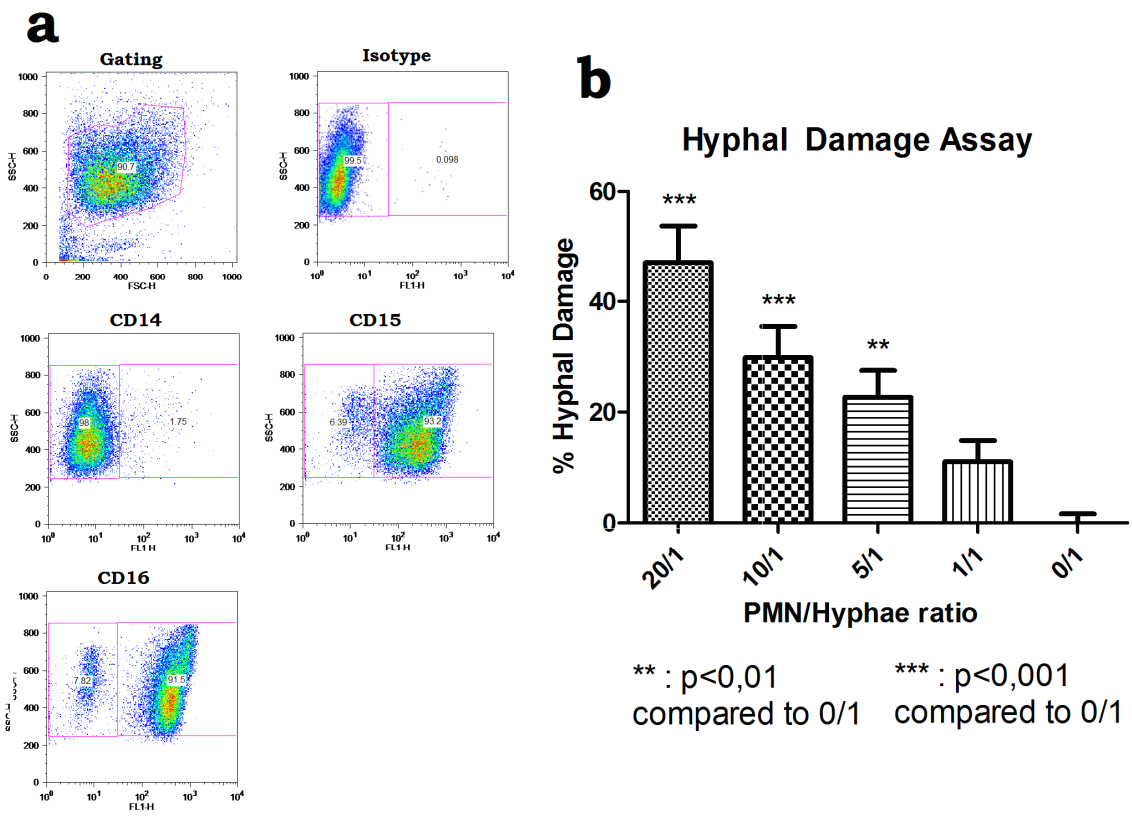

Figure C.5: Hyphal damage assay with human neutrophils. Immunostaining of isolated human cells from peripheral blood with surface markers confirms they are neutrophils (a). Establishment of hyphal damage assay with human neutrophils and hyphae of *Aspergillus fumigatus*, grown previously for 18 hours at 37oC & 5% CO2 in RPMI-complete (b). Statistical analysis in (b) was performed with the one way ANOVA test with Dunett's post test.

To assess the neutrophil damage on hyphae of *Aspergillus fumigatus*, we developed a previously described method [54], which uses XTT to assess

the loss of metabolic activity of hyphae after co-incubation with neutrophils. In Figure C.5 (b) the results of 2 hour incubation of freshly isolated human neutrophils with mature hyphae of *Aspergillus fumigatus* strain 293 are shown.

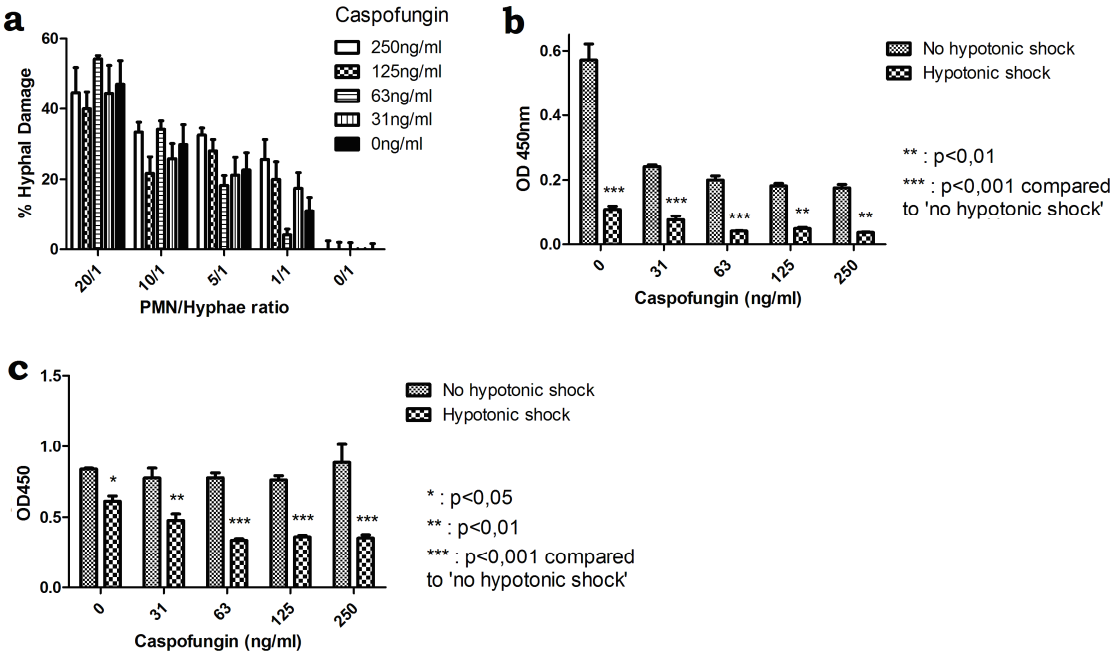

Figure C.6: Effect of caspofungin on hyphal damage. Hyphal damage of human neutrophils on *Aspergillus fumigatus* 293 hyphae, grown previously for 18 hours at 37oC & 5% CO2 in RPMI-complete with different concentrations of caspofungin (a). Damage of *Aspergillus fumigatus* 293 hyphae, grown previously for 18 hours at 37oC & 5% CO2 in RPMI-complete with different concentrations of caspofungin caused by hypotonic shock performed with water for injection directly after the shock (b) and after overnight incubation in PBS at room temperature (c). Statistical analysis was performed in (a) with the two way ANOVA test, and in (b) and (c) with the two way ANOVA test with Bonferroni post tests.

The y-axis shows the percentage of 'hyphal damage' which is the loss of metabolic activity of the hyphae, judging by the XTT conversion, after co-incubation with neutrophils, compared to the hyphae alone, while the x-axis shows the PMN/hyphae ratio, which is the number of neutrophils added on hyphae in each condition divided by the number of the resting conidia added in the well as an initial inoculum. As anticipated, the higher the PMN/hyphae ratio, the higher the hyphal damage compared to the 0/1 ratio, that is hyphae

without neutrophils. These differences were also statistically significant for the 20/1, 10/1 and 5/1 ratios but not for the 1/1 ratio.

Then, in order to see if *Aspergillus fumigatus* hyphae produced by resting conidia incubated with caspofungin are more susceptible to damage by human neutrophils, we performed the hyphal damage assay with different concentrations of caspofungin added to the culture plate before germination of the conidia. As shown in Figure C.6 (a), 4 different concentrations of caspofungin were tested along with the non-caspofungin treated hyphae, 250ng/ml, 125ng/ml, 63ng/ml and 31ng/ml, and even though the graph shows that there is more hyphal damage in the higher PMN/hyphae ratios as expected, there does not seem to be any difference in hyphal damage between the different concentrations of caspofungin for the same PMN/hyphae ratio, suggesting that the presence and concentration of caspofungin during the germination of *Aspergillus fumigatus* strain 293 conidia does not have any effect on the damage caused by neutrophils on the mature hyphae.

Interestingly, we noticed that mature hyphae are vulnerable to damage induced by osmotic shock caused by hypotonic lysis with water, as shown in Figure C.6 (b) and (c). In this figure, the optical density at 450nm, which reflects the metabolic activity of hyphae, is plotted on the y-axis, and the concentration of caspofungin used during the germination of the fungus, is shown in the x-axis. Filled bars show the conditions that were not subjected to hypotonic shock and the dotted bars show the conditions that were subjected to hypotonic shock. As shown in Figure C.6 (b), hypotonic shock to mature hyphae leads to a statistically significant reduction of the metabolic activity of hyphae, judging by their reduced ability to reduce XTT when the solution containing the XTT was added to the hyphae immediately after the hypotonic shock. Furthermore, the same figure confirms our previous findings that pre-exposure to caspofungin leads to a lower biomass for the concentrations shown, namely 31ng/ml, 63ng/ml, 125ng/ml and 250ng/ml. This reduction of metabolic activity due to caspofungin, albeit smaller, is also seen in hyphae that had been subjected to hypotonic shock, as also seen in Figure C.6 (b).

However, when the same hyphae that were subjected to the osmotic shock, were again tested with XTT after been previously incubated overnight at room temperature in an isotonic solution, their ability to reduce XTT was dramatically different as shown in Figure C.6 (c), which shows that the hyphae that were not submitted to hypotonic shock present the same ability to reduce XTT, since their supernatants have the same optical density at 450nm, while the hyphae that had been submitted to hypotonic shock have statistically significant decreased metabolic activity when compared to the hyphae that were not submitted to hypotonic shock, and this decreased metabolic activity is higher as caspofungin concentration increases from 31ng/ml to 63ng/ml and then is the same from 63ng/ml to 250ng/ml.

D. DISCUSSION

Evidence from clinical studies in humans, and preclinical data from studies in animal models of invasive aspergillosis indicate that echinocandins are effective antifungal agents for treatment of invasive aspergillosis [59, 65, 66, 67, 68, 69, 70, 71]. However, these compounds possess mediocre activity against *Aspergillus in vitro*, since they fail to inhibit fungal growth even at relatively high concentrations [51, 67]. The reasons that account for this discordance between the *in vivo* and the *in vitro* effect of this category of drugs are currently unknown.

In this study, we attempted to decode the echinocandin paradox that is the difference in *in vivo* and *in vitro* activity of echinocandins. An *in vitro* environment that simulates the *in vivo* environment was established, and susceptibility test of a representative strain of *Aspergillus fumigatus* to caspofungin, showed a tremendous reduction of fungal growth. Additionally, hyphae pre-treated with caspofungin below the MEC, showed a remarkably enhanced exposure of β-1,3 glucan, an immunogenic fungal molecule able to trigger host immune responses, even though there was no evidence for an additive hyphal damage of neutrophils and caspofungin. Furthermore, caspofungin treated hyphae presented significantly more damage after osmotic shock compared to the untreated controls. Collectively, our work could explain, at least partially, the echinocandin paradox and emphasizes on the limitations of *in vitro* studies in dissecting the complexity and dynamics of host-pathogen interplay during *in vivo* infection.

D1: *In vitro* activity of echinocandins against *Aspergillus fumigatus* under conditions simulating *in vivo* growth

The difference between the *in vitro* and the *in vivo* efficacy of echinocandins comprises the echinocandin paradox. To address this paradox, a systematic attempt to dissect the differences between the *in vitro* and the *in vivo*

environment should be performed. A major difference between the *in vitro* environment at which the susceptibility tests are performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) [78] and the human tissues are the temperature, the CO2 concentration and the pH, since the CLSI guidelines suggest performing the susceptibility test at 35oC without CO2 at pH 7, while the tissue environment at which *Aspergillus fumigatus* will germinate *in vivo* has a temperature of 37oC, 5% CO2 and pH 7,4.

To address the question whether the difference in the temperature, the pH and the presence of CO2 could have any effect in the susceptibility of *Aspergillus fumigatus* to caspofungin, we used a well characterized *Aspergillus fumigatus* strain. To have a clear readout of the fungal susceptibility to antifungal drugs, beyond the standard visual and microscopical determination of fungal growth, we adapted a previously well described colorimetric assay that relies on the reduction of XTT by actively metabolizing fungal cells [77], and confirmed its accuracy to measure *Aspergillus* biomass, through measuring its metabolic activity.

To further simulate the tissue environment, a complete medium that also contained a buffer solution to exclude large deviations of the pH from 7,4, was used in the susceptibility tests. As additional controls, the susceptibility of 2 different strains of *Candida albicans* at 35oC without CO2, at caspofungin was tested, as well as the susceptibility of *Aspergillus fumigatus* strain 293 to other antifungal agents that also target the fungal cell wall but do not belong in the caspofungin family, both at 35oC without CO2 and at 37oC with 5% CO2. Interestingly, even though caspofungin seems to have a mild effect on fungal growth of *Aspergillus fumigatus* in *in vitro* conditions, as also shown in previous studies [67, 72, 73], the response of the fungus in the conditions that mostly simulate the *in vivo* environment is completely different, since there is a significant reduction of fungal growth that reaches even 90%. Along with this significant difference in the growth of *Aspergillus fumigatus*, a difference in the

pH of the complete medium that was used was also noticed. Even though the pH of the medium was 7,4 at the beginning of the experiment, after 48 hours, the pH of the medium at 37oC and 5% CO2 was still 7,4 contrary to the same medium at 35oC without CO2, where the pH had increased to higher than 9. This difference in the pH, could be related to the difference seen in the fungal growth of *Aspergillus fumigatus* with caspofungin, however this cannot be associated with the response of *Aspergillus fumigatus* to all the antifungal compounds, since amphotericin B and voriconazole showed similar response in the two different environments tested. On the other hand, this lag in *in vitro* activity cannot be a general characteristic of echinocandins at higher pH either, since two strains of *Candida albicans* that were tested, were susceptible to caspofungin and presented complete inhibition of growth with relatively low concentrations of the drug. It could be that high pH somehow affects the activity of caspofungin against *Aspergillus fumigatus*, and this could explain the mediocre activity of the drug against *Aspergilli in vitro*, however, in our knowledge, there is no evidence in the literature suggesting that caspofungin is affected by pH, even though there is evidence suggesting that the activity of some antifungal drugs could be affected by the pH of the medium, as well as from other environmental factors, like hypoxia, and the presence of CO2 [85, 86, 87]. Indeed, in two of these studies, the susceptibility of *Aspergillus fumigatus* to two echinocandins, anidulafungin and micafungin, was higher in hypoxic conditions, even allowing for MIC determination at relatively low concentrations of the antifungal drugs [86, 87]. These studies, that also occupy an environment that simulates the infected tissues of the host, with the low oxygen and the presence of CO2, are in line with our findings, and imply a role for the specific environmental conditions, at which the growth of *Aspergillus fumigatus* takes place *in vivo*, in the activity of echinocandins. Furthermore, they also suggest that the current *in vitro* susceptibility testing conditions are far from perfect for assessment of activity of these antifungal drugs.

Indeed, this paradoxical increase of caspofungin activity against *Aspergillus fumigatus* in the *in vitro* conditions that simulate the *in vivo* growth in our hands, could even allow the use of the term 'MIC' for susceptibility testing against caspofungin under these conditions, since the alternative endpoint for the susceptibility testing of *Aspergilli* to this class of antifungal drugs, MEC, is of ambiguous clinical significance, and the correlation between MEC and clinical response of invasive aspergillosis should not be taken for granted. Indeed, other studies use different methods to define the MEC, such as the cut-off point for the induction of macrophage activation [74], implying the need for a more reliable, and clinically relevant method to assess the susceptibility of *Aspergillus fumigatus* to echinocandins.

Alternatively, performing the susceptibility test of *Aspergillus fumigatus* to echinocandins in the environment that simulates the *in vivo* conditions could be indicative of the susceptibility of *Aspergillus fumigatus* to these antifungal drugs in terms of clinical practice, which could also be reflective of the concentration of the antifungal needed to achieve a therapeutic effect in the case of invasive aspergillosis.

Furthermore, even though this was not tested in this study, this difference in activity of echinocandins in conditions with and without CO2 could also apply for susceptibility testing of other molds. Importantly, susceptibility testing in 5% CO2 conditions is easily applicable to most microbiology labs and could be a way to improve susceptibility testing of echinocandins by determining MEC values that reflect on their clinical activity.

On the other hand, the reason for this increase in the susceptibility of *Aspergillus fumigatus* to caspofungin in the presence of CO2 is not clear. It is tempting to suppose that CO2 acts by stabilizing the pH of the culture medium, and that the effect of echinocandins on *Aspergillus fumigatus* is pH dependent, but this has to be further evaluated with susceptibility tests with media at different pH values. However, the fact that echinocandins were effective against *Candida albicans in vitro*, suggests that if the effect of echinocandins against *Aspergillus fumigatus* is pH dependent, then it solely applies to this

fungus. If this is the case, the reason could have to do with intracellular events in *Aspergillus fumigatus*. Indeed, it is known that even though antifungal compounds have a major target, like the fungal cell wall, they also trigger several intracellular stress response pathways [88]. Importantly, in a study using *Saccharomyces cerevisiae*, several genes conferring resistance or susceptibility to caspofungin were identified [89], with some of them belonging in the biosynthesis pathway of the fungal cell wall and ergosterol, which could be explained by the fact that treatment with echinocandins leads to reduction of β-1,3 glucan and structural rearrangements of the fungal cell wall [90, 91], while other genes are associated with vacuole function, transportation of vesicles, transcription and other functions, implying a role for these pathways in the susceptibility or resistance to echinocandins. Furthermore, microarray and proteome analysis of *Aspergillus fumigatus* treated with caspofungin revealed down-regulation of genes and proteins associated with mitochondrial hypoxia stress response and up-regulation of genes and proteins involved in protein synthesis compared to untreated *Aspergillus* [92]. If the environment of the fungal growth somehow affects these biological pathways in *Aspergillus fumigatus*, it would be reasonable to expect some differences in the susceptibility of *Aspergillus fumigatus* in different environments. Indeed, culture of *Aspergillus fumigatus* in hypoxic conditions revealed increased expression of proteins related to oxidative and nitrosative stress response [93] as well as altered expression of genes related to oxidative metabolism and cell wall biosynthesis [94, 95], implying that caspofungin treatment along with the hypoxic conditions in the *in vivo* environment could have an additive effect that could cause profound damage to the fungi, even though, this remains to be tested in future studies. Thus, it could be interesting to examine whether essential fungal responses are blocked in high CO2 conditions of growth in future studies.

D2: Immunomodulatory properties of echinocandins that could explain their improved *in vivo* activity via exposure of immunostimulatory cell

wall β-glucans, and effect of echinocandins in neutrophil activity against *Aspergillus fumigatus* hyphae

Previous studies reported on the immunomodulatory properties of echinocandins that are mediated via increased exposure of the immunogenic molecule β-1,3 glucan on the wall of *fungi including A. fumigatus* [35, 74] *Candida albicans* and *Saccharomyces cerevisiae* [75, 76]. Importantly, β-1,3 glucan exposure occurs at concentrations of echinocandins below the MIC, suggesting this immunomodulatory action could be of clinical relevance [34, 74]. In *Aspergillus fumigatus*, *ex vivo* studies with murine bone marrow macrophages and human neutrophils found that this β-1,3 glucan unmasking induced by echinocandins resulted in increased cytokine release and enhanced killing of hyphae [35, 74].

In agreement with these studies, we confirmed a significant increase in β-1,3 glucan exposure in *Aspergillus fumigatus* cell wall that occurred selectively in the hyphal form of the fungus, both *in vitro* and in the environment that simulates the *in vivo* growth of *Aspergillus fumigatus*. Importantly, since neutrophils are the major population of white blood cells that migrate in the infected lung, and the only phagocytes specialized to attack hyphae [17, 19, 34], we investigated the effect of echinocandins in neutrophil activity *ex vivo*. By adapting a previously described assay that has as a read out the hyphal damage induced by neutrophils [54], with neutrophils isolated from human blood and mature hyphae of *Aspergillus fumigatus* grown in the *in vitro* environment that simulates the *in vivo* growth, we opted to see if pre-exposure of hyphae to caspofungin at concentrations below the MEC were leading to increased hyphal damage from neutrophils. Interestingly, in contrast to a previous study, we did not find an appreciable increase in neutrophil killing upon echinocandin pre-exposure [35]. Nonetheless, in our studies we noticed that hypotonic lysis following incubation with neutrophils had a more pronounced effect in hyphae that had been exposed to caspofungin. Therefore, in contrast to the aforementioned study in calculations of neutrophil

induced damage, we normalized for the effect resulting from increased susceptibility to osmotic stress in caspofungin-treated hyphae.

Besides, β-1,3 glucan mediated immunomodulation is not limited to direct neutrophil antifungal activity. For example, activation of β-1,3 glucan sensing receptors could result in increased release of proinflammatory cytokines and chemokines with critical function in the course of the infection with *Aspergillus fumigatus* [27]. Obviously, these effects are difficult to evaluate by *in vitro* or *ex vivo* studies. Furthermore, although β-1,3 glucan has been proposed as the predominant cell wall molecule with immunostimulatory properties, cell wall damage mediated by echinocandins could also result in exposure of other immunostimulatory molecules. This could be an interesting future research direction.

On the other hand, a beneficial role of immune augmentation in the outcome of invasive aspergillosis is difficult to predict. For example, unabated inflammatory responses are not always beneficial for infection resolution [96, 97, 98]. In fact, recent studies in the CGD model of invasive aspergillosis clearly demonstrate that unrestricted inflammation, because of impaired function of regulatory T cells and the lack of anti-inflammatory action of ROS are key pathogenetic mechanisms [99, 100, 101]. Of interest, paradoxical inflammatory responses upon neutrophil recovery with clinical worsening have been described in patients receiving echinocandins [102]. It is tempting to speculate that echinocandin–mediated immunomodulation is implicated in these detrimental responses, even though further studies are needed to confirm this hypothesis. Therefore, although we failed to prove that immunomodulation mediated by echinocandins was important for direct antifungal function of human phagocytes, it is plausible that activation of other innate immune signaling pathways could be of significant importance *in vivo*.

Surprisingly, during a hypotonic lysis step that was performed in our experimental settings, we noticed that hyphae of *Aspergillus fumigatus* were being damaged from the osmotic shock, and that this damage was not reversed in the caspofungin treated hyphae, meaning that beyond the other

effects of caspofungin on *Aspergillus fumigatus*, namely, the growth inhibition in the environment that simulates the *in vivo* growth and the increased exposure of immunostimulatory β-1,3 glucan, caspofungin could probably increase the susceptibility of *Aspergillus fumigatus* hyphae to osmotic stress. In our knowledge, this is the first evidence showing that hyphae of *Aspergillus fumigatus* are susceptible to osmotic shock leading to a profound reduction of their metabolic activity, even though there is evidence suggesting that hyphae exposed to caspofungin are susceptible to osmotic shock [103]. It is tempting to hypothesize that treatment with caspofungin, at concentrations way below the MEC is able to render hyphae of *Aspergillus fumigatus* susceptible to damage from environmental stress factors, as this would probably render hyphae susceptible to the hostile environment of the tissues after the recruitment of neutrophils and other leukocytes that would release their toxic intermediates, in their surrounding environment.

Collectively, this work comprises an attempt to explain the echinocandin paradox that is the difference in *in vivo* and *in vitro* activity of echinocandins. In an *in vitro* environment that simulates the *in vivo* fungal growth, *Aspergillus fumigatus* was found more susceptible to caspofungin, with a tremendous reduction of fungal growth, while hyphae pre-treated with caspofungin exposed significantly higher amounts of the immunogenic molecule β-1,3 glucan, even though there was no additive hyphal damage of neutrophils and caspofungin. On the other hand, caspofungin treated hyphae were more susceptible to severe damage after osmotic shock. This work could explain, at least partially, the echinocandin paradox and also emphasizes on the limitations of *in vitro* studies in dissecting the complexity and dynamics of host-pathogen interplay during *in vivo* infection. Thus, in the long term, this study could lead to introducing an alternative, more physiologically relevant way of susceptibility testing of *Aspergilli* to echinocandins, which could be easily adapted in routine practice of most microbiology labs and provide useful endpoints for the clinical practice.

Further experiments are needed to prove that this is also the case with other members of the echinocandin family, as well as with other strains of *Aspergillus fumigatus*, to repeat the susceptibility tests with different media, different content of serum and at different pH.

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