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Melanin targets LC3-associated phagocytosis (LAP): A novel pathogenetic mechanism in fungal disease

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ABSTRACT

Intracellular swelling of conidia of the major human airborne fungal pathogen *Aspergillus fumigatus* results in surface exposure of immunostimulatory pathogen-associated molecular patterns (PAMPs) and triggers activation of a specialized autophagy pathway called LC3-associated phagocytosis (LAP) to promote fungal killing. We have recently discovered that, apart from PAMPs exposure, cell wall melanin removal during germination of *A. fumigatus* is a prerequisite for activation of LAP. Importantly, melanin promotes fungal pathogenicity via targeting LAP, as a melanin-deficient *A. fumigatus* mutant restores its virulence upon conditional inactivation of *Atg5* in hematopoietic cells of mice. Mechanistically, fungal cell wall melanin selectively excludes the CYBA/p22phox subunit of NADPH oxidase from the phagosome to inhibit LAP, without interfering with signaling regulating cytokine responses. Notably, inhibition of LAP is a general property of melanin pigments, a finding with broad physiological implications.

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Melanins are ubiquitous pigments with a broad range of actions and uncharacterized molecular targets. In airborne fungi, dihydroxynaphthalene (DHN) cell wall melanin is a virulence factor that prevents killing of inhaled conidia (spores) by professional phagocytes. As a proof of this concept, pigmentless mutants of the major human fungal pathogen *Aspergillus fumigatus* that are defective in polyketide synthase responsible for the initial step in DHN melanin biosynthesis are avirulent in mice. There is evidence that DHN melanin promotes pathogenicity via a dual mode of action (i) by shielding pathogen-associated molecular patterns (PAMPs) from sensing by pattern recognition receptors and (ii) by conferring cell wall rigidity and physical resistance to degradation by the effector mechanisms of phagocytes. In addition, although *A. fumigatus* DHN melanin is immunologically inert in terms of direct modulation of pro-inflammatory cytokine responses, previous studies demonstrated that this molecule interferes with fungal phagosome biogenesis via uncharacterized mechanisms and molecular site(s) of action.

Recently, a noncanonical autophagy pathway called LC3-associated phagocytosis (LAP) was identified as the molecular link between activation of certain pattern recognition receptors and phagosome maturation. Importantly, activation of LAP promotes killing of extracellular pathogens and also regulates a broad range of physiological functions including, antigen presentation, apoptotic cell clearance, type I IFN signaling in plasmacytoid dendritic cells, and recycling of photoreceptor outer segment by retinal pigment epithelium. LAP is a noncanonical pathway distinct from classical macroautophagy, in many aspects including (i) the absence of double-membrane formation, (ii) the participation of certain autophagy proteins such as BECN1, ATG5, ATG7 and ATG3 in the absence of the pre-initiation complex and other autophagy protein members, (iii) the involvement of unique molecules such as RUBCN/Rubicon that is dispensable for macroautophagy, and (iv) distinct signaling requirements. Of interest, RUBCN is a master regulator of LAP that confers (i) stabilization of CYBB/NOX-2 for subsequent ROS production and (ii) sustained PtdIns3P phagosomal localization, 2 fundamental signaling requirements for conjugation of lipidated LC3-II to the phagosome membrane.

Importantly, LAP is activated by certain pathogens including *A. fumigatus*. Intriguingly, dormant conidia of *A. fumigatus* are immunologically inert because of the concealing of fungal PAMPs by a surface layer of hydrophobic rodlet proteins and melanin. In line with this concept, we have previously reported that activation of LAP by *A. fumigatus* occurs only upon intracellular swelling of the conidial cell wall that leads to β-glucan surface exposure and subsequent activation of CLEC7A/Dectin-1-SRC-SYK kinase-NADPH oxidase signaling and ROS production.

Although concealing of pro-inflammatory PAMPs is a prevailing mechanism to avoid immune activation in fungi, we reasoned that surface molecules that are removed during conidial cell wall remodeling (swelling) could also directly inhibit

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LAP. In order to dissect the complexity of surface cell wall rearrangements during conidial swelling we followed a genetic approach of sequential removal of surface cell wall layers in dormant conidia of *A. fumigatus*, namely rodlet hydrophobin protein layer (rodA) and melanin, and assessed activation of LAP. Surprisingly, we noticed that while removal of rodA results in surface PAMP exposure and triggers robust pro-inflammatory cytokine release, it fails to activate LAP. In contrast, cell wall melanin removal is a prerequisite to enable β-glucan-dependent activation of LAP.

Mechanistically, fungal cell wall melanin does not interfere with SYK kinase signaling regulating pro-inflammatory cytokine production but displays a selective mechanism of inhibition of LAP via blocking NADPH oxidase-dependent ROS production. Although melanin possesses well-characterized ROS scavenging properties, its inhibitory effect on LAP occurs through active inhibition of NADPH oxidase assembly via the exclusion of the CYBA subunit from the phagosome membrane.

Physiologically, melanin-induced LAP blockade promotes fungal virulence, as the attenuated pathogenicity of the pigmentless *A. fumigatus* mutant is reversed upon conditional inactivation of *Atg5* in hematopoietic cells in vivo and in macrophages ex vivo. In addition, ATG5-dependent LAP is important for immunity against wild-type *A. fumigatus*. These findings lead to a new pathogenesis model by demonstrating that 2 fundamental events that occur concomitantly during the intracellular lifecycle of airborne fungi, PAMP exposure and melanin removal, are required for LAP-dependent phagosome maturation and killing.

Intriguingly, we found that the ability of melanin to block LAP is not restricted to fungal cell wall melanin but is a general property of other melanin pigments tested. Of interest, melanin does not inhibit macroautophagy induced by rapamycin (unpublished data) but rather specifically inhibits NADPH oxidase-dependent activation of LAP. In view of the major differences in chemical composition and structure of various melanins it remains unclear how these molecules target LAP. Although we cannot formally rule it out, interaction with a common inhibitory receptor seems unlikely as a mechanism of inhibition of LAP by different forms of melanin. Instead, common physical and structural properties, such as electrostatic interactions, may underlie the inhibitory action of melanin on CYBA phagosome localization and NADPH oxidase assembly. In addition, it remains uncertain whether melanin has additional molecular targets upstream or downstream of NADPH oxidase. In view of the constant interaction of dormant conidia of airborne fungi with innate immune cells, dissecting the precise mechanism of CYBA exclusion by melanin is of major importance for the design of novel therapeutic strategies for prevention and treatment of invasive fungal diseases.

Given the physiological role of LAP in diverse immunological and cellular processes it is conceivable that melanin-induced LAP blockade could be an important mechanism implicated in diseases beyond the fungal pathogenesis field. Of interest, human melanin present in human tissues does not have a clearly defined role other than protection from UV irradiation. Recently, the role of LAP in recycling retinal epithelial receptors was identified. Because melanin is highly enriched in the retina it will be important to assess whether retinal melanin interferes with LAP. Moreover, melanin-induced LAP blockade could be relevant in physiological responses of human epithelia, and deregulated melanin production could be related to LAP-dependent disease development. Finally, developing targeted therapeutic strategies based on the ability of melanin to inhibit LAP-dependent inflammation could be a promising research direction.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.