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The cell biology of the innate immune response to *Aspergillus fumigatus*

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The development of invasive aspergillosis is a feared complication for immunocompromised patients. Despite the use of antifungal agents with excellent bioactivity, the morbidity and mortality rates for invasive aspergillosis remain unacceptably high. Defects within the innate immune response portend the highest risk for patients, but detailed knowledge of molecular pathways in neutrophils and macrophages in response to this fungal pathogen is lacking. Phagocytosis of fungal spores is a key step that places the pathogen into a phagosome, a membrane-delimited compartment that undergoes maturation and ultimately delivers antigenic material to the class II MHC pathway. We review the role of Toll-like receptor 9 (TLR9) in phagosome maturation of *Aspergillus fumigatus*—containing phagosomes. Advanced imaging modalities and the development of fungal-like particles are promising tools that will aid in the dissection of the molecular mechanism to fungal immunity.

Keywords: innate immunity; Dectin-1; Toll-like receptor; phagosome; macrophage; TLR9

Introduction

For breath is life, and if you breathe well you will live long on earth.

—Sanskrit proverb

Exposure to *Aspergillus fumigatus* is a universal phenomenon. In immunocompetent individuals, inhalation of these fungal spores is taken up by neutrophils and macrophages that quickly neutralize any potential invasion or infection.¹ However, through the advances of medical care, a growing number of patients have acquired immunological defects either by cellular deficiencies/dysfunction or administration of therapies that modulates the immune response.² These immunologically “fragile” patients possess a higher risk for development of clinically significant invasive fungal infections, including aspergillosis. Indeed, over 10% of patients who receive allogeneic bone marrow transplantation develop invasive aspergillosis during their clinical course.³ Antimicrobial therapies that have excellent *in vitro* activity against *Aspergillus* species have

been developed and are routinely used for both prophylaxis and treatment in these patients.⁴ Despite their widespread use and the increased awareness of this infectious complication by clinicians, the morbidity and mortality rate for immunocompromised patients with invasive aspergillosis remains unacceptably high. These clinical observations support the following statements:

- Persons with normal immune systems successfully keep *A. fumigatus* from developing invasive infections.
- Innate immunity is critical, as neutropenic patients have the highest risk of invasive aspergillosis.
- Successful recovery from invasive aspergillosis requires antimicrobial therapy coupled with some contribution from the host immune system.

These clinical observations lead to the fundamental question of what components of the immune response are critical to neutralize *A. fumigatus* quickly

and efficiently. Knowledge of the basic pathways of antifungal defense in both normal and immunocompromised animal models will likely elucidate the fundamental pathways necessary to develop novel therapeutics against this invasive fungal pathogen.

Innate immunity to *Aspergillus*

To provide a conceptual framework, the immune system can be divided into two principal branches: innate and adaptive immunity.⁵ The innate immune response includes neutrophils, monocytes/macrophages, and dendritic cells (DCs) whose primary role is to engulf microorganisms before they cause harm.⁶ To detect their presence, these phagocytic cells express both surface-disposed and endosomal Toll-like receptors (TLRs) that activate the cell in the presence of infectious agents. Instead of relying on highly specific signatures from pathogens TLRs sense the presence of common building blocks of microorganisms as triggers.⁷ The outcome of TLR signaling is determined, in part, by cell lineage and the selective use of signaling adaptors.⁸ Both ligand recognition by TLRs and the functional outcome of binding are governed, in part, by the subcellular location of the TLRs.⁹

In addition to TLRs, C-type lectin receptors (CLRs) compose a large family of receptors that bind to carbohydrates.¹⁰ The lectin binding activity of these receptors is mediated by conserved carbohydrate-recognition domains, and members of this family of proteins participate directly in the host defense against fungal infections.¹¹ Dectin-1, a surface type II membrane protein and CLR, is highly expressed on phagocytes.¹² Dectin-1 recognizes the carbohydrate epitope β -1,3-glucan, which constitutes the major cell wall component of multiple pathogenic fungi, including *Candida albicans* and *A. fumigatus*.¹³ Dectin-1 is required for proper modulation of immune responses against fungal pathogens, and patients with mutations in Dectin-1 are at higher risk for invasive fungal infections.^{14,15} The cytoplasmic tail of Dectin-1 contains an immunoreceptor tyrosine-based activation (ITAM)-like motif.¹⁶ Upon ligation of the extracellular domain of Dectin-1 the tyrosine residue within the cytoplasmic ITAM motif is phosphorylated. This phosphorylation event results in recruitment of spleen tyrosine kinase (Syk). Ultimately, this pathway leads to the activation of NF- κ B, production of reactive oxygen species (ROS), and elaboration

of proinflammatory cytokines, including TNF- α and IL-12, which leads to recruitment of additional immune cells to the site of infection.¹⁷ While the kinetics of this response is rapid (minutes to hours), the innate immune system does not provide memory to a given infection. In contrast, adaptive immunity consists of T and B lymphocytes that become activated after initiation of innate immunity and focus on specific peptides derived from pathogen-encoded proteins.¹⁸ In order to extract pathogen-specific information, newly enveloped microorganisms are shuttled into membrane-delimited compartments termed *phagosomes*, delivered through the endocytic pathway by modification of the membrane proteins and intraphagosomal environment (*phagosome maturation*), and targeted to lysosomes for degradation.¹⁹ Class II major histocompatibility complex (MHC) molecules intersect with these pathogen-containing compartments and relevant peptides derived from the pathogen are loaded into the MHC antigen binding clefts for delivery to the cell surface.^{5,20} Much of our understanding of phagosomal biology has come from live cell imaging of professional antigen-presenting cells (APCs).²¹

Novel imaging tools to visualize host-pathogen interactions

Application of live-cell imaging tools to the interface of immunology and infectious disease is in its infancy though preliminary observations challenge our view of long-held beliefs about immune responses to microorganisms. As an example, to control *Mycobacterium tuberculosis*, the host forms granulomas, histologically distinct structures regularly seen in clinical tissue of infected persons. Conventional wisdom suggested that macrophages, T cells, and occasionally DCs form a static barrier that envelops organisms to control their spread. The use of intravital two-photon microscopy in the liver of mice infected with *M. bovis* demonstrated that immune cells showed remarkable dynamic movements to initiate and maintain these granulomas.²² TNF- α -derived signals were required to recruit uninfected macrophages to these structures and preserve the involvement of T cells.

A long-term goal of our work is to understand how initial interactions between pathogens and phagocytic cells influence the degree of success of the host response. In order to understand this first step,

it is important to visualize subcellular interactions of host innate immune cells with pathogenic organisms in order to observe phagocytosis. Successful phagocytosis not only neutralizes the threat from the pathogen, but also generates antigenic material crucial for the ensuing adaptive immune response. The regulation of phagocytosis and phagosome formation/maturation has significant implications in specific immune responses to pathogens.²³ To carry out these experiments, we have assembled both spinning-disk confocal microscopy and optical trapping on the same platform. The power of spinning disk confocal microscopy lies in the ability of the pinhole to exclude out-of-focal-plane fluorescence emission from biological specimens, allowing the high-contrast imaging of an optically sectioned slice. The scanning of confocal excitation light is achieved through two spinning disks in which one contains thousands of pinholes and the other contains an equal number of microlenses to focus the laser beam into the pinholes. This enables fast, multicolor, three-dimensional live cell imaging. In combination with submicron Z-plane scanning of the sample by use of a precise motorized stage, this system permits complete sectioning of the specimen to create a three-dimensional image.^{20,24–27}

While this system generates illustrative images of APCs interacting with pathogens, spinning-disk confocal microscopes lack the capacity to exert control on the precise timing of these interactions. Commonly, investigators rely on serendipitous mixing of immune cells with pathogens to initiate phagocytosis. To overcome these obstacles, we adopted optical trapping to position actively live fungal pathogens at any arbitrary time and at any arbitrary location relative to immune cells, which allows real-time observation of the entire process using spinning disk confocal microscopy housed in an environment to mimic *in vivo* conditions.^{28,29}

TLR9 is recruited to phagosomes containing *A. fumigatus*

The generation of fusion proteins of TLR/CLR appended to fluorescent proteins (including GFP and mCherry) coupled with live cell imaging has provided valuable insight into the dynamic nature of protein redistribution in phagosome formation/maturation.^{30,31} While the majority of the data regarding TLR specificity and function have focused on bacterial and viral-derived ligands, our

understanding of fungal interactions with TLRs is accumulating.¹ Much of our understanding of TLR function is derived from infection experiments in mice lacking specific TLRs.³² TLR2- and TLR4-defective mice show a decreased recruitment of neutrophils and a reduced cytokine response to *A. fumigatus* conidia and hyphae.³³ The complex fungal cell wall component zymosan triggers expression of proinflammatory cytokines via Dectin-1 and crosstalk with TLR2 and TLR4.³⁴ During swelling and germination of *A. fumigatus* conidia, β -1,3 glucans become exposed to the surface and can be targeted by cells of innate immune system expressing TLR2, TLR6, and Dectin-1, including macrophages and DCs.^{35,36}

Comprehensive reviews of TLRs and the role of TLRs in fungal infections have been recently published.^{1,6,8,18,37} We will focus on the role of TLR9 in the host defense of *A. fumigatus*. The subcellular localization of TLR9, which engages and signals to unmethylated CpG DNA, is tightly regulated and receptor activation is a multistep process.³⁸ TLR9 is translated into the endoplasmic reticulum in its mature, full-length form and then passes through the Golgi to the endolysosomal compartment where its ectodomain is proteolytically cleaved to generate a functional receptor.^{39,40} In the endolysosomal compartment, ligand binding to preassembled TLR9 dimers induces a conformational change that allosterically initiates signal transduction.⁴¹ While the truncated form of TLR9 can be found in the endolysosomal compartment of unstimulated cells, TLR9 trafficking has been shown to be a highly regulated, dynamic process.³¹ The extent to which there is dynamic movement of TLR9 between subcompartments and the underlying processes regulating TLR9 trafficking remain poorly understood.

Although the best-known ligand for TLR9 is unmethylated bacterial and viral CpG-rich DNA, TLR9 has also been implicated in antifungal defense.^{42,43} A role for TLR9 in host defense against *A. fumigatus* has been suggested by experiments in murine models of invasive pulmonary aspergillosis and allergic bronchopulmonary aspergillosis, where TLR9 modulates the innate immune response in the lung.⁴⁴ Although most studies have focused on the importance of TLR2 and TLR4 in defense against *A. fumigatus*, a polymorphism study associated increased susceptibility to ABPA with a polymorphism in the TLR9 gene.⁴⁵ Intranasal CpG, a known TLR9

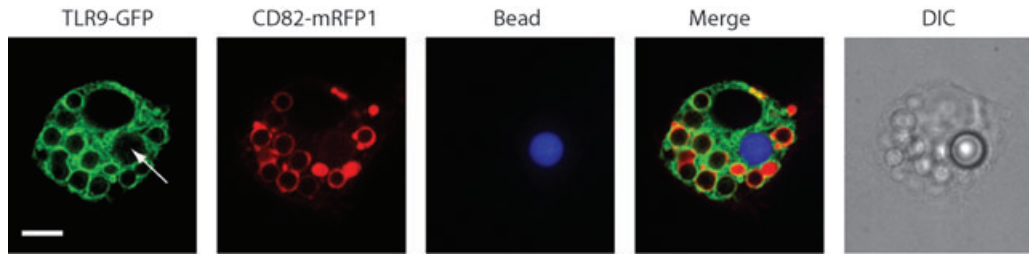


Figure 1. TLR9 is specifically recruited to phagosomes containing *A. fumigatus*. Mouse macrophages expressing TLR9-GFP (green) and CD82-mRFP1 (red) were exposed to both live *A. fumigatus* (Af293) and fluorescent polystyrene beads (blue 5 μ m). Twenty minutes after phagocytosis, macrophages had taken up both particles, but only phagosomes containing *A. fumigatus* recruited both CD82-mRFP1 and TLR9-GFP. Scale bar equals 5 μ m, and arrow denotes internalized polystyrene bead.

ligand, had a therapeutic effect during established murine fungal asthma, implicating a requisite role for TLR9 in this disease process.⁴⁶ However, the cell biological processes underlying TLR9-mediated *A. fumigatus* immune responses are still largely unresolved. TLR9-mediated recognition of *A. fumigatus* DNA by human and murine cells induced proinflammatory cytokines,⁴⁷ but the intracellular processes that enable *A. fumigatus* antigen recognition by TLR9 in professional APCs remain uninvestigated.

To gain insight into the intracellular fate of TLR9 when immune cells are exposed to *A. fumigatus* conidia, we investigated the spatiotemporal regulation of TLR9 compartmentalization after phagocytosis of *A. fumigatus* conidia by murine macrophages. We found that the presence of *A. fumigatus* phagosomes resulted in a dramatic change of the subcellular distribution of TLR9 to a bright, ring-shaped compartment around the *A. fumigatus* conidia (Fig. 1).²⁵ TLR9 recruitment was specifically induced by *A. fumigatus* but not by bead-containing phagosomes, indicating that the composition of the phagocytosed content dictates recruitment of TLR9 to the phagosomal membrane. We demonstrated that *A. fumigatus*-induced TLR9 recruitment was independent of *A. fumigatus* spore germination stage. Expression of TLR2, TLR4, and the TLR signaling adaptors MyD88 and TRIF were not required for successful *A. fumigatus* phagosomal TLR9 recruitment. Further investigation of the requirements for proper intracellular trafficking of TLR9 revealed that the TLR9 N-terminal proteolytic cleavage domain was critical for accumulation of TLR9 in CpG-containing compartments and *A. fumigatus* phagosomal membranes.

In addition to *A. fumigatus*, we have demonstrated that specific triggering of TLR9 recruitment to the macrophage phagosomal membrane is a conserved feature of fungi of distinct phylogenetic origins, including *C. albicans*, *Saccharomyces cerevisiae*, *Malassezia furfur*, and *Cryptococcus neoformans*.²⁷ The capacity to trigger phagosomal TLR9 recruitment was not affected by a loss of fungal viability or cell wall integrity. TLR9 deficiency has been linked to increased resistance to murine candidiasis and to restriction of fungal growth *in vivo*. Macrophages lacking TLR9 demonstrate a comparable capacity for phagocytosis and normal phagosomal maturation compared to wild-type macrophages. We have shown that TLR9 deficiency increases macrophage TNF- α production in response to *C. albicans* and *S. cerevisiae*, independent of yeast viability.²⁷ The increase in TNF- α production was reversible by functional complementation of the TLR9 gene, confirming that TLR9 was responsible for negative modulation of the cytokine response. Consistently, TLR9 deficiency enhanced the macrophage effector response by increasing macrophage nitric oxide production. Moreover, microbicidal activity against *C. albicans* and *S. cerevisiae* was more efficient in TLR9 knockout macrophages than in wild-type macrophages.

In conclusion, our data have demonstrated that TLR9 is compartmentalized selectively to fungal phagosomes and negatively modulates macrophage antifungal effector functions. Our data support a model in which orchestration of antifungal innate immunity involves a complex interplay of fungal ligand combinations, host cell machinery rearrangements, and TLR cooperation and antagonism.

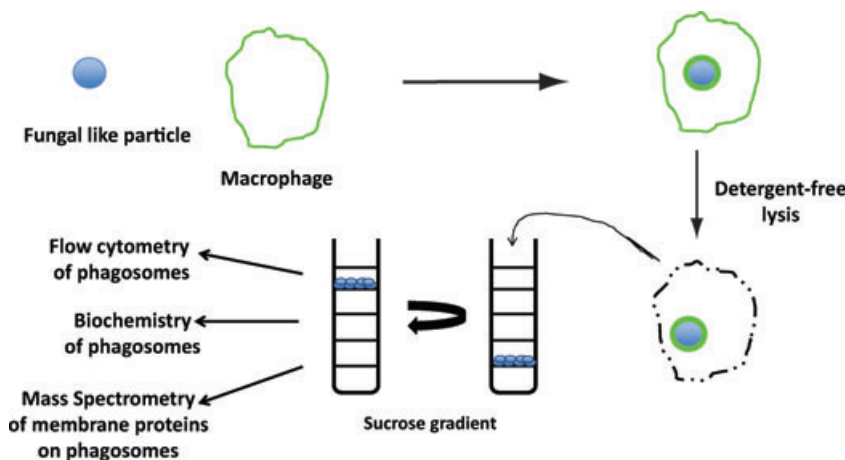


Figure 2. Cartoon depicting method to isolate and analyze phagosomes containing fungal-like particles. Polystyrene beads with fungal-derived carbohydrates covalently attached to the surface are incubated with macrophages. After a defined period, detergent-free lysis releases cellular content including intact phagosomes. Lysates are added to discontinuous sucrose gradients and then are subjected to ultracentrifugation. Phagosomes are easily isolated as a result of the buoyant properties of polystyrene beads and then analyzed by flow cytometry, Western blot, or mass spectrometry of membrane proteins.

Reducing the complexity: development and use of fungal-like particles

The mammalian immune response to invasive fungal pathogens, including *A. fumigatus*, is a complex and dynamic process that serves to protect the host from invasive infection. Indeed, the generation of phagosomes and maturation of these organelles in part dictates the ensuing immune response to the pathogen. Our ability to understand the rules that govern this response is limited by two principal factors. First, the cell wall of *A. fumigatus* is a dynamic structure that undergoes both constitutional and morphologic changes, triggering additional responses from the APCs. *A. fumigatus* can swell inside the phagosome and reveal new antigenic determinants that are effectively shielded during the engulfment phase. Second, the resulting immune response from a complex particle such as *A. fumigatus* is difficult to dissect and to attribute specific responses to discrete cell wall components.

To overcome these obstacles, we set out to develop defined synthetic particles that displayed a single or fixed combination of fungal cell wall constituents on a size-matched polystyrene sphere.⁴⁸ In this way, we preserve the geometry of the native pathogen, an important feature to phagocytosis.⁴⁹ Synthetic fungal-like particles have multiple advantages as a substrate to probe the immune system. First, these particles do not vary during the phago-

somal maturation process. Second, the inflammatory response triggered by these particles can now be attributed to specific fungal ligands. Third, intrinsic properties of polystyrene beads permit the rapid recovery of these particles from APCs permitting direct interrogation of these phagosomes to probe the mammalian contribution of membrane proteins.⁵⁰ Polystyrene beads have a low buoyant density such that they float in concentrated sucrose solutions, allowing for the efficient separation of phagosomes from other cellular structures after cell lysis. Analysis of these phagosomes can be done by the use of flow cytometry (PhagoFACS),⁵¹ Western blot analysis to determine the presence and relative quantity of a specific protein on the phagosome, and proteomic-based analysis to determine the composition of the mammalian phagosome generated by a synthetic ligand (Fig. 2).

As proof of principle, we have generated fungal-like particles that display β -1,3 glucan on the cell surface.⁴⁸ These fungal-like particles were characterized using differential interference contrast microscopy, immunofluorescence, and transmission-electron microscopy. The covalent interaction of the β -1,3-glucan layer to the surface of the bead was confirmed by a series of increasingly stringent detergent treatments. Purity of the β -1,3-glucan layer was also determined by incubating the beads with laminarinase, a specific β -1,3-gluconase.

By stimulating bone marrow–derived macrophages with conjugated β -1,3-glucan beads, we observed a dose-dependent response of TNF- α produced when compared to soluble β -1,3-glucan, uncoated beads, and soluble β -1,3-glucan mixed with uncoated beads. Finally, β -glucan coated fungal-like particles triggered dynamic and specific recruitment of GFP-Dectin-1 to nascent phagosomes in living mouse macrophages, demonstrating that the beads retained the desired biological properties.⁴⁸

By providing a platform to probe directly immune responses to β -1,3-glucan, we can dissect the critical steps in the early recognition of pathogen-derived fungal carbohydrate antigens in innate immunity. Additionally, this system permits the addition of other relevant pathogen-derived carbohydrates to polymeric beads to analyze the specific contribution of these ligands in the immune response.

Concluding remarks

Three clinical outcomes from inhalation of *A. fumigatus* exist: rapid removal of conidia without overt clinical symptoms (neutralization), exuberant Th2-biased responses characterized as allergic responses (allergic), or local tissue destruction that can lead to systemic infection (invasion). The outcome of this host–pathogen interaction appears to be dictated largely by host factors. Our understanding of the rules that govern the innate immune response to *A. fumigatus* conidia is relatively simple at this time, but this area is critical to the elucidation of the critical immune pathways that require modulation in order to add to our armamentarium against this clinically relevant pathogen. Novel tools utilizing subcellular imaging and synthetic fungal-like particles are critical developments to elucidate the molecular basis of *A. fumigatus*–host cell interactions.

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Conflicts of interest

The authors declare no conflicts of interest.

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