HHS Public Access

Author manuscript

Curr Opin Microbiol. Author manuscript; available in PMC 2018 December 01.

Published in final edited form as:

Curr Opin Microbiol. 2017 December ; 40: 40–45. doi:10.1016/j.mib.2017.10.010.

The mucormycete-host interface

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Abstract

Mucormycosis is a fungal infection with fulminant angioinvasion leading to high morbidity and mortality in susceptible individuals. The major predisposing conditions are uncontrolled diabetes, neutropenia, malignancies, receipt of a transplant and traumatic injury [1]. Over the past decade, mucormycosis has become an emerging fungal infection due to the increase in patient groups presenting with these pre-disposing conditions and our medical advances in diagnosing the infection [2-4]. Yet, we currently lack clinical interventions to treat mucormycosis effectively. This in turn is due to a lack of understanding of mucormycosis pathogenesis.

Here, we discuss our current understanding of selected aspects of interactions at the mucormycete-host interface. We will highlight open questions that might guide future research directions for investigations into the pathogenesis of mucormycosis and potential innovative therapeutic approaches.

Innate immune responses during mucormycosis

Once a pathogen has overcome our non-specific barriers (e.g. skin and mucosal layers), innate immune effectors such as macrophages and neutrophils are our first cellular response against the foreign attack. Many fungal pathogens (e.g. *Cryptococcus, Candida*, *Coccidioides* species and *Histoplasma capsulatum*) have been recognized as intracellular pathogens of phagocytes (reviewed in [5]). Similarly, there is growing evidence that pathogenic Mucorales species can adapt an intracellular life style within these innate immune effectors.

The infectious particles for mucormycosis are asexual sporangiospores found ubiquitously within the environment. These resting spores can swell and germinate to produce fast-

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growing hyphae during their natural life cycle (Figure 1) [6]. Germination and filamentous growth within a host causes angioinvasion, vessel thrombosis and necrosis [7-9].

Monocytes, macrophages and natural killer (NK) cells can recognize and damage, but are unable to kill, hyphae. Conversely, filamentous forms are effectively killed by human polymorphonuclear leukocytes (PMNs) *in vitro* [10-13]. Invasive fungal growth activates pro-inflammatory signaling. Hyphae interact with TLR-2 on the surface of human PMNs inducing transcription of the proinflammatory cytokines TNF-α and IL-1β [14]. Human monocyte derived dendritic cells recognize β-glucan exclusively expressed on the hyphal surface through the pattern recognition receptor dectin-1 to induce IL-23, IL-1 and TNF-α [15]. Damage and killing is mediated by oxidative means after monocyte or neutrophil attachment to fungal filaments [16-18], through degranulation, and release of cationic peptides or perforin by rabbit and human neutrophils or NK cells, respectively [12,18-20]. Hydrocortisone treatment inhibits neutrophil or macrophage induced hyphal damage [18,21] and macrophages from diabetic mouse have reduced ability to adhere to hyphae [17]. Even in healthy hosts, the extent of hyphal damage depends on the extent of fungal biomass [12,22].

Mucormycetes are extremely fast-growing fungi and thus are likely to outcompete our immune response when it is in state of suppression. Hyphal growth is essential for virulence in yeast-locked mutants of *Mucor circinelloides*. Inhibition of the calcineurin pathway that regulates hyphal growth chemically through the calcineurin inhibitor FK506 or by mutation of the calcineurin regulatory subunit cnbR significantly reduced virulence of *M. circinelloides* in wax moth larvae [23]. Mucorales species with fast germination rates (e.g. *Cunninghamella bertholletiae*) are significantly more virulent than species with slower germination rates (e.g. *Rhizopus oryzae*, *R. microspores*, *M. circinelloides*) in a neutropenic rabbit model of pulmonary mucormycosis. The increased virulence is characterized by higher lung burden, amplified angioinvasion and lower survival [24]. Likewise, *M. circinelloides* isolates with larger spores germinate faster and are more virulent in the wax moth larva and a murine intraperitoneal infection model [25]. Thus, a protective immune response might require spore clearance before onset of filamentous growth.

After infection with mucormycete spores, phagocytes are recruited rapidly to the site of infection to internalize and form tight clusters around spores in rabbit [26,27], mouse [9,28,29] and zebrafish larval models of disease [30]. A lack or delay of this early inflammatory response renders diabetic hosts susceptible to infection leading to disease dissemination [17,27,30]. Yet, phagocytes are not able to kill resting spores *in vitro* or *in vivo* in vertebrate [9,29,30] and non-vertebrate model systems [31]. To establish within the phagocytic niche, mucormycete spores must either withstand the harsh environment or subvert phagocyte anti-microbial mechanisms. It has been demonstrated that *Rhizopus oryzae* downregulates the transcription of host defense genes (e.g. immune-inducible peptides) in infected fruit flies [31]. Resting spores are not able to elicit a pro-inflammatory cytokine response in dendritic cells [15] whilst hyphae also inhibit IFN-γ expression by IL-2 stimulated human natural killer cells [12,13]. The human macrophage-like cell line THP-1 failed to express proinflammatory cytokines in response to *M. circinelloides* or *R. oryzae* compared to *A. fumigatus* or *C. albicans* [32]. The oxidative burst elicited from PMNs by

mucormycete spores is strain dependent and reflects the virulence potential. For example, intermediate virulent strains belonging to the *Rhizopus* genus induce a smaller reactive burst than the low virulence strain *Lichtheimia corymbifera* [33,34]. Resting spores are resistant to cationic peptides released from neutrophils *in vitro* [19]. Although phagocytes fail to kill spores, they effectively prevent spore germination in healthy murine hosts [17,35,36]. Rat alveolar macrophages, but not the human macrophage cell line THP-1, inhibit spore germination through nitric oxide [37]. In susceptible mice with induced diabetes or treated with corticosteroids, inhibition of spore germination by bronchoalveolar macrophages fails allowing for filamentous growth [17,36].

Interestingly, disease can be reactivated from granulomatous clusters during acute diabetic acidosis in rabbits [26]. This opens the possibility of latent infections with Mucorales and disease reactivation in previously healthy hosts after acquired immunosuppression. Yet, we have little knowledge on the virulence factors that enable Mucorales spores to reside insight phagocytes and granulomas. At the same time, the unique enhanced susceptibility of uncontrolled diabetics and DKA patients indicates that immune responses to Mucorales are distinct from other fungal pathogens and/or Mucorales possess virulence traits that enable them to thrive in such hosts (Table 1). Thus, we need a better understanding of the mechanisms employed to establish intracellular survival within phagocytes and the phagocytic defects induced by predisposing conditions that allow spore germination.

Platelets are known to play a role in antimicrobial host defense against several pathogens by secretion of platelet microbicidal proteins [38]. Platelets were shown to adhere to Mucorales, induce time dependent damage to fungal hyphae and suppress hyphal elongation through a granule dependent mechanism [39].

Taken together, protection from mucormycosis by the innate immune system relies on the control of spores residing in phagocytes and granulomatous clusters to inhibit spore germination. In susceptible individuals, this control is lost leading to filamentous fungal growth. Increasing evidence, supporting Mucorales as intracellular pathogens within granulomas, poses the possibility of latent infections. This might offer new therapeutic strategies targeting resting spores before onset of fulminant hyphal growth in prophylactic approaches.

Adaptive immunity during mucormycosis

There is limited evidence for a major role of the adaptive immune system in combating mucormycosis. HIV alone is not a predisposing condition for disease, though cases have been reported in this patient population in association with intravenous drug or corticosteroid use and neutropenia [40]. Similarly, T-lymphocyte depletion in mice does not increase susceptibility to mucormycosis [41].

As with the innate immune response, $CD4^+$ and $CD8^+$ T-cell are only produced in response to hyphae [42] and during invasive mucormycosis [43]. However, these T-cells are lost soon after resolution of infection [43]. Both sets of T-cells produce a range of cytokines including IL-4, IFN-γ, IL-10 and IL-17 [43]. $CD4^+$ cells are predominant and show cross-reactivity

with a range of other fungal pathogens (*Aspergillus fumigatus*, *Penicillum chrysogenum* and *C. albicans*) in healthy individuals [42]. Although spores can persist in hosts, clearance of *R. pusillus* from lungs of infected mice has been reported after approximately 30 days [35]. This indicates some relevance of an adaptive immune response that warrants further investigation and might be relevant for future development of immunotherapeutic approaches against the disease.

The mucormycete-epithelial and mucormycete-endothelial interface

There has not been much work conducted on studying the interactions of mucormycetes and epithelial cells, despite these interactions representing some of the earliest events during infection. A study linked outbreak of food poisoning due to intake of yogurt to contamination with *Mucor circinelloides* [32]. This study demonstrated that Mucorales produce secondary metabolites that are toxic to the gastrointestinal mucosa. Similarly, dead Mucorales can cause considerable host cell damage *in vitro* supporting the presence of toxins [44]. It is possible that these toxic substances are responsible for the clinical feature of extensive tissue necrosis. It is also known that *Rhizopus* spores can adhere to extracellular matrix proteins such as laminin and type IV collagen [45] that embeds epithelial or endothelial cells.

Unlike epithelial cells, considerable work has been conducted on interactions of Mucorales and endothelial cells because of the angioinvasive nature of the disease. It was found that Mucorales adhere to, and invade human umbilical vein endothelial cells through specific and unique binding capacity to the heat shock glucose-regulated protein 78 (GRP78) [46]. This interaction occurs via the unique cell surface CotH invasins (Figure 2) [47] and results in a substantial injury to the endothelium *in vitro* [46]. CotH proteins are universally present in Mucorales and absent from other pathogens [48]. Interestingly, elevated glucose, iron, and β-Hydroxy butyrate (BHB) concentrations (relevant to levels seen in diabetic ketoacidosis patients) induces endothelial cell invasion and damage by *Rhizopus* and promotes virulence in mice due to surface overexpression of both GRP78 and CotH proteins [46,47,49]. It appears that during these interactions acquisition of host iron via several mechanisms (e.g. high affinity iron permease, and ferrioxamine receptors) is critical in determining the fate of infection [50-52]. Importantly, antibodies targeting GRP78/CotH interactions reduce Mucorales-induced invasion and injury of endothelial cells and protect mice from mucormycosis [46,49]. These results provide insights into why patients with diabetic ketoacidosis are uniquely predisposed to mucormycosis infections and point to potentially novel immunotherapeutic interventions.

Clinical relevance and application

Much of the focus in understanding the immune responses to mucormycosis is focused on invasive disease. While this knowledge is critical in our understanding on how mucormycosis progressively develops into a disseminated infection and ultimately will help in designing adjunctive therapies to improve outcome, understanding early events in the course of infection is likely to add therapeutic strategies that act synergistically with strategies targeting angioinvasion. Further, understanding early infection events can develop

preventative measures in targeted populations. For example, this review highlights the inability of innate immune effectors in susceptible hosts to inhibit the transition to filamentous growth and the quick growing nature of Mucorales hyphae as the main contributors to the high mortality during mucormycosis. Together with the possibility of latent infections of this emerging intracellular pathogen, development of new treatments can focus on either inhibiting the fungal ability to undergo germination or enable protective immunity targeting spores before onset of invasive disease.

Although we know a range of environmental factors that initiate spore germination (e.g. pH, nutrient availability, hydrophobicity), we currently lack an understanding of the genetic regulation of this developmental process. Likewise, we have little information on the virulence determinants enabling spores to survive within phagocytes. Whilst research has been hindered by lack of genetic tractability of Mucorales, a range of tools has become available in recent years. Whole genome projects and comparative genomics have revealed a genome wide duplication and gene family expansions for ergosterol synthesis pathway (e.g. lanosterol 14α-demethylase), GTPases, secreted proteases and cell wall synthesis enzymes that could support resistance to antifungals and adaptation to changing environments [48,53]. In addition, targeted gene attenuation in *Rhizopus* can reliably be achieved using RNAi techniques [47,51,52]. Finally, the community will benefit from a recently published RNAi-based knock out library of *M. circinelloides* enabling screens for genes involved in germination and virulence [54].

Protective immunity could be achieved by correcting immune deficiencies in susceptible patients or inhibition of virulence strategies employed by Mucorales (e.g. neutralization of CotH with antibodies [47]). In the context of mucormycosis, adjuvant cytokine treatments have proven some efficacy. GM-CSF and GM-CSF in combination with IFN-γ increase antifungal activity of PMNs by increasing the oxidative burst *in vitro* [33,34], whilst GM-CSF in combination with liposomal amphotericin B improved the survival of mice with systemic mucormycosis [55]. Recovery of normal blood pH in mice with β-Hydroxy butyrate (BHB) induced acidosis through bicarbonate treatment significantly increased survival of mucormycosis in prophylaxis or therapeutic mouse models [49]. Lastly, isolation and proliferation of T-cells increased phagocytic capacity and reactive oxygen burst in response to mucormycetes *in vitro* and might offer the possibility of adoptive immune cell transfer in the future [42,56]. The timing of any clinical intervention and immunomodulation should be considered carefully in the context of mucormycosis.

Conclusion and Future Research Directions

The rise of the number of susceptible individuals together with current lack of effective treatment requires further research into the host-pathogen interactions during mucormycosis and will enable us to devise new and more effective treatments for this debilitating disease.

Acknowledgments

ASI was supported in part by Public Health Service grant R01 AI063503. KV was supported by a Wellcome Seed Award (108387/Z/15/Z).

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Highlights

- The innate immune system controls mucormycete spores by inhibiting spore germination
- There is limited evidence for adaptive immunity in combating mucormycosis
- Host iron acquisition is the determining factor for progression of mucormycosis on the endothelial interface

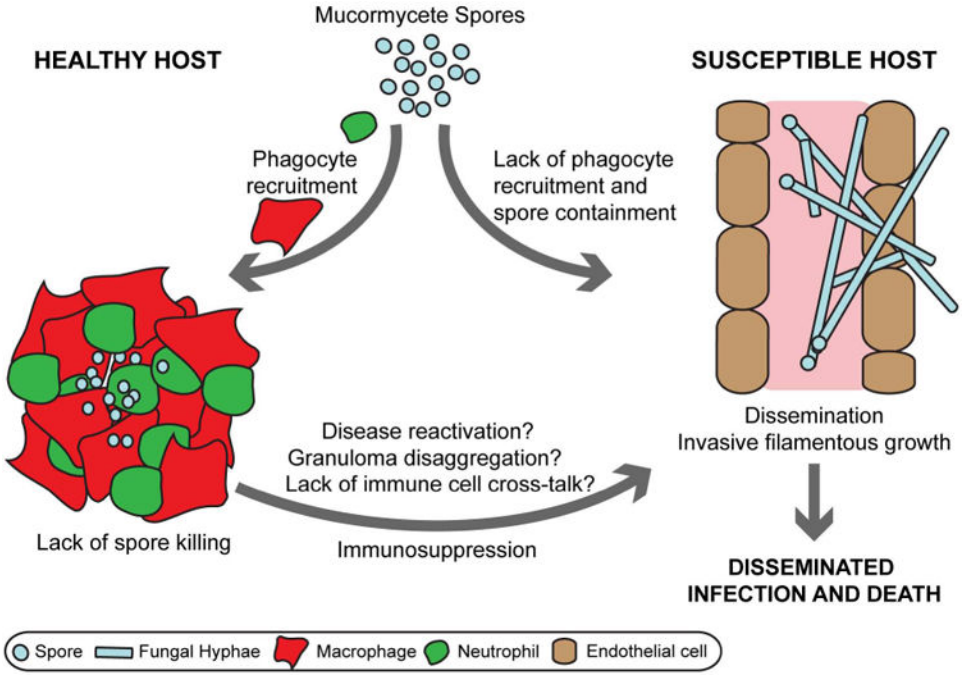

Figure 1. Spore germination and filamentous growth of *Rhizopus microsporus*

Resting spores start to swell shortly after incubation in rich media. First germ tubes are produced after approximately 7 hours incubation with a hyphal network established at 13 hours incubation. Scale bar 50 μm.

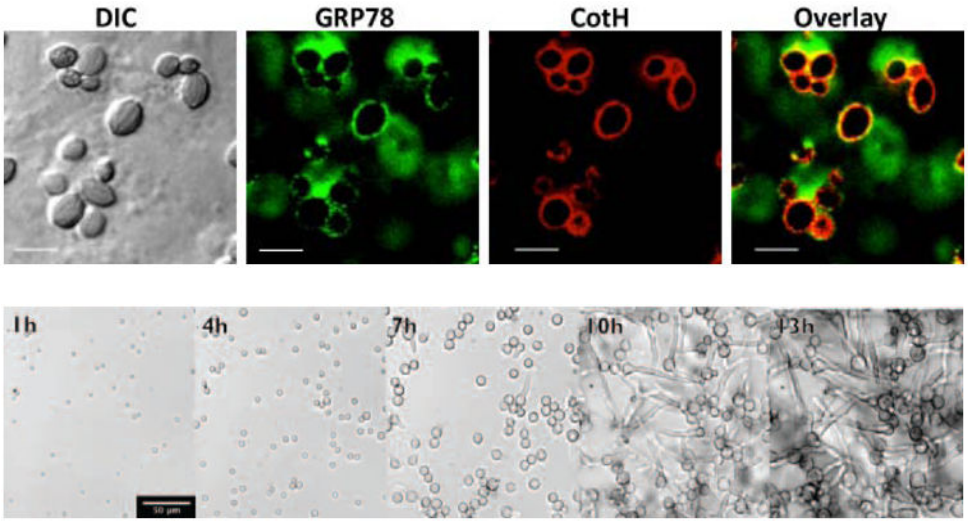

Figure 2. Colocalization of host cell GRP78 and *R. delemar* CotH during invasion of human umbilical vein endothelial cells

GRP78 (green) is labeled with Alexa Fluor 488, CotH (red) is labeled with Alexa Fluor 658. Merged image show colocolization (yellow) of endocytosed fungal swollen spores ~60 min after incubation. Scale bar 10 μm.

Table 1

Proven virulence traits of Mucorales

Virulence trait	Function	References
High affinity iron permease (Ftr1p)	Acquisition of host iron	[51,57]
Ferrioxamine receptors (Fob1 and Fob2)	Acquisition of iron from ferrioxamine	[52]
Fungal Spore coating protein (CotH)	Invasion of the endothelium	[47]
Host Glucose regulated protein 78 (GRP78)	Invasion of the endothelium	[46,49]
Host Platelet-derived growth factor receptor (PDGFR)	Invasion of host cells	[48]
Spore size	Faster germination	[25]
calcineurin pathway	Regulation of hyphal growth	[23,58]
Uncharacterized toxins	Host cell damage and possible induction of inflammatory response	[32,44]