Trends in Microbiology

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Review

Convergent evolution of innate immune-modulating effectors in invasive fungal pathogens

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Invasive fungal infections pose a major threat to human health. Bacterial and protozoan pathogens secrete protein effectors that overcome innate immune barriers to promote microbial colonization, yet few such molecules have been identified in human fungal pathogens. Recent studies have begun to reveal these long-sought effectors and have illuminated how they subvert key cellular pathways, including apoptosis, myeloid cell polarization, Toll-like receptor signaling, and phagosome action. Thus, despite lacking the specialized secretion systems of bacteria and parasites, it is increasingly clear that fungi independently evolved effectors targeting pathways often subverted by other classes of pathogens. These findings demonstrate the remarkable power of convergent evolution to enable diverse microbes to infect humans while also setting the stage for detailed dissection of fungal disease mechanisms.

Highlights

Invasive fungal infections are a growing threat to human health.

Secreted effector proteins are a ubiquitous strategy used by diverse microbial pathogens to manipulate host biology and enable pathogen replication.

Recent work has revealed secreted protein effectors produced by human fungal pathogens.

Secreted fungal effectors target apoptosis, myeloid cell polarization, Toll-like receptor signaling, and phagosome action.

Despite lacking specialized secretion systems, fungi have evolved secreted effectors that subvert many host pathways targeted by highly host-adapted bacteria and parasites.

Secreted effectors overcome host barriers to microbial colonization

Invasive fungal infections cause 1.5 million deaths each year, with effective treatment challenged by poor diagnostics and limited therapeutic options [1,2]. Such infections are an escalating biomedical concern as susceptible immunocompromised patient populations grow, drug-resistant isolates emerge, and environmental fungi adapt to a warming global climate that selects for tolerance to mammalian body temperature [1,3,4]. Improving therapies requires a molecular understanding of fungal pathogenesis, yet human fungal pathogens remain severely understudied relative to bacteria and viruses.

Evading or suppressing host immunity is critical for pathogenicity, and pathogens often accomplish these goals using secreted **effector proteins** (see Glossary) that neutralize host blocks to infection. Bacterial effectors are often injected into the host cytosol via molecular needles corresponding to one of several specialized secretion systems (e.g., types III, IV, and VI secretion systems) [5]. Among numerous functions, effectors can modulate host membrane trafficking to construct an intraphagosomal niche (e.g., by preventing fusion with the lysosome) and co-opt host ubiquitin signaling to promote bacterial survival (e.g., by suppressing antibacterial immune defenses) [6–8]. In the Apicomplexa, a phylum of intracellular parasitic protozoa that includes *Toxoplasma gondii* and the malaria parasites *Plasmodium* spp., protein effectors traffic through the endomembrane system and are housed within secretory organelles that release them at precise timepoints during infection [9,10]. *T. gondii* effector functions include arrest of the host cell cycle and suppression of antimicrobial pathways such as interferon-γ [10], whereas most known *Plasmodium* effectors remodel the host erythrocyte, such as by increasing cell rigidity to promote adherence to the microvasculature and avoid splenic clearance [9]. Plant fungal pathogens secrete effectors that are taken up by host cells through poorly understood mechanisms, and these proteins have critical host-modulatory functions that include degradation of the plant cell wall,

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manipulation of immune-activating phytohormones, and sequestration of the **pathogen-associated molecular pattern** (**PAMP**) chitin [11–14].

In human fungal pathogens, secreted polysaccharides from *Cryptococcus neoformans* and *Aspergillus fumigatus* have well-established, albeit mechanistically poorly understood, immunomodulatory functions [15]. The secreted dihydroxynaphthalene melanin (DHN-melanin) pigment from *A. fumigatus* also plays important roles in pathogenesis [16]. In contrast to bacteria and parasites, few protein effectors with clear host-acting, immunomodulatory functions are known, despite the identification of several secreted fungal proteins with important roles in pathogenesis [17]. Now, due to advances in fungal genetics, genomics, and proteomics, novel immunomodulatory effector proteins have recently been described in four different human fungal pathogens [18–21]. Here, we highlight the methodologies that led to the discovery of these effectors, the key experiments elucidating their functions, and the striking convergence of fungal effector functions with those of bacterial and protozoal effectors.

Induction of macrophage apoptosis by *Histoplasma capsulatum* Cbp1

H. capsulatum is a thermally **dimorphic fungus** that causes respiratory disease in immunocompetent individuals and at least 100 000 annual cases of life-threatening disseminated disease in immunocompromised patients [2]. HIV/AIDS-associated histoplasmosis is a neglected disease that frequently goes undiagnosed or misdiagnosed as tuberculosis, resulting in treatment delays and mortality rates ranging from 10% to 60% [22,23]. Human infection with *H. capsulatum* begins following inhalation of environmental **spores** that undergo a programmed developmental switch to a pathogenic **yeast** form upon exposure to mammalian body temperature [24]. Alveolar macrophages engulf *Histoplasma* Yeast, which proliferate intracellularly within phagosomes and induce cell lysis to disseminate to new host cells [25]. The molecular mechanisms by which *H. capsulatum* modifies the macrophage phagosome, evades host immunity, and controls host cell death remain poorly defined.

Recent work has established that macrophage lysis is driven by an effector protein, calcium binding protein 1 (Cbp1), that is an abundant yeast-specific secreted protein required for pulmonary colonization [18,26–31]. Cbp1 is dispensable for proliferation within macrophages but is required for *H. capsulatum*-driven macrophage lysis [31]. Notably, *cbp1Δ* yeast eventually exceed the intracellular burden at which wild-type yeast induce lysis; thus, lysis is an active Cbp1-driven process rather than a mere consequence of fungal burden [31]. Consistently, a Cbp1 ortholog from the related macrophage-lytic fungus *Paracoccidioides americana* complements the macrophage lysis defect of *cbp1Δ H. capsulatum* [32], whereas Cbp1 orthologs from related nonlytic fungi fail to complement [18].

Cbp1-dependent lysis occurs via apoptosis. Lysis is preceded by multiple hallmarks of apoptosis, including reduced levels of the negative apoptotic regulator phospho-Akt and induction of initiator caspase-8 and executioner caspases-3 and -7 [31,32]. Macrophages lacking Bax and Bak, which oligomerize in the mitochondrial outer membrane to induce its permeabilization and initiate the intrinsic apoptosis pathway, are partially protected from *H. capsulatum*-mediated lysis [31]. In contrast, macrophages lacking essential components of the necroptosis and pyroptosis pathways are not protected from lysis [31]. Transcriptional profiling revealed that macrophages infected with *H. capsulatum* exhibit Cbp1-dependent transcriptional stress signatures [31]. This stress response was not due to induction of the host unfolded protein response (UPR) [32], which is frequently induced by intracellular pathogens [33]. Instead, *H. capsulatum* infection induces eIF2α phosphorylation, upregulation of the stress-responsive transcription factor ATF4, and induction of the ATF4 downstream targets *CHOP* and the *TRIB3* [32], all of which are

Glossary

Commensal: a microbe that lives in association with a host without causing significant harm or benefit.
Conidia: nonmotile, asexual spores produced by some fungi.
Dimorphic fungus: a fungus that can exist as either a filamentous mold or a single-celled yeast depending on environmental conditions.
Effector protein: a protein secreted by a pathogen or commensal to manipulate host functions.
Exaptation: an organismal characteristic with a use that either (i) is different from that which was originally shaped by natural selection or (ii) natural selection had not previously acted upon.
Hypha: a multicellular fungal morphotype characterized by long, branching filaments that grow from their tips.
Pathogen-associated molecular patterns (PAMPs): conserved microbial molecular structures recognized by host pattern-recognition receptors to initiate innate immune responses.
Phosphoinositide: a phosphatidylinositol lipid phosphorylated at one or more of three head group positions to regulate membrane trafficking, signaling, and other cellular processes.
Rab GTPases: a family of small GTP-binding proteins that regulate intracellular membrane trafficking.
Saprophyte: an environmental organism that obtains nutrients by decomposing dead organic matter.
Spore: a single-celled, sexually or asexually produced fungal reproductive structure that is released into the environment and germinates upon exposure to suitable conditions.
Type 1 immune response: an immune polarization state characterized by activation of T-helper 1 (Th1) cells and production of proinflammatory cytokines, typically involved in defense against intracellular pathogens.
Type 2 immune response: an immune polarization state characterized by activation of T-helper 2 (Th2) cells and production of anti-inflammatory cytokines, typically associated with allergic immunity and defense against helminth parasites.
Yeast: a single-celled fungal morphotype that reproduces asexually by budding or fission.

hallmarks of the integrated stress response (ISR). The ISR is activated by diverse cellular stresses, including amino acid starvation, proteostasis defects, viral infection, and oxidative stress, and induces apoptosis in situations where cells are unable to remediate those stresses [34]. Supporting a model wherein *H. capsulatum*-driven apoptosis occurs via ISR induction, macrophages lacking the ISR components *CHOP* or *TRIB3* display reduced caspase-3/7 activation and reduced lysis during *H. capsulatum* infection *in vitro* [32]. *CHOP*-deficient mice are less susceptible to *H. capsulatum* infection, displaying reduced lung and spleen yeast burden and improved survival compared to wild-type mice [32], suggesting that subversion of this pathway influences pathogen fitness *in vivo*.

How Cpb1 triggers the ISR to promote host cell lysis is unclear. Cbp1 fractionates with cytosolic markers and localizes to discrete puncta throughout the macrophage [18], raising the possibility that it might directly modulate cytosolic factors to induce the ISR and drive apoptosis. Affinity purification-mass spectrometry of Cpb1 identified an additional yeast-phase specific protein, Yps-3, as an interacting partner, and co-elution of Yps-3 and Cbp1 during size exclusion chromatography suggests that the two form an effector complex during infection [18]. While the function of Yps-3 is unknown, *yps-3Δ* yeast induced delayed macrophage lysis *in vitro* and reduced virulence *in vivo* [18], indicating that it is important for Cpb1-driven lysis.

While the precise mechanism by which Cbp1 triggers ISR activation remains to be elucidated, these data indicate that *H. capsulatum* deploys a secreted effector to the host cell cytosol to activate a stress response pathway and trigger apoptosis (Figure 1). Host cell death pathways

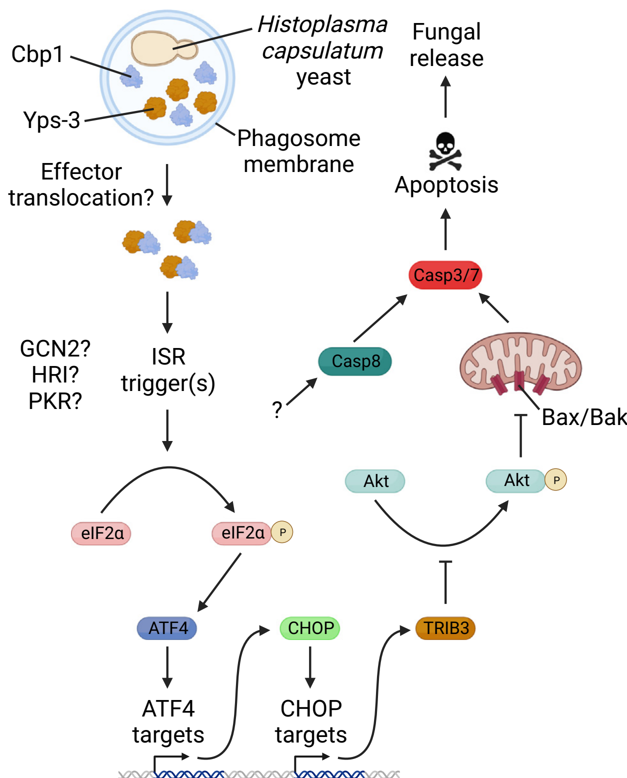

Figure 1. Model for induction of apoptosis by *Histoplasma capsulatum* Cbp1. Phagosomal *H. capsulatum* translocates Cbp1 and Yps-3 protein effectors into the host cytosol through unknown mechanisms. These proteins form an effector complex that induces one or more integrated stress response (ISR) triggers to initiate eIF2α phosphorylation which induces translation of the mRNA encoding the transcription factor ATF4. ATF4 drives expression of the transcription factor CHOP, which drives expression of the pseudokinase TRIB3. TRIB3 inhibits phosphorylation of Akt, thereby allowing oligomerization of Bax and Bak in the mitochondrial outer membrane to induce mitochondrial permeabilization and activation of the intrinsic apoptosis pathway. Additionally, through unknown mechanisms, Cbp1 also appears to induce apoptosis through caspase-8 activation. Abbreviations: GCN, general control nonderepressible; HRI, heme-regulated inhibitor; PKR, protein kinase RNA-activated.

critically influence infection outcomes and are targets of numerous bacterial effector proteins that either induce or inhibit them depending on the specific needs of the pathogen [35,36]. The effectors Map and EspF from enteropathogenic *Escherichia coli* (EPEC) and related pathogens, for example, directly interact with host mitochondria to disrupt mitochondrial membrane potential and induce apoptosis [37–40]. Multiple bacterial effectors induce the UPR during infection, and a subset of these influence cell death [33]. The *Brucella abortus* effector VceC, for example, interacts with the endoplasmic reticulum (ER) chaperone BiP to induce ER stress, which, in turn, promotes *CHOP*-dependent placental trophoblast cell death and abortion in a pregnant mouse infection model [41,42]. Induction of host cell death is thus a conserved paradigm among pathogens, and *H. capsulatum* has evidently evolved a secreted protein to target this pathway through a novel mechanism.

Modulation of macrophage polarization by *Cryptococcus neoformans* Cpl1

The opportunistic pathogen *C. neoformans* is the most common cause of fungal meningitis and is responsible for 112 000 annual deaths, representing nearly 20% of HIV/AIDS-related mortality worldwide [43]. This yeast is globally distributed in the environment, and human infection begins with inhalation of spores or desiccated yeast that initially colonize the lungs [44]. Immunocompetent individuals readily control infection, but under conditions of immune compromise yeast can disseminate to the central nervous system and cause meningitis that is uniformly fatal if untreated. One striking feature of cryptococcal infection is induction of a **type 2 immune response** characterized by pulmonary eosinophilia, alternatively activated (M2) macrophages, and production of the cytokines IL-4, IL-5, and IL-13 [45–49]. *In vivo*, **type 1 immune responses** are critical for control of cryptococcal infection whereas type 2 responses promote fungal survival [50], indicating that modulating this immune axis is an important virulence attribute. *C. neoformans* is a facultative intracellular pathogen that can replicate within acidified macrophage phagolysosomes [51,52], and *in vitro* studies indicate that M2-polarized macrophages are permissive for cryptococcal growth [53–56], providing one possible mechanism underlying the benefits that type 2 immune responses confer to fungal growth. Host recognition of fungal chitin is one determinant of type 2 immune response induction [49], yet other fungal components that influence phagocyte polarization are unknown.

To address this question, our group sought to identify the cryptococcal determinants of type 2 immune response induction in macrophages. Stimulating murine bone marrow-derived macrophages (BMDMs) with *C. neoformans* induced expression of the M2 macrophage marker arginase-1 [19]. This response occurred even when yeast were separated from macrophages by transwell inserts, implicating a soluble factor [19]. A screen of 4401 *C. neoformans* deletion strains constructed in our laboratory found that the secreted protein Cpl1 is necessary for arginase-1 induction in BMDMs. Recombinant Cpl1 produced in the yeast *Pichia pastoris* was sufficient to induce arginase-1, and costimulation with Cpl1 and IL-4 further boosted induction, indicating a potential role for Cpl1 in amplifying M2 signals initiated through the IL-4 signaling pathway.

M2 gene expression programs are induced by the transcription factors STAT3 and/or STAT6 when cytokines, including IL-10 and IL-4, among others, engage their cognate receptors and activate Janus kinases (JAKs) that phosphorylate the receptors' cytosolic domains. This results in recruitment, phosphorylation, dimerization, and nuclear translocation of STAT proteins to promote transcription of target genes [57,58]. Concordantly, recombinant Cpl1 induced STAT3 phosphorylation and enhanced IL-4-driven STAT6 phosphorylation in BMDMs [19]. These data are reminiscent of an arginase-1 induction mechanism in *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), which produces yet-unidentified microbial products that stimulate Toll-like receptors (TLRs) 2 and 4 to induce secretion of IL-6, IL-10, and G-CSF, which in turn activate STAT3 via autocrine/paracrine signaling [59,60]. Concordantly, induction of arginase-1 by Cpl1

similarly required TLR4 (but not TLR2) and its downstream adaptor Myd88 [19]. While TLR4 canonically serves as a receptor for bacterial lipopolysaccharide (LPS) to induce inflammatory responses, the allergens ovalbumin and house dust mite antigen drive allergic inflammation through TLR4 via small amounts of contaminating LPS [61–63]. Several experiments, including the identification of a single amino acid substitution in Cpl1 that abrogates its activity, ruled out LPS contamination as the source of TLR4-dependent arginase-1 induction [19]. These data support a model (Figure 2) in which Cpl1 signals through TLR4–Myd88 on macrophages to induce expression of STAT3-activating cytokines. STAT3 upregulates IL-4Rα, which may contribute to potentiation of arginase-1 induction through the IL-4-STAT6 pathway by virtue of increased receptor availability. However, STAT3 also induces IL-4Rα-independent arginase-1 expression *in vitro*, as STAT3 is necessary for Cpl1-induced arginase-1 in BMDMs whereas IL-4Rα is not [19].

This pathway influenced pathogenicity *in vivo*, as intranasally administered *C. neoformans* induced arginase-1 expression in pulmonary interstitial macrophages in a Cpl1-dependent manner [19]. Cryptococci preferentially associated with arginase-1-expressing interstitial macrophages compared to non-expressing macrophages [19], suggesting that *C. neoformans* deploys Cpl1 to construct a replicative niche during infection. Concordantly, STAT6-deficient mice displayed reduced arginase-1 induction in interstitial macrophages and reduced pulmonary fungal burden [19]. Whereas wild-type *C. neoformans* achieved higher lung burdens in wild-type mice compared to STAT6-deficient mice, presumably due to an inability to induce type 2 immune responses in the absence of STAT6, *cpl1Δ* yeast achieved similar lung burdens in wild-type and STAT6-deficient mice [19]. This observation indicates that Cpl1 functions by modulating STAT6-driven immune manipulation *in vivo*.

Co-opting STAT3 and/or STAT6 signaling to manipulate macrophage polarization is an emerging mechanism observed in several host-pathogen systems [64,65]. Beyond the TLR2/4-dependent mechanism utilized by *M. bovis* BCG mentioned above [59,60], *Salmonella enterica* serovar

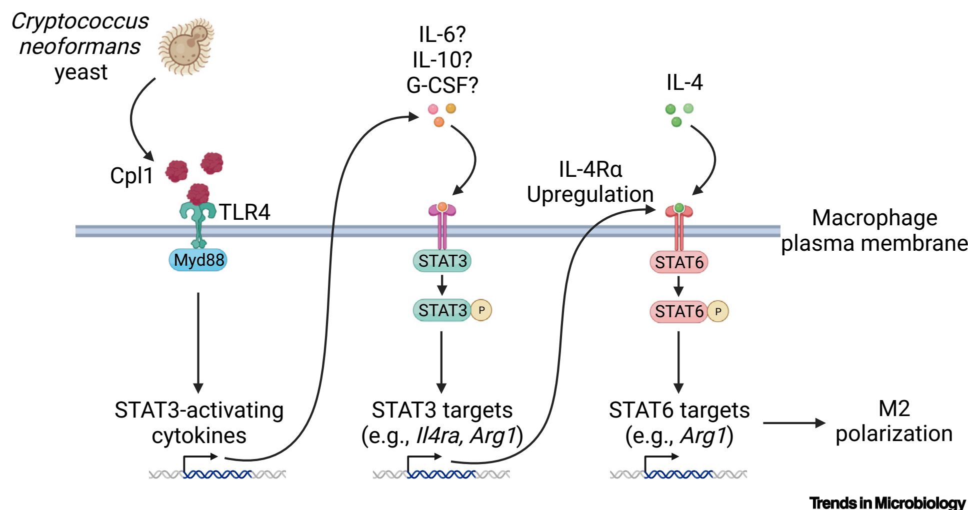

Figure 2. Model for manipulation of macrophage polarization by *Cryptococcus neoformans* Cpl1. Secreted *C. neoformans* Cpl1 binds TLR4 on macrophages to induce STAT3-activating cytokines, which potentially include IL-6, IL-10, and G-CSF. *In vitro*, STAT3 activation drives upregulation of both *Il4ra* and *Arg1*, although the *in vivo* relevance of STAT3-driven *Arg1* induction remains unclear. STAT3 activation potentiates IL-4-driven STAT6 activation, presumably due to increased IL-4Rα receptor availability, to enhance *Arg1* expression and type 2 immune polarization. Abbreviations: G-CSF, granulocyte colony-stimulating factor; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor.

Typhimurium manipulates STAT signaling using the secreted effector SteE, which binds and modulates the substrate specificity of the host kinase GSK3 to enable phosphorylation of the noncanonical substrate STAT3 and thus bypass the typical receptor-based signaling mechanism [66–69]. Similarly, the *Bartonella henselae* effector BepD promotes STAT3 phosphorylation by co-opting a different noncanonical host kinase, c-Abl [70]. The *T. gondii* effector kinase ROP16 directly phosphorylates both STAT3 and STAT6 to induce M2 polarization [71–75]. Thus, macrophage polarization state is a host vulnerability commonly targeted by pathogen effectors. Strikingly, *C. neoformans*, which is not thought to have coevolved with mammals, has converged upon a similar strategy. The mechanism by which Cpl1 induces type 2 immune activation is distinct from SteE, BepD, and ROP16, which function intracellularly to induce noncanonical phosphorylation of STAT3 and/or STAT6. Instead, Cpl1 likely acts extracellularly, potentially by direct binding and activation of TLR4. MD-2 is a coreceptor that binds LPS and complexes with TLR4 to enable signaling [76–78], and the house dust mite antigen component Der p 2 structurally mimics and replaces this protein to drive allergic inflammation [62,79–81]. Whether Cpl1 similarly mimics MD-2 or activates TLR4 signaling through an alternative mechanism is of key interest to understand how an environmental fungal pathogen evolved the ability to manipulate mammalian immune polarization states.

Inhibition of dendritic cell responses by *Candida albicans* Lip2

C. albicans is the most common cause of fungal infection, annually causing 134 million cases of recurrent vulvovaginal candidiasis, 3.3 million cases of oral or esophageal candidiasis in HIV/AIDS patients, and 750 000 cases of invasive candidiasis [2]. In contrast to the environmental fungi *H. capsulatum* and *C. neoformans*, *C. albicans* is a mammalian **commensal** that stably colonizes the gut, skin, and genitourinary tract and can benefit the host by enhancing immunity against a subset of bacterial and fungal pathogens [82]. Candidiasis typically occurs under conditions of gut dysbiosis, mucosal barrier breach, or immune suppression and ranges from superficial infection of mucosal tissues (mucocutaneous candidiasis) to life-threatening disseminated disease [83,84]. *C. albicans* undergoes morphological transitions between yeast, a form typically associated with commensalism, and **hyphae**, which are associated with tissue invasion and disease [85,86]. Despite the importance of the yeast-to-hypha switch, systematic screens found that nearly half of *C. albicans* mutants defective in fitness in the host are dispensable for this process [87], suggesting that numerous virulence mechanisms remain uncharacterized.

Recent work found that a secreted *C. albicans* lipase, Lip2, is required for kidney colonization in a bloodstream infection model but dispensable for hyphal morphogenesis [20]. Yeast lacking *LIP2* induced higher *Il17a* expression in infected renal tissue compared to wild-type yeast [20], indicating a potential role for Lip2 in suppressing IL-17 signaling. IL-17 is primarily secreted by lymphocytes and promotes immunity to extracellular bacteria and fungi by inducing target cells to produce antimicrobial peptides, proinflammatory cytokines, and neutrophil-recruiting chemokines [88,89]. IL-17 signaling has well-established roles in defending against both mucocutaneous and systemic candidiasis in mice [83,90,91], whereas human patients with genetic deficiencies in the IL-17 pathway or undergoing anti-IL-17 therapeutic treatment display increased susceptibility to mucocutaneous candidiasis but not disseminated disease [92–95]. Growth and virulence of the *lip2Δ* mutant are completely restored in mice lacking IL-17A and IL-17F, the two IL-17-family cytokines accounting for most known IL-17 functions, indicating that *C. albicans* Lip2 suppresses the mouse IL-17 response during systemic infection in mice [20].

γδ T cells are the primary source of IL-17 in *lip2Δ*-infected kidneys [20]. IL-17 production by lymphocytes is typically induced by macrophage- or dendritic cell (DC)-derived IL-23, suggesting the hypothesis that Lip2 acts on these upstream myeloid cells. Indeed, wild-type *C. albicans* infection

drove *Il23* upregulation in renal DCs *in vivo*, and this response was exacerbated in infections with *lip2Δ* yeast [20]. Bone marrow-derived DCs (BMDCs) secreted IL-23A in response to *lip2Δ* yeast, but not wild-type yeast, in a TLR2- and TLR4-dependent manner [20]. Both *lip2Δ*-induced IL-23A and suppression of this response by wild-type yeast occurred when yeast were separated from BMDCs by transwell inserts, indicating that soluble factors mediate both DC activation and suppression [20]. Exogenous palmitic acid, but not stearic or linoleic acids, suppressed *lip2Δ*-induced IL-23A production in BMDCs, and Lip2 catalytic activity is necessary for its activity [20]. This suggests that Lip2-derived palmitic acid, presumably produced from triglyceride hydrolysis, drives Lip2-dependent phenotypes.

These data support a model wherein host DCs sense soluble *C. albicans* PAMPS via TLR2 and TLR4 and, under normal circumstances, secrete IL-23 to activate γδ T cells, which then secrete IL-17 to activate antifungal responses (Figure 3). Secreted Lip2 counteracts this circuit by producing palmitic acid to inhibit DC activation. How palmitic acid acts is unknown. One model is

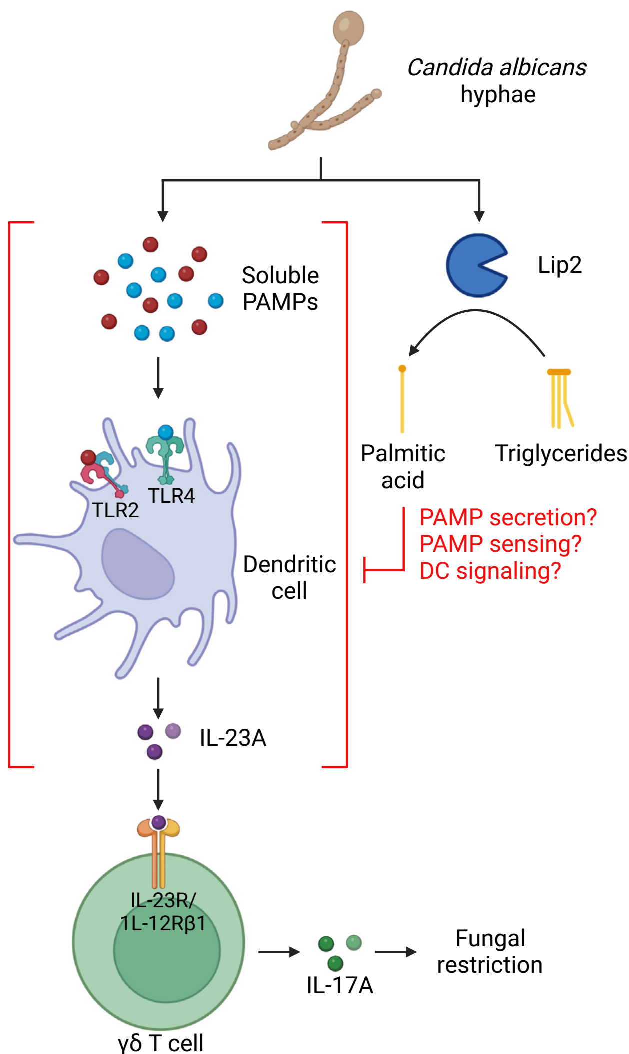

Figure 3. Model for suppression of dendritic cell responses by *Candida albicans* Lip2. Secreted *C. albicans* Lip2 produces palmitic acid, presumably by triglyceride hydrolysis, to inhibit activation of dendritic cells (DCs) by soluble fungal pathogen-associated molecular patterns (PAMPs). It remains unclear whether palmitic acid acts via (i) inhibition of PAMP secretion by *C. albicans*; (ii) inhibition of PAMP sensing by DCs; or (iii) disruption of DC signaling. In the absence of Lip2, DCs sense fungal PAMPs and secrete IL-23A, which induces γδ T cells to secrete IL-17 and suppress fungal growth.

that it blocks binding of yeast PAMPs to TLR2 and TLR4, thereby inhibiting activation. Alternatively, palmitic acid might induce anti-inflammatory responses that counteract TLR2/4-derived activation signals. Another possibility is that palmitic acid acts on *C. albicans* to prevent PAMP production. Notably, although lipases as protein effectors have been observed in other pathogen classes, *C. albicans* Lip2 seems to function in a manner that is mechanistically distinct. For example, while the secreted lipase Geh from the Gram-positive bacterial pathogen *Staphylococcus aureus* also suppresses TLR2-dependent innate sensing, it does so by cleaving acyl chains from bacterial lipoproteins that are TLR2 agonists, thereby preventing host recognition of these important PAMPs [96]. *Fusarium graminearum*, a fungal pathogen of wheat, secretes a lipase, Fgl1, that cleaves lipids to produce the free fatty acids linoleic acid and α-linoleic acid [97]. These fatty acids inhibit the enzyme that synthesizes the β-(1,3)-glucan polymer callose [97], which the plant deposits to inhibit pathogen invasion. Thus, whereas Geh directly modifies microbial PAMPs to inhibit innate immune sensing and Fgl1 inhibits downstream immune effector functions through its fatty acid products, Lip2 utilizes its product palmitic acid to disrupt upstream innate sensing.

Redirection of phagosome trafficking by *Aspergillus fumigatus* HscA

A. fumigatus is an environmental filamentous fungus that causes a range of clinical diseases including 4.8 million annual cases of allergic bronchopulmonary aspergillosis (ABPA), 3 million annual cases of chronic pulmonary aspergillosis (CPA), and 300 000 annual cases of invasive aspergillosis (IA) [2]. Human infection begins with inhalation of **conidia**. Neutrophils and alveolar macrophages play a central role in clearing these fungal cells from the airways, and the ability of conidia to evade phagocytic killing mechanisms is an important determinant of infection outcomes [98]. LC3-associated phagocytosis (LAP) is a pathway by which PAMP sensing promotes phagosome maturation and killing of ingested pathogens in a manner dependent on a subset of autophagy components and the NADPH oxidase complex [99,100]. LAP is important for killing phagocytosed *A. fumigatus* spores in experimental systems [101], and previous work showed that the conidial cell wall pigment DHN-melanin inhibits LAP by depleting intraphagosomal calcium, resulting in reduced NADPH oxidase recruitment to the phagosome membrane [102,103]. Despite a major role for DHN-melanin in evasion of killing by phagocytes, some nonmelanized conidia were nonetheless able to survive inside phagosomes [102,104], suggesting that *A. fumigatus* encodes additional mechanisms to subvert this important host defense.

To identify fungal proteins that interact with host cells, a biotinylation approach was employed to label surface-exposed *A. fumigatus* proteins and to identify those that bound cultured lung epithelial cells [21]. This identified the Hsp70-family chaperone HscA as a candidate interactor, and indeed recombinant HscA, but not recombinant *A. fumigatus* Hsp70, bound to cells of multiple human and mouse epithelial cell lines [21]. Conidia lacking *HscA* displayed two phenotypes. First, *ΔhscA* conidia bound host cells to a lesser extent than wild-type conidia, suggesting a role in attachment [21]. Second, after internalization by host cells, more *ΔhscA* conidia stained positive for phagosome maturation markers compared to wild-type conidia, suggesting that HscA plays a role in preventing phagosome maturation [21]. Notably, both phenotypes could be rescued by addition of recombinant HscA but not recombinant Hsp70. Furthermore, HscA-coated latex beads displayed reduced association with the phagosome maturation marker Rab7 compared to BSA-coated bead controls [21], suggesting that HscA is sufficient to inhibit phagosome maturation in the absence of other fungal components.

Unbiased proteomics identified human p11, also called S100A10, as an HscA binding partner [21]. p11 is a multifunctional protein that forms a heterotetrameric complex with the phospholipid-binding protein annexin A2 (AnxA2). This protein complex has been localized both intracellularly to the cytosolic face of endosomes and the plasma membrane, as well as

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extracellularly to the plasma membrane, and it influences a diverse array of cellular processes including endosome trafficking, transmembrane protein localization, actin dynamics, tight junction maintenance, and plasmin activation [105–108]. Validating an HscA–p11 interaction, both p11 and AnxA2 coprecipitated with HscA-GFP, and recombinant HscA could not bind host cells lacking p11 [21]. Deletion of p11 both reduced attachment of wild-type conidia and increased the association of internalized conidia with phagosome maturation markers [21], confirming a role for p11 in the attachment and inhibition of phagosome maturation phenotypes attributed to HscA. Phagosomes lacking maturation markers were instead shunted to a recycling pathway, as wild-type conidia were associated with recycling endosome markers (e.g., Rab11) to a greater extent than HscA-deficient conidia [21]. Concordantly, internalized HscA-coated beads stained positive for recycling endosome markers whereas Hsp70-coated beads did not [21]. These data support a model in which HscA binds human p11 to prevent phagosome maturation and shunt phagosomes to a recycling pathway (Figure 4).

Functionally, deleting *hscA* from conidia or p11 from host cells reduced the percentage of germinated conidia within phagosomes [21], suggesting a role for modulation of phagosome trafficking in fungal development during infection. Additionally, deletion of *hscA* reduced exocytosis of conidia from host cells [21]. Screening for human p11 single-nucleotide polymorphisms (SNPs) associated with risk of invasive pulmonary aspergillosis (IPA) in a cohort of hematopoietic stem cell transplant recipients and their donors revealed one SNP in the first intron of p11 in donors associated with significantly reduced IPA risk [21]. Engineering this protective SNP into cultured

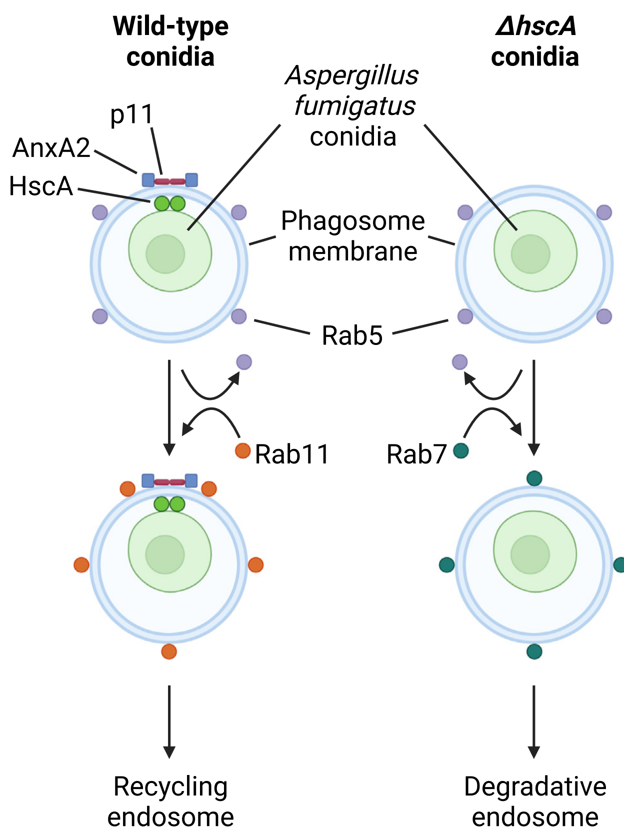

Figure 4. Model for manipulation of phagosome trafficking by *Aspergillus fumigatus* HscA. HscA on the surface of phagosomal *A. fumigatus* conidia recruits p11–AnxA2 heterotetramers to the cytosolic face of the phagosomal membrane through an unknown mechanism. This prevents accumulation of the late endosome marker Rab7 and allows recruitment of the recycling endosome marker Rab11, which promotes fungal survival by promoting exocytosis or germination within the phagosome. In the absence of HscA, p11 and AnxA2 are not recruited, and Rab7 accumulates on the phagosomal membrane to drive it to an antifungal degradative pathway. Note that the topology of HscA-p11–AnxA2 interactions remains to be elucidated, and there is likely a population of lumenal p11–AnxA2 (not shown) arising from HscA interactions with surface p11–AnxA2 during attachment and engulfment. The relative contributions of cytosolic versus lumenal p11-AnxA2 to phagosome redirection are unclear.

epithelial cells reduced the percentage of conidia associated with phagosome maturation markers and increased the percentage associated with recycling endosome markers [21].

Destruction by the phagolysosome is one of the most common challenges to infection that successful pathogens must overcome, and there are numerous microbial effectors dedicated to escaping the phagolysosome or preventing its maturation [109]. Effectors modulating phagosome trafficking act through diverse biochemical mechanisms but tend to functionally converge on modifying critical determinants of phagosome identity, such as **Rab GTPases** and **phosphoinositides**, to construct an intraphagosomal environment that facilitates pathogen survival [109]. *A. fumigatus* HscA functions similarly by preventing the accumulation of phagosome maturation markers and promoting the accumulation of recycling endosome markers. The HscA-interacting partners p11 and AnxA2 influence multiple membrane-related processes in the cell, but how they contribute to phagosome redirection is unclear. The topology of the HscA-p11 interaction is of particular importance to elucidate, as the current data suggest somewhat of a paradoxical model. During internalization of conidia by host cells, HscA on the conidial surface likely binds extracellular p11 on the plasma membrane. Phagocytosis would presumably internalize extracellular p11 and AnxA2 to the phagosome lumen, but it seems unlikely that lumenal HscA-p11 interactions could coordinate Rab turnover and phagosome redirection. Cytosolic p11-AnxA2 more probably carries out these functions, but it is then unclear how lumenal HscA interfaces with these factors across the phagosome membrane.

Like the effector targets described for other fungal pathogens above, there are multiple examples of infectious microbes coopting p11 and AnxA2. AnxA2 is reported to interact with the *S.* Typhimurium effectors SopD2 and PipB2 and may be required for the SopD2-dependent positioning of the *Salmonella*-containing vacuole near the host nucleus [110]. Paralleling the dual roles for p11-AnxA2 in both attachment and phagosome trafficking during *A. fumigatus* infection, this complex is also recruited to the site of *S.* Typhimurium invasion in a manner partially dependent on the secreted phosphatase SopB to promote actin-dependent membrane ruffling and bacterial internalization [111]. The effector EspL2 from enterohemorrhagic *E. coli* (EHEC) binds AnxA2 and activates its actin cross-linking activity [112], and extracellular AnxA2 has been implicated as a host cell attachment factor for *Rickettsia australis* and *S. aureus* [113]. Additionally, p11 and AnxA2 are critical to the life cycles of multiple viruses, with reported roles in attachment, entry, replication, virion assembly, and release [114]. Thus, diverse pathogens target this protein complex to promote their infectious lifestyles.

Concluding remarks and future perspectives

Microbial pathogens utilize secreted effector proteins to counteract host barriers to infection. While few such proteins had been identified in human fungal pathogens, the recent findings reviewed here make it clear such proteins are widespread. While the fungal effectors described to date have diverse biochemical functions and host target pathways, they nonetheless modulate key host pathways that are often subverted by other classes of pathogens. Because the fungal pathogens described here are primarily commensals or environmental **saprophytes**, the sophisticated mechanisms by which they subvert these critical host pathways could be viewed as paradoxical. However, many bacterial pathogens are also found in the environment and lack a known animal reservoir (e.g., *Pseudomonas aeruginosa* and *Legionella pneumophila*). It has been proposed that the predation by eukaryotes in the environment drove the evolution of pathogenicity in these systems [115,116], and similar hypotheses have been made for human fungal pathogens [117]. Additionally, in cases of opportunistic fungi, such as *C. neoformans* and *A. fumigatus*, we speculate that proteins involved purely in these organisms' environmental lifestyles might occasionally, by chance, encode functions that happen to promote survival during

Outstanding questions

How does *H. capsulatum* Cbp1 access the host cytosol?

Is Cbp1 sufficient to initiate the ISR and apoptosis in the absence of other *Histoplasma* machinery?

How does Cbp1 initiate the ISR?

Does *C. neoformans* Cpl1 directly interact with TLR4?

What is the functional consequence Cpl1-mediated manipulation of interstitial macrophage polarization during *in vivo* infection?

What is the mechanism by which Lip2-produced palmitic acid suppresses recognition of *C. albicans* by dendritic cells?

How is *A. fumigatus* HscA directed to the conidial surface?

How does HscA, which is thought to localize on the conidial surface in the phagosome lumen, access p11, which likely resides in the cytosol?

infection. *C. neoformans* Cpl1, for example, also promotes capsule assembly [118] and is part of a family of five secreted proteins that are developmentally regulated and are thought to influence fungal morphogenesis and stress tolerance in different environmental conditions [119]. *A. fumigatus* HscA is an Hsp70-family chaperone which might have evolved for any number of functions unrelated to pathogenesis. These proteins' roles in host colonization may be examples of **exaptation**, in which a characteristic that evolved in one environment, with or without a specific function, subsequently promotes fitness in a new environment [120]. Rather than anomalies, effectors that arise through exaptation might be an underappreciated source of raw materials for the evolution of pathogenesis from environmental microbes. While horizontal gene transfer, especially in bacteria, is undoubtedly a major mechanism for the acquisition of host-modulatory functions, retooling existing proteins that encode effector functions by chance could be an alternative strategy for a saprophyte to establish a mammalian host as a new niche. Regardless of the underlying forces, the recent work makes clear that secreted fungal effectors have evolved to enable infection of mammals via manipulation of key pathways including apoptosis, myeloid cell polarization, Toll-like receptor signaling, and phagosome activity. We imagine that they represent the tip of an iceberg whose depths will soon be revealed (see Outstanding questions).

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Declaration of interests

No interests are declared.

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