Cryptococcus neoformans induces antimicrobial responses and behaves as a facultative intracellular pathogen in the non mammalian model *Galleria mellonella*

Nuria Trevijano-Contador[1,†], Inés Herrero-Fernández[1,†], Irene García-Barbazán[1], Liliana Scorzoni[1,2], Cristina Rueda[1], Suélen Andreia Rossi[1,2], Rocío García-Rodas[1,#], and Oscar Zaragoza[1,*]

[1]Mycology Reference Laboratory; National Center for Microbiology; Instituto de Salud Carlos III; Carretera Majadahonda-Pozuelo, Km2; Majadahonda, 28220, Madrid, Spain; [2]Laboratório de Micologia Clínica; Faculdade de Ciências Farmacêuticas; R. Expedicionários do Brasil, 1621, CEP. 14801–902, Araraquara, São Paulo, Brazil

[†]These authors contributed equally to this work.
[#]Present Address. Institute of Biology Valrose (iBV) UMR CNRS7277 – INSERM1091 – University of Nice-Sophia Antipolis. Center de Biochimie, 06108 Nice, FRANCE

Cryptococcus *neoformans* is an encapsulated opportunistic fungal pathogen that is found in multiple niches in the environment and that can cause fatal meningoencephalitis in susceptible patients, mainly HIV+ individuals. *Cryptococcus* also infects environmental hosts such as nematodes, insects and plants. In particular, *C. neoformans* can kill the lepidopteran *Galleria mellonella*, which offers a useful tool to study microbial virulence and drug efficacy. *Galleria mellonella* immunity relies on innate responses based on melanization, accumulation of antimicrobial peptides, and cellular responses as phagocytosis or multicellular encapsulation. In this work we have investigated the immune response of *G. mellonella* during cryptococcal infection. We found that *G. mellonella* infected with *C. neoformans* had a high lytic activity in their hemolymph. This response was temperature- and capsule-dependent. During interaction with phagocytic cells, *C. neoformans* behaved as an intracellular pathogen since it could replicate within hemocytes. Non-lytic events were also observed. In contrast to *Candida* species, *C. neoformans* did not induce melanization of *G. mellonella* after infection. Finally, passage of *C. neoformans* through *G. mellonella* resulted in changes in capsule structure as it has been also reported during infection in mammals. Our results highlight that *G. mellonella* is an optimal model to investigate innate immune responses against *C. neoformans*.

Introduction

Cryptococcus neoformans is an opportunistic fungal pathogen that is usually found in the environment, in particular soils and pigeon excreta. Infection begins by inhalation of infectious particles. In immunocompetent people the infection can remain in a latent state, or be cleared by the host. However, in immunosuppressed patients (mainly HIV infected patients and transplant recipients), the yeast can disseminate and invade the central nervous system causing a fatal meningoencephalitis.[1,2] *Cryptococcus neoformans* is responsible for over 600,000 deaths per year, which makes this pathogen a major global threat.[3] Defense against *C. neoformans* largely depends on innate response and Th1 polarization.[2]

Cryptococcus neoformans has several virulence factors that have been associated with host damage, such as the polysaccharide capsule and melanin production.[4,8] Moreover, *C. neoformans* has developed mechanisms that prolong its survival in the host including the ability to form titan cells and to change the size of the capsule.[9,11] *Cryptococcus neoformans* behaves as an intracellular fungal pathogen, since it can survive and replicate inside phagocytic cells.[12,13]

Cryptococcal infection is not human-specific, and this fungus can cause disease in a wide variety of hosts, including amoebas, insects, nematodes, koalas, birds, dolphins, and even plants.[14,20] These environmental interactions have important implications to understand how *C. neoformans* has acquired virulence traits required for host adaptation.[21] For this reason, the interaction between alternative models and pathogenic fungi is becoming a field of interest. The use of these hosts also offers bioethical advantages because it reinforces the application of the "3 Rs" rule to reduce animal suffering and pain. One of the main model host used to investigate fungal pathogenesis is the lepidopteran *Galleria mellonella*.[22,23] This insect offers important advantages to investigate microbial virulence,[24] so

it has been successfully used to study the pathogenicity of some bacteria and fungi.[22,25,33] *Cryptococcus neoformans* can also infect and kill *Galleria mellonella*.[34] Virulence of this fungus in this model depends on the temperature and on the capsule. *Cryptococcus neoformans* can enlarge the capsule and produce titan cells in *G. mellonella*.[35] Recently, a multihost approach using *Caenorhabditis elegans* and *G. mellonella* has identified new genes required for cryptococcal virulence.[36] After challenge with microorganisms, *G. mellonella* induces melanization around the exogenous particles.[37] This insect contains hemocytes with phagocytic activity in the hemolymph, that also produce antimicrobial peptides.[38,44]

Since some aspects of the *G. mellonella* innate immunity are similar to those elicited by mammals in response to *C. neoformans*, we have characterized the response of *G. mellonella* after challenge with this pathogenic yeast. We demonstrate that *C. neoformans* induce the accumulation of antimicrobial peptides in the hemolymph, but does not induce early melanization of the *G. mellonella*. We also show that this fungus behaves as an intracellular fungal pathogen in *G. mellonella* hemocytes. In conclusion, these findings support the use of non-mammalian models to investigate the immune response to *C. neoformans*.

Results

Lytic activity of the hemolymph

We first studied the accumulation of antimicrobial peptides in the hemolymph using a lytic assay based on agar plates containing the bacteria *Micrococcus luteus*. As shown in **Figure 1A**, *C. neoformans* induced an increase in the lytic activity of the hemolymph. This induction was noticeable 6 hours after infection, and reached a maximal response after 24 h (**Fig. 1A**). The increase was similar to the one observed after infection with the pathogenic yeast *C. albicans* (**Fig. 1B**). We decided to characterize this phenomenon in detail and investigated if the capsule played a role in this induction. When we performed the same experiment with the acapsular mutant *cap59*, there was a slight increase in the lytic activity of the hemolymph compared to induction observed in *G. mellonella* infected with its parental strain B3501 (**Fig. 2A**). This result suggested that the capsule played a role in the recognition of the yeast by *G. mellonella*, so we investigated the effect of capsular polysaccharide on the induction of antimicrobial peptides. For this purpose, we purified the main polysaccharide of the capsule (glucuronoxylomannan, GXM) and injected different doses in *G. mellonella*. We found that there was a dose-response effect, so that 50 µg induced a significant increase in the lytic activity of the hemolymph (**Fig. 2B**). Interestingly, greater amounts of GXM did not induce the lytic activity. Next, we examined if cryptococcal cells with different capsule sizes induced different larval responses. However, we did not find any difference in the accumulation of lytic peptides when *G. mellonella* was challenged with cells of small or large capsule size (**Fig. 2C**).

Cryptococcus neoformans is more virulent at 37°C than at 30°C, so we studied if the incubation temperature of infected *G. mellonella* played a role on the induction of lytic peptides. We observed that there was a trend to a higher induction of the lytic activity of the hemolymph when *G. mellonella* were placed at 37°C (data not shown). Induction of this larval response depended on living yeast, since dead cells did not show a significant effect on the lytic activity (data not shown).

Intracellular replication

Cryptococcus neoformas behaves as an intracellular fungal pathogen in murine and human macrophages. So we investigated if after phagocytosis by hemocytes, *C. neoformans* also replicated inside these cells. We infected *G. mellonella* larvae, and after 3 hours of incubation at 37°C, we isolated the hemocytes from the hemolymph and visualized internalized *C. neoformans* cells overnight at 28°C. As shown in **Figure 3A** and supplemental video 1, we found hemocytes in which *C. neoformans* replicated several times, indicating that this yeast behaves as a facultative intracellular pathogen. Moreover, we also found some cases in which internalized fungal cells were extruded from the hemocytes (**Fig. 3B** and supplemental
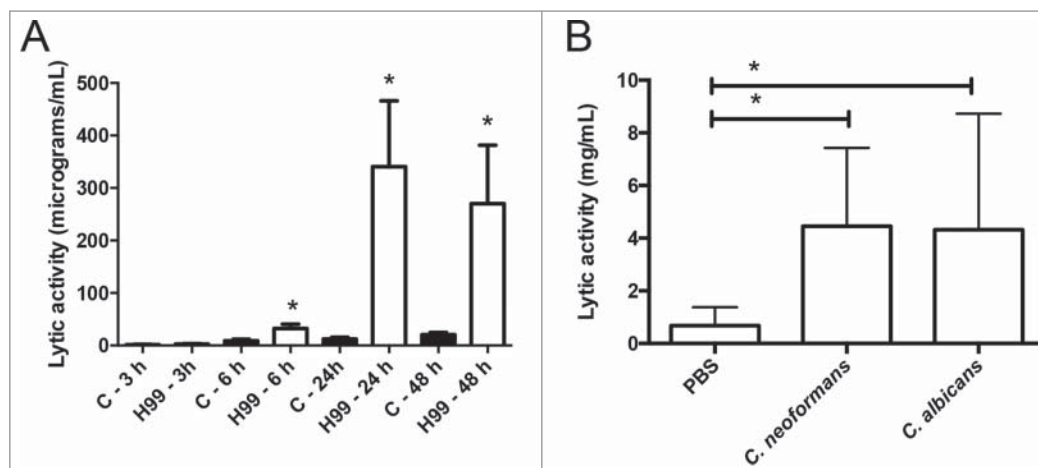

Figure 1. Effect of *C. neoformans* on the antimicrobial activity of the *G. mellonella* hemolymph. (**A**) Groups of 10 *G. mellonella* were infected with *C. neoformans* H99 strain (10^6 cells/*G. mellonella*) or with PBS (**C**), and then they were incubated at 37°C. The lytic activity of the hemolymph was evaluated as described in Material and Methods after different timepoints. Asterisks denote statistical difference between the sample and the corresponding control *G. mellonella* larvae treated with PBS. (**B**) *G. mellonella* larvae were infected with *C. neoformans* H99 or *C. albicans* SC5314 strain (10^5 cells per *G. mellonella*) and incubated at 37°C overnight. Then, the lytic of the hemolymph was evaluated. Asterisks denote statistical difference between the sample and the control *G. mellonella* treated with PBS.
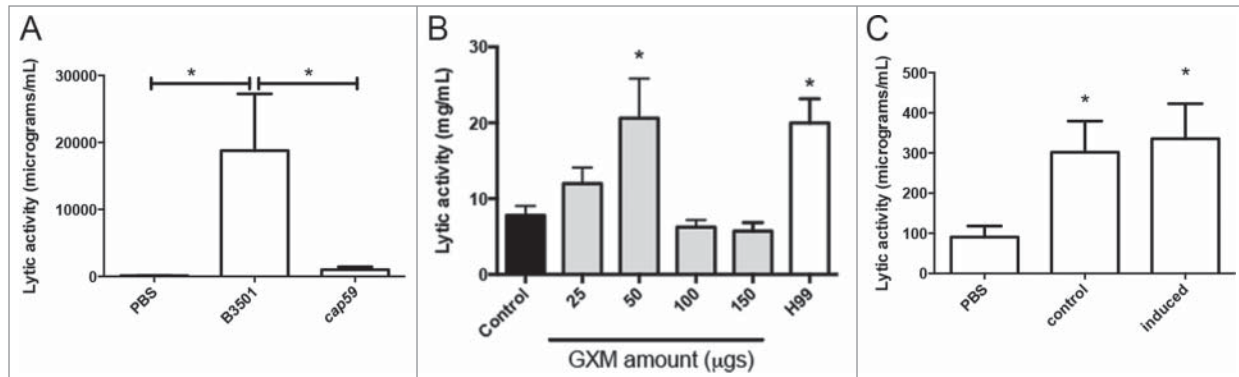
Figure 2. Role of the capsule on the accumulation of antimicrobial peptides in the hemolymph. (**A**) *Galleria mellonella* larvae were infected with approximately 10^5 cells from *B3501* or *cap59* strains as described in Material and Methods, and placed at 37°C for 24 h. Lytic activity of the hemolymph was assessed as described in M&M. Asterisks denote statistical difference. (**B**) Groups of 10 *G. mellonella* were infected with 10^6 H99 cells prepared in water or with different amounts of purified GXM. In parallel, *G. mellonella* were injected with sterile water. After 24 h of incubation at 37°C, the antimicrobial activity of the hemolymph was determined. Asterisks indicate statistical difference between the sample and the control treated with water. (**C**) Cells from H99 strain with different capsule size were obtained after growth in Sabouraud (Control, small capsule) or 10% Sabouraud, pH 7.3 (Induced, large capsule). Then, *G. mellonella* were infected with 10^5 cells from these cultures or with PBS, and lytic activity in the hemolymph was determined after 24 h of incubation at 37°C. Asterisks denote statistical difference between the sample and the larvae treated with PBS.

video 2), which is another phenomenon also found after phagocytosis by mammalian macrophages and amoebas.

Cryptococcus neoformans did not induce hemolymph melanization

We then investigated how *C. neoformans* induced one of the early responses in *G. mellonella* after exposure to exogenous particles, which is melanization. This parameter can be monitored by measuring the OD of the hemolymph at 405 nm. We used as a positive control *Candida albicans*.[45] As shown in **Figure 4**, *C. albicans* induced melanization of the hemolymph as early as 1 hour after infection. In contrast, melanization was not observed when *G. mellonella* was infected with *C. neoformans*. This finding suggests that *C. neoformans* is differently recognized by *G. mellonella*.

Structural changes of the capsule during infection in *G. mellonella*

A typical feature of the capsule during infection is that it can change in size and structure. We have previously described that *C. neoformans* enlarges the capsule in *G. mellonella*.[35] In this work, we also investigated if the capsule suffered any structural change during infection. For this purpose, we tested if there was any change in the binding pattern of the GXM specific monoclonal antibodies to the capsule of yeast cells recovered from *G. mellonella* or grown in vitro in Sabouraud (small capsule) or under capsule enlargement conditions (10% Sabouradud, pH 7.3). We tested the binding of 2 IgG (18B7 and 2H1) and one IgM (2D10) mAbs. As shown in **Figure 5**, mAb 18B7 gave a similar fluorescent pattern in all the cases, although cells obtained from *G. mellonella* presented lower fluorescent intensity, suggesting
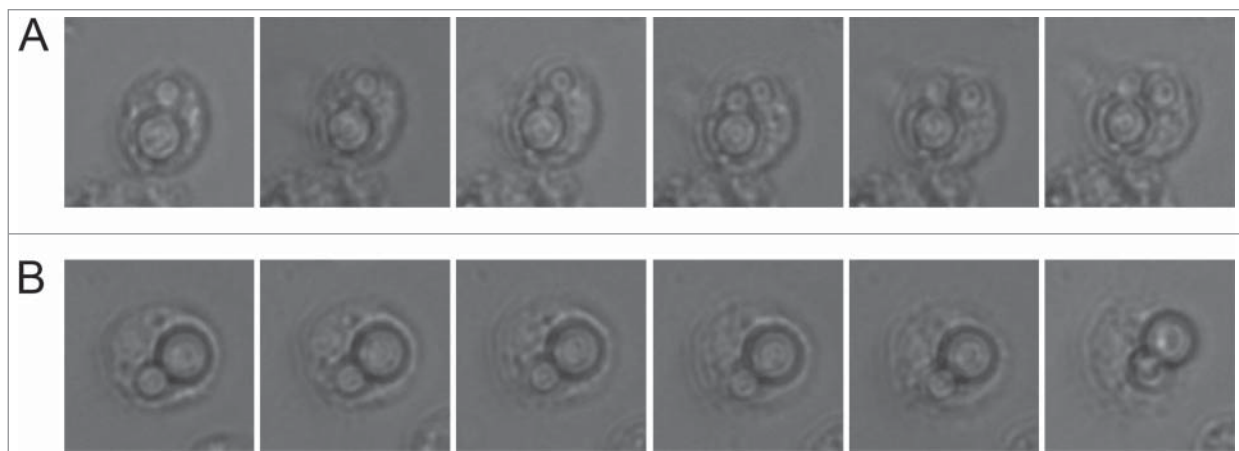

Figure 3. Behavior of *C. neoformans* inside hemocytes. *Galleria mellonella* were infected with 10^6 cryptococcal cells (H99 strain) and after 3 h of incubation at 37°C, the hemolymph was collected and hemocytes placed in a 96-well plate under the microscope. (**A**) Intracellular replication event; (**B**) Non lytic exocytosis of the cryptococcal cells from the hemocytes. In each case, the time difference between the pictures of the panels is 9 min.
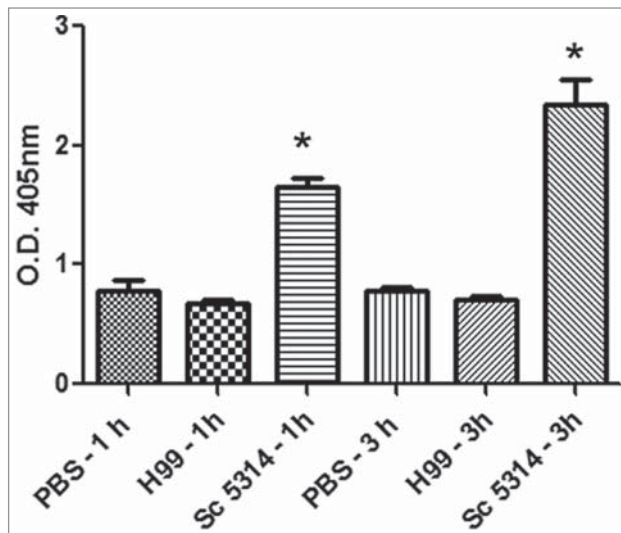
Figure 4. Early melanization of *G. mellonella* after infection with *C. neoformans* and *C. albicans*. Groups of 10 *G. mellonella* larvae were injected with PBS (A), *C. albicans* SC5314 strain (10^6 cells/*G. mellonella*) or *C. neoformans* H99 (10^6 cells/*G. mellonella*). Quantification of hemolymph melanization as described in M&M. Asterisks denote statistical difference between the sample and the control *G. mellonella* treated with PBS.

that the number of epitopes for this mAb were reduced after passage through *G. mellonella*. Monoclonal Ab 2H1 bound to cells grown in vitro in a homogenous way, showing an annular binding pattern. In contrast, we found that in cells isolated from *G. mellonella*, there was heterogeneity in the fluorescence binding pattern. While some cells also showed an annular pattern, in some others the fluorescence was not homogenously distributed throughout the capsule, and there was a trend to find higher fluorescence in the inner regions of the capsule (**Fig. 5**). Finally, mAb 2D10 showed a clear punctuate binding pattern to cells grown in vitro under capsule inducing conditions and isolated from *G. mellonella*. In contrast to the situation with mAb 18B7, cells obtained from *G. mellonella* gave higher fluorescence than cells grown in Sabouraud, but lower than in vitro cells with large capsule.

Discussion

Cryptococcus neoformans is a pathogenic yeast that can cause disease in susceptible patients. But it can also infect a wide variety of environmental hosts, such as amoebas, insects, nematodes, mammals and plants. Interestingly, the virulence mechanisms seem to be conserved independently of the host. For this reason, the use of alternative models to mammals has raised the interest of the scientific community. In particular, the lepidopteran *G. mellonella* is being largely used to investigate microbial pathogenesis. In this work, we have studied different aspects of the immune response of *G. mellonella* after infection with *C. neoformans*. Some of these responses, such as the induction of antimicrobial peptides and phagocytic cells, are also present in mammals.

Cryptococcus neoformans induced the production of lytic activity in the hemolymph, which is also another well-known response of *G. mellonella*. In this case, the capsule played a role, since acapsular mutants did not induce this process as encapsulated strains. However, *cap59* mutants are avirulent, and presumably, they replicate inside *G. mellonella* at a slower rate than encapsulated strains. Induction of antimicrobial peptides in the hemolymph depended on live yeasts and on the pathogen dose. So it is possible that the decrease response elicited by *cap59* mutant is due to the presence of a lower number of viable yeast cells during infection compared to wild type strains. The capsular polysaccharide has many effects on the immune response of mammals, and they produce immunomodulation, complement depletion, decreased Ab production and apoptosis.[46,51] So it could be argued that a similar situation might be found during cryptococcal infection in *G. mellonella*. We found that purified GXM produced a dose response effect, so intermediate amounts of polysaccharide induced the accumulation of antimicrobial peptides, but higher doses did not have any effect. This result suggests that this polysaccharide can also induce "immunological paralysis" in *G. mellonella*, as it has been also described in mammalian models (see reviews in[2,6]). Moreover, it cannot be discarded that higher GXM amounts are toxic. So, this lepidopteran provides an alternative model to investigate some aspects of the effect of the main cryptococcal virulence factor on the immune response. The induction of lytic activity is of interest because in mammals, some antimicrobial peptides also play a role in immune defense, such as defensins and histatins, which show anticryptococcal activity. An interesting aspect is that the induction of antimicrobial peptides did not protect *G. mellonella* from death, indicating that the natural role of this response might be to eliminate other type of pathogens, such as bacteria.

We noticed that the induction of antimicrobial activity is very variable between larvae, and even injection with PBS already induced accumulation of these peptides. This indicates that this response is very sensitive and tightly regulated, so minor changes in the hemolymph homeostasis are sensed by *G. mellonella*, resulting in an enhancement of innate responses. In agreement, physical stress is enough to prime the immune response of *G. mellonella* and the expression of some genes encoding lytic peptides.[44]

One of the most important aspects of the cryptococcal virulence is its interaction with phagocytic cells.[52,53] *Cryptococcus neoformans* is phagocytosed by hemocytes, but it was unknown whether it could behave as a facultative intracellular pathogen. We report that after phagocytosis, *C. neoformans* undergoes 2 of the outcomes described in mammalian phagocytic cells, which are intracellular replication and non-lytic exocytosis.[12,13,54,56] Similar phenomena have been described also in other non-mammalian hosts, such as amoebas and hemocytes from *Drosophila melanogaster*.[20,57,58] These results enforce the hypothesis that the intracellular pathogenesis is a virulence trait that *C. neoformans* has acquired after environmental interactions,[21] and allows the yeast to escape from death by macrophages in more complex hosts. Some entomopathogenic fungi, such as *Metarhizium anisopliae* or *Beauveria bassiana*, can also affect the phagocytic activity
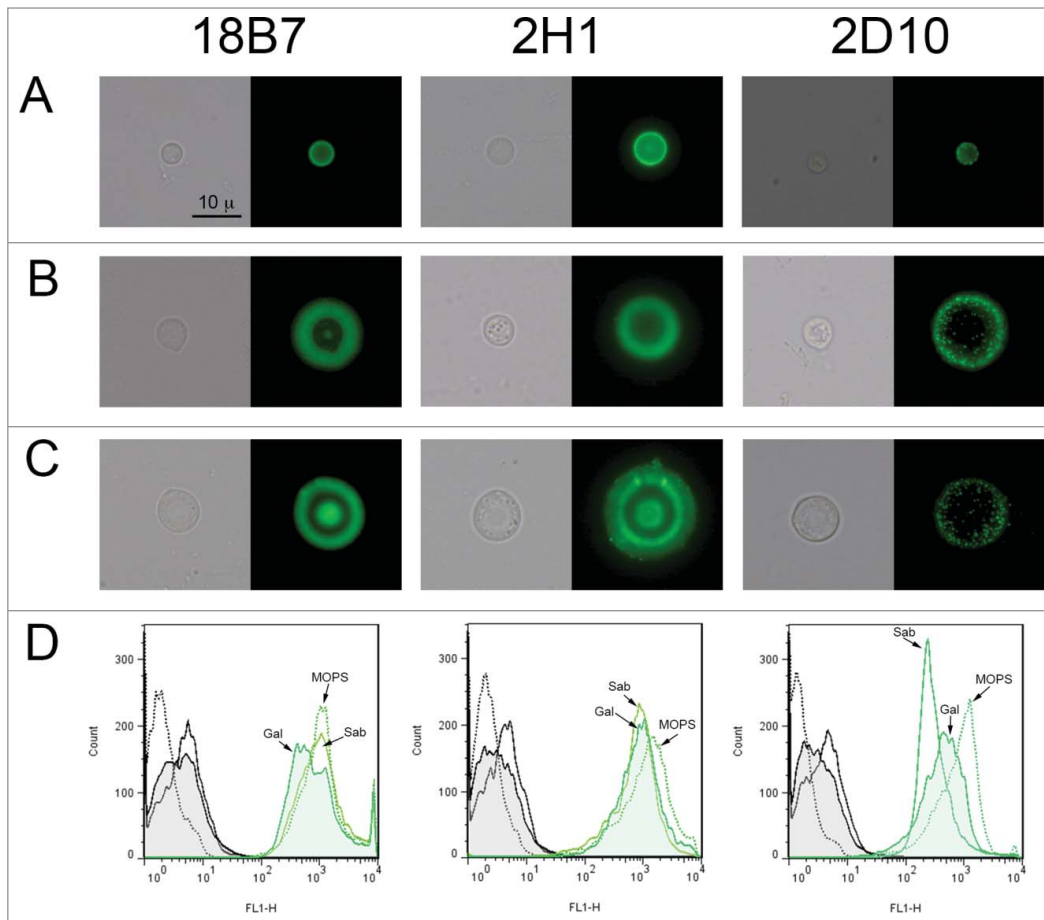
Figure 5. Structural features of the *C. neoformans* capsule in vitro and after passage through *G. mellonella*. Cells from H99 strain were obtained in vitro with different capsule sizes or from *G. mellonella*. (**A**) Cells with small capsule size after growth in vitro, (**B**) cells with enlarged capsule after incubation in 10% Sabouraud pH 7.3, (**C**) cells isolated from *G. mellonella*. These cells were incubated with different mAbs to the capsule (18B7, 2H1 and 2D10). mAb 18B7 was directly conjugated to Alexa488, and for the other 2, specific GAM-IgG (for 2H1) or GAM-IgM (for 2D10) were used. In each case, a representative cell is shown, including both light microscopy (left panel) and fluorescence (right panel). Scale bar in upper left panel applies to the rest of the pictures. (**D**) Quantification of the fluorescence of the cells shown in **A**, **B** and **C** by flow cytometry. Black histograms correspond to cells to which no primary anti-cryptococcal mAb has been added (negative controls), and green histograms indicate the fluorescence intensity of the cells to which both anti-cryptococcal and secondary Abs have been added. In all cases, straight line corresponds to cells grown in Sabouraud (Sab, small capsule *in vitro*), dotted line to cells incubated in 10% Sabouraud pH 7.3 (MOPS, large capsule *in vitro*), and filled histogram to yeast cells isolated from *G. mellonella* (Gal).

with acapsular mutants is barely above the control (data not shown). Since the cryptococcal cell wall has very low levels of β-glucans, this result suggests that the structural differences at the yeast surface determine the type of response elicited by *G. mellonella*. At the moment, it cannot be discarded that other factors play a role in this phenomenon. For example, *C. neoformans* can melanize when incubated in *G. mellonella* homogenates,[60] so maybe the fungus has the ability to act as a scavenger of melanin precursors from the hemolymph. When *G. mellonella* is infected by *Beauveria bassiana*, some strains also fail to induce larval melanization and in this case, this effect correlates with the production of toxins by the fungus.[37]

Capsule enlargement is a process that has a great impact on phagocytosis. In the case of mammalian macrophages, this transition inhibits complement-mediated phagocytosis and protects against free radicals.[61,62] Capsule growth also occurs during infection in *G. mellonella* and amoebas,[35,63] and this process impairs phagocytosis by hemocytes.[35] In this work, we have also shown

of hemocytes,[59] most probably due to the secretion of secondary metabolites that impair the recognition of the fungal cells. Our findings confirm that this lepidopteran can be used to investigate aspects of the fungal intracellular pathogenesis.

We have found that *C. neoformans* is recognized differently compared to other pathogenic yeast. After injection with *Candida* species, *G. mellonella* induces melanization of the hemolymph that is noticeable after 30 minutes postinfection. This response has been associated to β-glucans on the surface.[45] This early response was not observed after challenge with *C. neoformans*, although *G. mellonella* did melanize when they died (4–6 d after infection). The capsule could in fact inhibit the recognition of the yeast by *G. mellonella*, but preliminary results show that the melanization of the hemolymph measured after infection

that after passage through *G. mellonella*, the capsule of *C. neoformans* suffers rearrangements of its structure. This has been described both in vitro and in mice,[64, 65] and it seems to be a process associated with brain invasion.[65] Since the capsule plays an important role during phagocytosis[35] and in the induction of antimicrobial peptides in *G. mellonella*, we argue that capsular rearrangements might influence the recognition of the pathogen by the host and contribute to immune evasion.

Galleria mellonella is a versatile model host to study fungal pathogenesis. In this work, we have demonstrated that this lepidopteran can be used to investigate mechanisms of fungal immune evasion and intracellular pathogenesis. These new aspects open new perspectives in the fungal field for different reasons. First, we confirm that some of the cryptococcal virulence

mechanisms used in mammals also occur in this insect, which highlights the importance of environmental interactions in the acquisition of virulence traits. Moreover, using *G. mellonella* as model host, it has been possible to show that microbial virulence is a complex phenotype that cannot be explained by single virulence factors,[66] and that virulence must be considered from a holistic point of view. For this reason, the characterization of the immune response of *G. mellonella* during cryptococcal infection described in this work will contribute to the understanding of the pathogenesis of this microorganism. Second, our findings expand the use of *G. mellonella* to study fungal virulence. Classically, this model host has been used to investigate antifungal efficacy and differences in virulence between strains and species. Finally, our data expands the use of *G. mellonella* for more "sophisticated" studies, as it has been already shown in the case of some pathogenic bacteria, which demonstrated that this model host can be used to investigate brain infections and maternal transfer of immunity[25,67] (see comment in[68]).

Material and Methods

Yeast strains and growth conditions. In this work we used the following *C. neoformans* strains: H99 (variety *grubii*,[69]), a derivative from this strain which expresses the green fluorescent protein,[70] B3501 (variety *neoformans*,[71]) and the acapsular strain C536 (*cap59*,[72] derived from B3501). In addition, *C. albicans* SC5314 strain[73] was used in some experiments. The cells were routinely grown in Sabouraud solid (Oxoid, Reference CM0041) or liquid media (Oxoid, Reference CM0147). In the case of liquid media, the cultures were incubated at 30°C with moderate shaking (150 r.p.m.). In some experiments, the size of the capsule was induced by placing the cells overnight in 10% Sabouraud at pH 7.3 buffered with 50 mM MOPS (Sigma Aldrich, Reference M1254) buffer.[74] When indicated, the yeast cells were killed by incubation at 65°C for 30 min.

Insects and incubation conditions media. *Galleria mellonella* was obtained from Alcotán (Valencia, Spain). The larvae were selected by weight (0.2–0.3 g) and by the absence of dark spots on the cuticle. The larvae were maintained at room temperature, and the day before the experiment, they were transferred to the temperature at which the experiment was going to be performed (30 or 37°C, as indicated).

Injection

Galleria mellonella larvae were infected with different pathogen doses (ranging from 10^4 to 10^6 cells/larva as described[34] (a visual demonstration can be found in Youtube (https://www.youtube.com/watch?v=2XEu_5aF1qk)). The larva abdomen was cleaned with 70% ethanol and the yeast suspensions prepared in PBS (137 mM NaCl, 2,7 mM KCl, 10 mM Na$_2$HPO$_4$, 1,8 mM KH$_2$PO$_4$, pH 7,3) were injected in the hemocele through the last right pro-leg of the larvae using a 10 μL Hamilton syringe (Hamilton, USA, Reference 80366, distributed by Fisher Scientific, Reference W0166E).

Lytic activity of the hemolymph

The presence of antimicrobial peptides in the hemolymph was performed as described[67] with some modifications. This assay is based on the degradation of the bacteria *M. luteus* by lytic peptides and enzymes from the hemolymph (mainly lysozyme). Briefly, plates containing *M. luteus* (1.7 mg/mL, Sigma Aldrich, Reference M3770–5G), phosphate buffer (Na$_2$HPO$_4$, Panreac, Reference 121512, 67 mM, pH 6.4) and 1 % agar (BD, Reference 214010) were prepared, and 2 mm holes were punched in the agar. Then, 3 microliters of recently isolated hemolymph were placed in these wells. In parallel, the same volume of lysozyme prepared at different concentrations (0.25; 0.03 and 0.0039 mg/L, Sigma Aldrich, Reference L6876) was placed as internal standard in each plate. The plates were incubated for 4–5 hours at 37°C, and then, pictures were taken using a Nikon D60 digital camera. The width of the halos was measured with Adobe Photoshop (Adobe, San Jose, CA), and lytic activity was calculated for each sample using the internal controls of lysozyme present in each plate. To test the effect of capsular polysaccharide on the accumulation of antimicrobial peptides, GXM was purified as described.[75] After the final lyophilization, the polysaccharide was dissolved in sterile distilled water at 15 mg/mL. Serial dilutions were prepared (10, 5 and 2.5 mg/mL), and 10 μL were injected in groups of 10 larvae (corresponding to 150, 100, 50 and 25 μg/larva). For this particular experiment, the control larvae were injected with 10 μL of sterile distilled water.

In vivo microscopy of infected hemocytes

Galleria mellonella larvae were infected with 10^6 *C. neoformans* cells, and after 3 hours of incubation at 37°C, the hemolymph was collected and centrifuged in a bench centrifuge at 12,000 r.p.m. (13,800 g). The pellet was suspended in Schneider's medium (Sigma Aldrich, Reference S0146) supplemented with 10% of fetal bovine serum (HyClone-Perbio Thermo Fisher Scientific, Reference SV30160). Two hundred microliters of this suspension were placed in a 96-well plate (Corning Inc., Reference 3599), and videos were taken as described.[76] Briefly, the plate was placed under a Leica DMI 4000B microscope and observed with a 20x lens objective. This microscope had a temperature-regulated chamber, which was adjusted at 28°C. Pictures were taken every 3 min overnight. Pictures were exported as .avi documents and processed with ImageJ (http://rsb.info.nih.gov/ij).

Hemolymph melanization

Hemolymph melanization assay was performed as described.[29] Briefly, 10 larvae were infected with 10^6cells/larva of *C. neoformans* H99 or *C. albicans* SC5314 and incubated at 37°C for 1 and 3 hours. After the incubation period the hemolymph were collected and diluted 1:10 in cold PBS and centrifuged at 12,000 rpm (13,800 g) in a Heraus Fresco 21 bench centrifuge (Thermo Electron Corporation). The supernatants were placed in a 96 well microdilution plate (Corning Inc., Reference 3599), and optical density determined at 405 nm in a iEMS Reader MS (LabSystems, Thermo Scientific). PBS-injected larvae were used as control.

Binding of mAbs to the *C. neoformans* capsule after passage through *G. mellonella*

Galleria mellonella larvae were infected with 10^6 *C. neoformans* cells as described above and incubated at 37°C for 24 hours. After this time, the hemolymph was collected and centrifuged. The pellet, containing *C. neoformans* cells, was washed with PBS and suspended in PBS with 1% of bovine serum albumin (BSA, Sigma Aldrich, Reference A4503–50G). After 30 min of incubation at 37°C, mAbs to the capsule 18B7, 2H1 (both mouse IgG1) and 2D10 (mouse IgM)[77] were added at 1 μg/mL. Monoclonal antibody 18B7 was directly conjugated to Alexa 488 using the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Life Technologies, Reference A10235) following the manufacturer's recommendations. The cells were incubated for 30 min at 37°C, and washed with PBS/BSA. In the case of samples incubated mAb 2H1 and 2D10, a goat anti-mouse (GAM) IgG-Alexa 488 (Life Technologies, Reference A11001) or GAM IgM secondary Ab conjugated to Alexa 488 (Life Technologies, Reference A21042) was added at 1 μg/mL, respectively. The cells were washed with PBS/BSA and suspended in 50 μL of same buffer. Cells were placed on a glass slide and mounted with Fluoromount G (Southern Biotech, Reference 0100–01). Fluorescence binding pattern was visualized in a Leica DMI3000B fluorescence microscope coupled to a Leica DFC300 digital camera and pictures were taken using the LAS 3.3.1 software (Leica Microsystems). To quantify the fluorescence intensity, parallel samples without the primary antibodies, but with the secondary antibodies, were prepared. Fluorescence of the cells was analyzed in a FACS Calibur flow cytometer (BD) in the FL1 channel. Data was processed with FlowJo software (FlowJo, LLC). In addition, and as controls, Ab binding was evaluated in parallel in cells grown in Sabouraud and in capsule growth inducing conditions (10% Sabouraud, pH 7.3, see above).

Statistics

Data was analyzed with GraphPad 5.0 software (GraphPad Software, La Jolla California USA). Normality of the samples was assessed with the Kolgomorov-Smirnov (KS) test. In case of normality, data was analyzed with ANOVA and T-test. When samples were not normally distributed ($p < 0.05$ in the KS test), statistical differences were evaluated with the non-parametric tests Kruskal-Wallis and Mann-Whitney. In all cases, statistical differences were considered when $p < 0.05$.

Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website at http://dx.doi.org/10.4161/21505594.2014.986412

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