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SENSING THE THREAT POSED BY ASPERGILLUS INFECTION

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Abstract

The mammalian immune system can tune its inflammatory response to the threat level posed by an invading pathogen. It is well established that the host utilizes numerous 'patterns of pathogenicity', such as microbial growth, invasion, and viability, to achieve this tuning during bacterial infections. This review discusses how this notion fits during fungal infection, particularly regarding *Aspergillus fumigatus* infection. Moreover, how the environmental niches filled by *A. fumigatus* may drive the evolution of the fungal traits responsible for inducing the strain-specific inflammatory responses that have been experimentally observed will be discussed. Moving forward understanding the mechanisms of the fungal strain-specific inflammatory response due to the initial interactions with the host innate immune system will be essential for enhancing our therapeutic options for the treatment of invasive fungal infections.

Keywords

invasive aspergillosis; invasive fungal infections; Aspergillus fumigatus; fungal pathogenesis; fungal immunology; Innate immunity; inflammation

INTRODUCTION

It is estimated that approximately 2 million cases of life-threatening invasive fungal infections are reported worldwide each year [1]. While hundreds of species exist within fungal genera, only a handful have been shown to cause invasive mycoses in humans. The rapid rise in cases of life-threatening invasive fungal infections during the second half of the 20th century can be attributed to an increase in immunocompromised patients due to the advent of myeloablative chemotherapy, glucocorticoid usage for organ transplantation, and the AIDS pandemic. Moreover, the number of invasive fungal infection cases is predicted to continue to rise due to the sustained growth of the immunocompromised patient populations

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Declaration of interests

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and the ongoing emergence of drug resistance [1]. However, not all immunocompromised patients develop invasive fungal infections, polymorphisms in numerous innate immune sensing and signaling pathways alter the susceptibility of transplant patients to developing invasive fungal disease (reviewed in [2], [3] and [4]). Additionally, patients with primary immunodeficiencies in the NADPH oxidase complex, STAT3 signaling pathway, CARD9 signaling pathway, leukocyte adhesion deficiencies, and those with severe congenital neutropenia have been shown to be predisposed to developing invasive fungal infections (reviewed in [2] and [5]).

These polymorphisms and primary immunodeficiencies in the Innate immune sensing pathways demonstrate the importance of innate immunity in maintaining resistance against fungal pathogens, including *Aspergillus fumigatus*. Experimental studies using mouse models have demonstrated a number of these innate immune sensing and signaling pathways are essential for coordinating the inflammatory response to *A. fumigatus*. Alveolar macrophages are critical for killing inhaled conidia and contribute to the initial antifungal inflammatory response [6–8], although others have shown them to be dispensable [9]. In zebrafish, tissue macrophages are essential for maintaining resistance against only certain *A. fumigatus* strains, which might explain the discrepancy in the murine studies [10]. Lung-infiltrating $CCR2^+$ inflammatory monocytes are also critical in establishing the inflammatory milieu following *A. fumigatus* challenge [11, 12]. These cells serve as critical sources of IFNα [13] and IL-1α [14], which regulate the recruitment and activation of neutrophils whose timely recruitment are essential for maintaining host resistance [15]. $CCR2^+$ inflammatory monocytes also regulate recruitment of plasmacytoid dendritic cells who maintain resistance against *A. fumigatus* through the enhancement of neutrophil antifungal functions [16]. In addition to orchestrating the inflammatory cytokine response, $CCR2^+$ inflammatory monocytes can differentiate into monocyte-derived dendritic cells which are potent antifungal killers themselves [11]. The interplay of macrophage and neutrophil-dependent inflammatory responses is further highlighted in a zebrafish model where macrophages controls slow growing *A. fumigatus* strains, while neutrophils are critical for controlling rapidly growing strains [10]. Thus, the host can initiate a well-organized innate immune response necessary for the maintenance of host resistance against *A. fumigatus*.

In this review, we discuss the importance of sensing the growth, viability, and danger posed by these fungal pathogens to tune the inflammatory response to the threat posed by a given isolate. We highlight the importance of environmental niche evolution in the ability of *A. fumigatus* isolates to drive stress resistance needed for virulence in a host and how this intersects with the induction of the innate immune response. Overall, the balance between these host-pathogen interactions are essential for driving appropriately tuned immune responses to maintain host resistance against fungal pathogens without too much collateral tissue pathology.

STRAIN VARIABLITY: IMPACTS ON PATHOGENTICITY AND IMMUNITY

A. fumigatus is ubiquitously found throughout the environment, flourishing in a wide variety of environmental niches. Each of these environmental niches present their own unique

microbial and metabolic pressures. However, the most advantageous traits for surviving in these different environmental niches -- temperature tolerance, UV resistance, protease repertoire, hypoxic tolerance, resistance to amoeba predators, toxin production for inter-microbial competition -- may or may not reflect the pressures found within the mammalian lung.

Studies comparing *A. fumigatus* isolates have demonstrated that differences in metabolic and stress adaptation impact fungal pathogenesis, as well as the host inflammatory response. In experimental models of invasive aspergillosis, typically, one of two clinical isolates are commonly utilized: Af293 [17] or CEA10 [18]. Af293 is less virulent than CEA10 in immune competent mice [14, 19], immune competent zebrafish [10], and immunosuppressed mice [20]. Importantly, this phenomenon is not restricted to these two laboratory strains of *A. fumigatus* as a number of clinical and environmental isolates demonstrate a full range of virulence in mouse models [14, 20]. Interestingly, these differences in virulence are not seen in the chemotherapeutic (i.e. neutropenic) murine model of invasive aspergillosis [20]; thus, immunological pressure by neutrophils and metabolic changes within the respiratory environment likely drive the observed virulence differences.

One major pressure found in the lungs is tissue hypoxia which is most prevalent in the immunosuppressed model of invasive aspergillosis [21]. Hypoxic fitness of *A. fumigatus* isolates correlates with virulence in the immunosuppressed model of invasive aspergillosis [20]. Further experimental support for this correlation is provided by the experimental evolution of the Af293 strain, which has a poor fitness in hypoxic environments, resulting in a novel strain that had increased hypoxic fitness and was significantly more virulence than the parental Af293 strain in the immunosuppressed model of invasive aspergillosis [20]. The genetic basis underlying this phenotypic evolution is dependent on the *hrmA* gene [22]. Specifically, the hypoxia evolved variant of *hrmA* results in a transcriptional rewiring of the hypoxic response such that the strain is primed for rapid growth in low oxygen driving increased virulence in the immunosuppressed model of invasive aspergillosis. Moreover, *hrmA* variants had altered biofilm architecture and cell wall composition, which results in more robust inflammation. The more robust inflammation likely drives strain-specific induction of lung hypoxia due to the increased neutrophil accumulation. In addition to fungal adaptation to hypoxia, host hypoxia signaling in myeloid cells, through the transcription factor HIF-1α, is critical for murine survival in CEA10 [19, 23] but not Af293 infections [19]. Thus, not only does hypoxia impact the fungal biology and virulence but, also, alters the host inflammatory response in a strain-specific manner.

A second major pressure found in the lungs is the limited nutrient availability within the airways. A consistent phenotype is that more virulent strains of *A. fumigatus* are more rapidly able to undergo conidial germination both *in vitro* and *in vivo* [10, 14, 24]. The molecular mechanism behind why certain isolates of *A. fumigatus* initiate germination more rapidly within the nutrient limited environment of the airways remains unresolved. However, it is apparent that strains which undergo more rapid conidial germination drive a more robust and broader inflammatory response [14, 19]. Not only is the utilization of nutrients likely critical to initiate fungal germination and growth, but the controlled use of carbon sources

through CreA-mediated carbon catabolite repression is critical within the hypoxic environment of established infection for disease progression. Specifically, a *creA*-null mutant is unable to thrive in the low oxygen environment within the host, due to its inability to shift to a more hypoxic-based glycolytic metabolism [25]. Thus, the ability to utilize the limited nutrients found in the lung environment is critical for the growth throughout *A. fumigatus* infection.

Another major pressure found in the lungs is the host innate immune cells. Typically, *A. fumigatus* spores are rapidly engulfed and killed by host phagocytes through both oxidative and non-oxidative mechanisms [7]. In the natural environment *A. fumigatus* may be preyed upon by amoeba. Some of the interactions between *A. fumigatus* with amoebas resemble those occurring between *A. fumigatus* and phagocytes, which has led to a hypothesis that amoeboid predation may drive the evolution of animal virulence determinants in the fungi [26, 27]. One such virulence determinant could be 1,8-dihydroxynapthalene (DHN) melanin. Spores of *A. fumigatus* lacking DHN melanin are ingested at higher rates by macrophages [28] and the amoeba *Dictyostelium discoideum* [29]. Moreover, DHN melanin inhibits phagolysosome maturation and acidification which significantly impairs conidial killing by macrophages [30, 31]. In addition to DHN melanin, secondary metabolites are key determinants in the interactions between *A. fumigatus* and amoebas. Specific examples include gliotoxin which is important in killing *D. discoideum* [29] or fumagillin which inhibits *Entamoeba histolytica* growth [32], while these secondary metabolites can inhibit NADPH oxidase, innate immunity signaling hubs, or drive epithelial cell damage in mammalian systems [33–36]. In addition to gliotoxin and fumagillin, *A. fumigatus* express a wide array of secondary metabolites that could have critical roles in regulation the host-pathogens and microbe-environment interactions of *A. fumigatus* [37].

Heterogeneity within *A. fumigatus* due to its evolution within its natural environmental niche likely drives the virulence heterogeneity observed in mice. Additionally, nutritional conditions in which spore development occurs appears to be critical in the regulation of the conidial development program, dormancy, and cell wall architecture which could influence germination rates, cell wall carbohydrate exposure and interactions with host phagocytes [38]. Thus, much remains to be understood about the heterogeneity among *A. fumigatus* isolates and how that impacts the host inflammatory response.

PHAGOCYTOSIS AND ANTI-FUNGAL KILLING OF FUNGAL PATHOGENS

A. fumigatus enters the body through the inhalation of airborne resting conidia. Resting conidia of *A. fumigatus* are tightly covered by a hydrophobic layer of rodlet proteins. Rodlets conceal the fungal polysaccharide-rich cell wall which is composed of an outer DHN melanin layer, a core layer composed of β−1,3-linked glucans, mannoproteins, and galactomannan, and an inner layer of chitin (Figure 1a–c) (reviewed by [39]). The rodlets are tightly packed limiting the exposure of the polysaccharide-rich cell wall components limiting activation of the innate immune response [40]. Recently, the CcpA protein was also found in the outer proteinaceous layer of the resting spores. Resting spores lacking CcpA maintained normal cell surface structure of the rodlet layer but had enhanced immunostimulatory potential [41]. The fungal lectin FleA is also found in the outer

proteinaceous layer of the resting spore and is a ligand for fucosylated glycoproteins that are found in lung mucins and is critical for the recognition and uptake of resting conidia by macrophages [42].

Upon fungal spore uptake, the phagosome undergoes a highly regulated process of maturation to mediate antifungal killing (Figure 1d). Upon phagocytosis, conidial germination and stripping of both the rodlet and DHN melanin layers exposes the β−1,3-linked glucans within the cell wall [43]. Exposure of β−1,3-linked glucans results in the engagement of Dectin-1 driving Syk-dependent signaling which is necessary for phagosome maturation [43] and reprogramming macrophage metabolism to glycolysis which is necessary for antifungal immunity [44]. Dectin-1/Syk signaling results in the phosphorylation of the $p47^{phox}$ protein, which drives NADPH oxidase complex formation and activation [43]. Upon activation the NADPH oxidase complex generates reactive oxygen species (ROS) which is not only necessary for antifungal killing within the phagosome, but also enhances the incorporation of LC3 into the phagosomal membrane [43]. LC3-associated phagocytosis is essential for mediating host antifungal immunity [45]. Interestingly, corticosteroid treatment can dampen LC3-associated phagocytosis which could contribute to the immunosuppressive environment in those patients ultimately making them susceptible to invasive aspergillosis [46].

It has been well established that DHN melanin in *A. fumigatus* mediates resistance against phagocytic killing. The role of DHN melanin in protecting the fungal spores is two-pronged: 1) directly protecting the spores from the damaging effects of ROS [47] and 2) impairing the LC3-associated phagosome maturation process [43]. In this latter role DHN melanin appears to sequester phagosomal Ca^{2+} thereby preventing calmodulin-dependent signaling and phagosome maturation [48]. Ca^{2+}-dependent signaling through calcineurin and NFAT downstream of TLR9 activation in the phagosome through a MyD88-independent, but Btk-dependent signaling pathway is critical in antifungal immunity against *A. fumigatus* in mice [49]. The importance of Btk signaling in preventing invasive fungal infections in humans is highlighted by the observation that cancer patients treated with ibrutinib are at increased risk of developing these infections [50–53]. Interestingly, β−1,3-linked glucan polysaccharide activation of Dectin-1/Syk signaling is necessary for the recruitment of TLR9 to phagosomes containing fungal spores and the induction of an interferon (IFN)-dependent transcriptional signature [54]. LC3-associated phagocytosis directly enhances IFNα production downstream of CpG-induced TLR9 activation through the direct interaction of LC3 with IKKα [55]. Both type I and type III IFNs are critical in regulating the protective innate antifungal immune response after *A. fumigatus* challenge [13]. Much more work on the cross-talk between phagosome localized PRRs and LC3-associated phagocytosis is needed to understand the induction of antifungal killing and inflammation in the context of *A. fumigatus* infection, as therapeutic targeting of these pathways can modulate disease outcomes [56, 57].

SENSING OF PATTERNS OF PATHOGENESIS AND VITA-PAMPs ENHANCE THE INFLAMMATORY RESPONSE TO BACTERIAL PATHOGENS

Mammalian hosts must be able to distinguish between commensal and pathogenic interactions given their microbiota. This has led to an explosion of research into how innate immune sensing pathways can distinguish these two outcomes. Vance, Isberg, and Portnoy have proposed a theory based on “patterns of pathogenesis”, which include: 1) microbial growth, 2) cytosolic access, 3) disruption of host cytosolic function [58]. Microbial growth would be the result of the sum of microbial replication and microbial cell death. It has been well established that live microbes induce stronger and more diverse immune responses than dead microbes. This observation led Blander and colleagues to propose the hypothesis that certain PAMPs may acts at markers of vitality (vita-PAMPs). Vita-PAMPs that have been identified during bacterial infections include microbial RNA [59] and cyclic-di-adenosine monophosphate [60], which can drive enhanced type I interferon and IL-1β inflammation leading to more robust protective immunity. Similarly, cytosolic access of microbial products or microbial effectors leads to the activation of numerous different inflammasomes (reviewed by [61]). Some of these microbial effectors are critical for altering cytosolic cytoskeleton proteins critical in cellular function and phagocytosis, which can be sensed by guard proteins like pyrin [62]. Overall, the mammalian innate immune system is well designed to assess the risk of each microbial insult and tune the overall inflammatory response in such a manner to deal with the pathogenic insult.

THE FUNGAL-MENTALS OF PATHOGENESIS FOR PREVENTING INVASIVE FUNGAL INFECTIONS

The patterns of pathogenesis framework is likely critical in understanding how the mammalian innate immune response is tuned to the threat posed by fungal species as well, given the emergence of the importance of the host mycobiome in the intestinal tract [63], as well as the fact that the respiratory tract is continually exposed to ubiquitous environmental fungal spores in the air. The former is critical in regards to *Candida* infections as it has recently been demonstrated by Hohl and colleagues that the bloodstream *Candida* spp. isolate from invasive candidiasis patients who had undergone allogeneic hematopoietic cell transplant originated in the intestinal mycobiome [64]. In agreement with the patterns of pathogenesis hypothesis both the interferon response [4, 13, 65] and inflammasome-dependent IL-1β production [66–71] are critical for maintaining host resistance against fungal pathogens. Thus, we will now highlight the role of fungal patterns of pathogenesis in tuning the inflammatory response to fungal pathogens, particularly focused on *A. fumigatus*.

Sensing of A. fumigatus growth.

One of the key features of *A. fumigatus* growth that can be sensed by the innate immune system is the observed changes in the fungal cell wall (Figure 1e). Sensing of these changes in the *Aspergillus* cell wall have been shown to be critical in regulating the antifungal immune response. For example, exposure of the β−1,3-glucans upon swelling by *A. fumigatus* conidia is recognized by Dectin-1 (*Clec7a*) [72, 73]. Additionally, Dectin-2 (*Clec4n*) can recognize both swollen conidia and germlings of *A. fumigatus* [74, 75]. While

the exact fungal ligand driving Dectin-2 activation by *A. fumigatus* remains unknown; in other fungi, cell wall mannans and high mannose structures have been shown to drive Dectin-2 activation [76, 77]. Both Dectin-1 and Dectin-2 therefore enable the host to sense the early growth of *A. fumigatus* directly upon conidial swelling. The hyphal-specific galactosaminogalactan carbohydrate can also modulate the inflammatory response [78–80], but how galactosaminogalactan interacts with the immune system remains unknown. Moreover, germlings/hyphae from *A. fumigatus* are known to secrete numerous hydrolytic enzymes (proteases and lipases) and secondary metabolites, which likely influence the inflammatory response. Our laboratory has shown that *A. fumigatus* germlings drive much stronger and broader inflammation, in particular driving IL-1α and LTB4 secretion resulting in increased neutrophil recruitment [14, 19]. The exact fungal triggers of this increased inflammatory response are unknown. Thus, the biochemical changes in the cell wall and metabolic status are important rheostats for the inflammatory response against *A. fumigatus*.

Inflammasome sensing and IL-1β secretion.

During fungal infections inflammasome activation and IL-1β secretion are critical for host antifungal immunity. Specific to *A. fumigatus*, both the NLRP3 and AIM2 inflammasomes can be activated [66]. β−1,3-glucans are critical in the priming and activation of the NLRP3 inflammasome [71, 81]. Moreover, ROS and K^+ efflux may contribute to the activation of NLRP3 [68, 71]. The activation of the AIM2 inflammasome is driven by fungal DNA [66]. This brings about an interesting paradox whereby the fungal ligands for both the NLRP3 and AIM2 inflammasomes are typically found within the phagosome, but the receptors are localized to the cytosol of the cell; thus, how do these ligand get transferred into the cytosol for detection by these inflammasome sensors? Recently, Kanneganti and colleagues demonstrated that IRGB10 induces damage to *A. fumigatus* hyphae to enhance the release of β−1,3-glucans from the fungal cell wall driving NLRP3 [81], but the exact mechanism by which this ligand gets transferred to the cytosol remain unresolved.

In other fungal systems it has been shown that phagosome rupture by hyphal growth can play an important role in NLRP3 pyroptosis and IL-1β release [70, 82, 83]. Analogously, challenging bone marrow-derived macrophages with *A. fumigatus* germlings results in greater IL-1α and IL-1β secretion compared to spores and these germlings had to be alive to elicit this response [14]. More recent work using several mutant collections demonstrate that hyphal growth is not absolutely required for NLRP3-depednent pyroptosis and IL-1β secretion [82, 84–87]. One important pathway identified using these mutant collections is the ergosterol biosynthetic enzymes [85, 86]. Moreover, ergosterol-containing liposomes were sufficient for the induction of NLRP3 inflammasome-dependent IL-1β secretion [86]. Our laboratory has also observed NLRP3 inflammasome activation using ergosterol crystals (unpublished observation). Thus, multiple mechanisms appear to exist in which fungal-derived ligands necessary for inflammasome activation can escape the phagosome and drive IL-1β secretion, which was dependent on metabolically active fungi surviving within the phagosome.

Regulation of the interferon response.

Studies in mice have shown that both type I and type III interferons (IFNs) are crucial for maintaining host resistance against *A. fumigatus* [13]. In this model, upon *A. fumigatus* challenge type I IFNs are rapidly produced by $CCR2^+$ monocytes, which promotes type III IFN signaling, expression, and induction of optimal antifungal activity of neutrophils [13]. However, the fungal triggers and host receptors responsible for initiating the IFN response following *A. fumigatus* challenge remains largely unresolved. Our recent work has described an essential role for Mda5 in maintaining host resistance against *A. fumigatus* [88]. Mda5 is a cytosolic receptor for double-stranded RNA and is critical for the induction of antiviral IFN responses [89, 90]. We found that double-stranded RNA from *A. fumigatus* was sufficient for Mda5 activation and that the type I IFN response was only partially dependent on Mda5, while the type III IFN response was entirely dependent on Mda5 [88]. Thus, other innate immune sensing receptors likely participate in inducing the type I IFN response, such as Dectin 1 and TLR9 [54, 91].

Similarly following *Candida albicans* infection IFNAR-depending signaling is essential for host resistance [4, 65, 92]. Following *C. albicans* challenge, both fungal nucleic acids [92, 93] and β−1,3-glucans [65] are involved in the induction of the type I IFN response. Induction of IFN-β by Dectin-1 requires Syk-dependent activation of Irf5 [65], while type I IFN induction by fungal nucleic acids is partially dependent on TLR7 and TLR9 [92, 93]. Additionally, in humans polymorphisms in the *IFIH1* gene are associated with altered risks for developing systemic *Candida* infections [94]. However, *Ifnb* expression in murine bone marrow derived macrophages was largely independent of Mda5 [94]. Thus, both type I and type III interferons are critical in maintaining host resistance against fungal pathogens, but much remains unknown about the initiation of these cytokines during fungal infection.

CONCLUDING REMARKS

As highlighted in this review, *A. fumigatus* induces inflammatory responses in mice that are tightly tuned to the virulence of the infecting fungal isolate. These differences in virulence appear to be due to the ability of an isolate to adapt to the stresses found in the infected lung, such as hypoxia, nutrient availability, and engulfment by host phagocytes. The ability of a strain to adapt or not to the stresses imposed within the mammalian lungs will result in changes in a strains metabolic activity, ability to survive, and ability to grow. These changes in the fungi are interrogated by the host innate immune system to maintain resistance against the fungal pathogen while hopefully minimizing collateral tissue damage. By understanding the regulation of these processes by both the fungi and mammalian immune response we should be able to develop better therapeutic avenues for these difficult to treat invasive fungal pathogens.

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HIGHLIGHTS

- Host-fungal interactions tune the innate inflammatory response to maintain host resistance against distinct fungal isolates
- Fungal growth, viability, phagosome escape, and cell wall changes are critical ‘patterns of pathogenesis’ during fungal infection
- Environmental evolution and growth conditions drive isolate-specific virulence traits

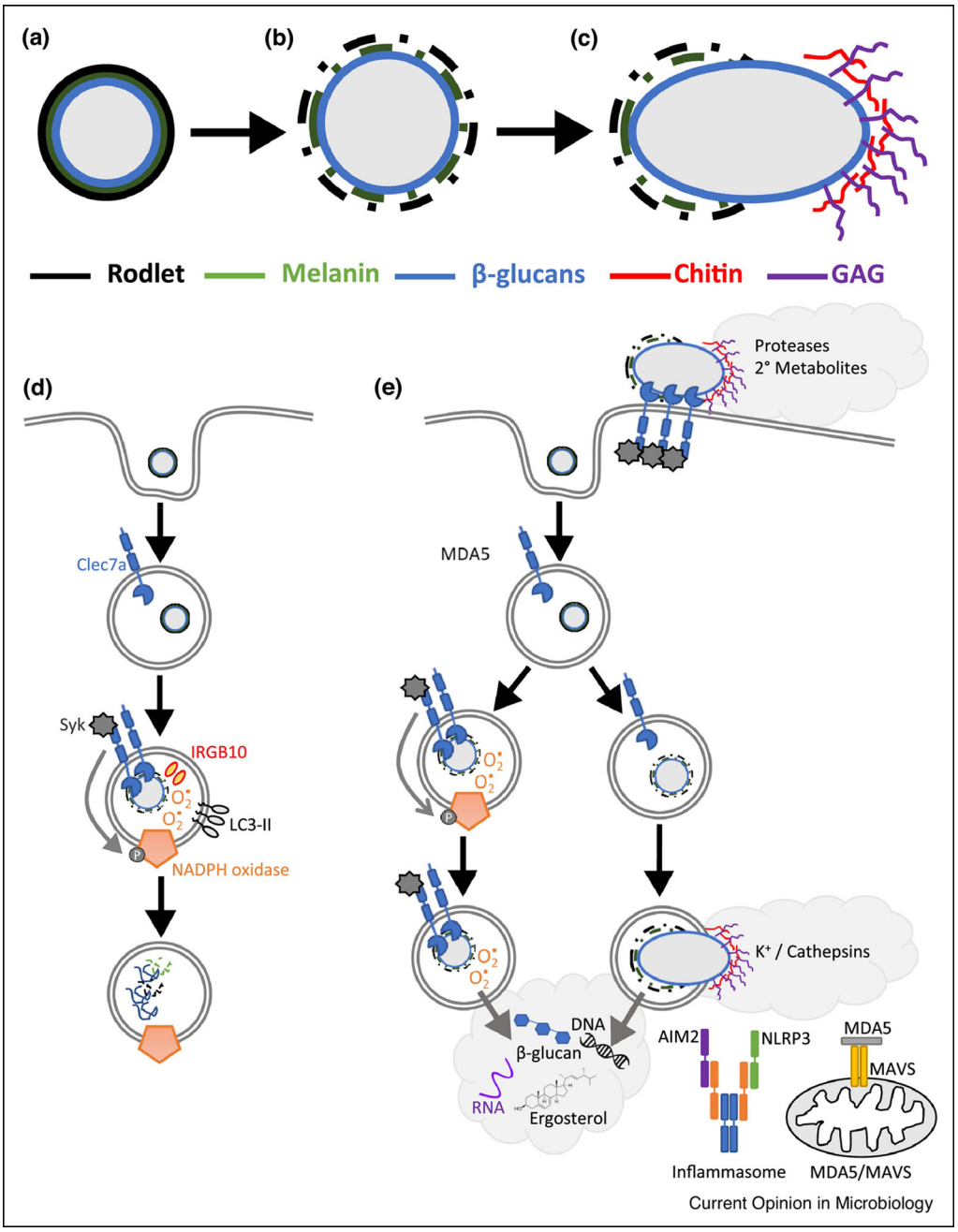

Figure 1. Phagosome maturation and the patterns of pathogenesis for regulating the innate immune response induced against *Aspergillus fumigatus*.

Immune sensing of *A. fumigatus* is highly dependent on the interplay between the changes in the fungal cell wall as the *Aspergillus* grows and the host innate immune cells and its phagosome. (a) Resting conidia of *A. fumigatus* have tightly packed rodlet hydrophobin and melanin layers, which limit the exposure of the core cell wall carbohydrates. (b) Upon osmotic swelling the rodlet hydrophobin and melanin layers are broken down exposing the underlying core carbohydrates, predominately β−1,3-glucans. (c) Upon germ tube emergence further changes occur, particularly the expression and integration of galactosaminogalactan on the outer surface of the cell wall. (d) During phagosome

maturation *A. fumigatus* spores that were engulfed can undergo conidial germination and stripping of both the hydrophobin rodlet and DHN melanin layers resulting in the exposure of the β−1,3-linked glucans that drive Dectin 1 signaling. Activation of Dectin1 results in NADPH oxidase activation and enhanced LC3-associated phagocytosis for the optimal killing of *A. fumigatus*. (e) For sensing the patterns of pathogenesis during fungal infection, several key events can be sensed by the host innate immune cells including cell surface recognition of germlings, phagosome rupture, or the release of fungal components into the cytosol. Release of fungal components, such as fungal DNA, β−1,3-glucans, and ergosterol or fungal RNA, drive the activation of the inflammasome for IL-1β release or Mda5/MAVS-dependent IFN production, respectively.