Mechanisms of Dendritic Cell Lysosomal Killing of Cryptococcus

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Cryptococcus neoformans is an opportunist pulmonary fungal pathogen that disseminates to the CNS causing fatal meningitis in immunocompromised patients. Dendritic cells (DCs) phagocytose C. neoformans following inhalation. Following uptake, cryptococci translocate to the DC lysosomal compartment and are killed by oxidative and non-oxidative mechanisms. DC lysosomal extracts kill cryptococci in vitro; however, the means of antifungal activity remain unknown. Our studies determined non-oxidative antifungal activity by DC lysosomal extract. We examined DC lysosomal killing of cryptococcal strains, anti-fungal activity of purified lysosomal enzymes, and mechanisms of killing against C. neoformans. Results confirmed DC lysosome fungicidal activity against all cryptococcal serotypes. Purified lysosomal enzymes, specifically cathepsin B, inhibited cryptococcal growth. Interestingly, cathepsin B combined with its enzymatic inhibitors led to enhanced cryptococcal killing. Electron microscopy revealed structural changes and ruptured cryptococcal cell walls following treatment. Finally, additional studies demonstrated that osmotic lysis was responsible for cryptococcal death.

Cryptococcus neoformans is a ubiquitous encapsulated opportunistic fungal pathogen that causes pneumonia and meningitis in immunocompromised patients, especially in recipients of organ transplants or individuals with AIDS. The infection is acquired through the inhalation of desiccated yeast mostly from soil contaminated with pigeon droppings. Although C. neoformans is most often acquired via the pulmonary route, few patients present with a clinical picture of pneumonia (reviewed in ). The predominant protective immune response to C. neoformans is cell-mediated immunity (CMI) primarily by Th1-type CD4 T cells. However, the first line of defense against C. neoformans is the innate immune system. The adaptive immune system must be primed by innate antigen-presenting cells (APCs), most important dendritic cells (DCs), as DCs are the predominant APC capable of antigen presentation to naive T cells. DCs are considered sentinels of the immune system, and can be especially important in surveillance at mucosal sites, such as the lung. DCs phagocytose, degrade, and process extracellular foreign organisms for antigen presentation primarily on class II MHC to CD4 T helper cells to initiate the adaptive immune response. In addition to their role as APCs, DCs and other innate cells can be involved in direct microbicidal activity against pathogens. DCs have been shown to be involved in killing C. neoformans as well as other pathogenic fungi.

In vitro, DCs phagocytose C. neoformans following opsonization with complement or antibody, and the organism can be killed by both oxidative and non-oxidative mechanisms. In vivo studies showed that C. neoformans can be phagocytosed by murine pulmonary DCs. After phagocytosis by DCs, C. neoformans enters the endosomal compartment and traffics to the lysosomal compartment, where it is degraded by the lysosomal components and processed for antigen presentation. Following cryptococcal uptake and processing, DC maturation is evident by increased surface expression of MHC II and the costimulatory molecules CD80 and CD86. These DCs are then able to present antigen to cryptococcal-specific T cells and induce T cell proliferation. Crude lysosomal extracts generated from murine bone-marrow-derived DCs (BMDCs) exhibit anticytotoxic activity in vitro in a dose-dependent manner.

Lysosomes are cellular organelles involved in the degradation of phagocytosed/endocytosed and intracellular material. They are the site of MHC class II loading and are important sites for killing and degradation of phagocytosed material, including pathogens. DC lysosomes contain an assortment of endopeptidases, exopeptidases, esterases, and reductases, but the majority of the proteases are the cysteine proteases such as cathepsin B, L, and S and the aspartate proteases cathepsin D and E. Other cysteine proteases are also present in lysosomes but are more closely related to the caspases. Cathepsins D and L act as endoproteases, and cathepsin B has poor endoproteolytic activity but is a strong carboxypeptidase; nonetheless, each has a role in antigen processing. Cathepsin L-deficient mice...
cannot process the invariant chain (II) and have defects in MHC II antigen presentation25. Cathepsins B and D also play a role in Ii processing and MHC II presentation23,24. Because of their importance in antigen degradation and processing, we primarily focused on the cathepsins as potential antifungal agents. In addition to enzymes, lysosomes also contain small peptides called antimicrobial peptides (AMPs), such as α- and β-defensins and the cathelicidins35–37. AMPs work by causing rupture of the membranes of bacteria, viruses, parasites, and fungi, or by interfering with DNA and RNA33–35.

In the present study, we determined DC lysosomal components that may play a role in the killing of Cryptococcus. Accordingly, we examined the antifungal activity of the DC lysosomal extract against four serotypes of Cryptococcus, tested the ability of purified lysosomal cathepsins to kill the organism, and blocked enzymatic activity of the cathepsins that demonstrated anti-cryptococcal activity. Finally, we investigated mechanisms of anti-cryptococcal activity by these lysosomal components.

Results
DC-derived lysosomal extract kills Cryptococcus strains in vitro. Previous studies have shown that DC-derived lysosomal extract can kill C. neoformans serotype A strain 145 in vitro20. To confirm DC lysosomal activity against other cryptococcal strains and serotypes, C. neoformans serotype A strain H99, C. gattii serotype B strain R265, C. gattii serotype C strain WSA87, and C. neoformans serotype D strain R4249 yeast cells were individually incubated in phosphate buffer alone or in phosphate buffer with lysosomal extract for 24 h at 37°C, following which the CFU in the wells were determined. We found that the DC-derived lysosomal extracts have significant antifungal activity against all cryptococcal strains tested, leading to complete killing of each strain of Cryptococcus (Figure 1). Because all strains and serotypes were equally affected, further studies were conducted using the highly pathogenic C. neoformans strain H99.

Purified lysosomal enzymes inhibit cryptococcal growth in vitro. Many lysosomal components of DCs can contribute to killing and degradation of phagocyted organisms. Lysosomal enzymes, such as cathepsins, are known to be involved in intracellular degradation and processing for antigen presentation36,37. Therefore, we chose to examine the role of cathepsins in the killing of C. neoformans strain H99. In order to examine the role of cathepsins in cryptococcal killing or inhibition, C. neoformans strain H99 yeast cells were incubated in phosphate buffer with 10 μg/ml or 50 μg/ml of cathepsin B (Figure 2A), cathepsin D (Figure 2B), or cathepsin L (Figure 2C) for 24 h at 37°C, following which the CFUs were determined. These concentrations were chosen to represent physiological concentration found in cell lysates (10 μg/ml38,39) and excess concentration (50 μg/ml). Results showed that cathepsin B significantly inhibited the growth of C. neoformans at 10 μg/ml, and at 50 μg/ml showed even greater inhibition almost to inocula levels (Figure 2A). Incubation of C. neoformans with cathepsin D at 10 μg/ml and 50 μg/ml had no effect on the growth when compared to growth in the phosphate buffer alone (Figure 2B). Cathepsin L inhibited the growth of C. neoformans at both 10 μg/ml and 50 μg/ml compared to the growth in the phosphate buffer alone, but this was not a statistically significant difference (Figure 2C). Because the only significant inhibition of C. neoformans growth was observed following incubation with cathepsin B, further studies focused only on cathepsin B.

Confirmation of the presence of cathepsin B in BMDC lysosomal extracts. In order to definitively confirm that cathepsin B was present in our DC lysosomal extract, the extract was examined for the presence of cathepsin B by Western blot analysis. Proteins from two separate preparations of DC lysosomal extract were separated by SDS-PAGE and transferred to a PVDF membrane. Blots were probed with anti-human/mouse cathepsin B monoclonal antibody. Recombinant mouse cathepsin B in the pro-form (39 kDa), was used as a positive control. Active cathepsin B is approximately 25–26 kDa. The Western blot showed the presence of cathepsin B in two separate preparations of BMDC lysosomal extract (Figure 3A). We next determined the concentration of cathepsin B in the BMDC lysosomal extracts by ELISA. Aliquots of each step during the lysosomal purification process were examined to quantify the amount of cathepsin B present. The ELISA showed that an average amount of 4000 pg/mg protein of cathepsin B is present in BMDC lysosomal extracts (Figure 3B).

Enzymatic inhibition of cathepsin B activity does not affect its ability to inhibit the growth of C. neoformans. Given our observations that the 50 μg/ml concentration of cathepsin B significantly inhibited the growth of C. neoformans strain H99 when compared to the growth in the phosphate buffer alone, we determined whether the inhibition of growth could be reversed by inhibiting the enzymatic activity of cathepsin B. For this, C. neoformans strain H99 yeast cells were incubated in phosphate buffer alone, in phosphate buffer with either cathepsin B, an inhibitor, or cathepsin B plus an inhibitor for 24 h at 37°C, following which the CFUs were determined. The inhibitors used were calpain inhibitor at 10 μg/ml, cathepsin B & L.
inhibitor at 50 or 100 μM, and CA-074 at 20 μM. Calpain inhibitor is only soluble in DMSO, which is toxic to C. neoformans at higher concentrations, so higher concentrations of the calpain inhibitor could not be used in our assay (data not shown). CA-074 is a specific inhibitor of only cathepsin B, cathepsin B&L inhibitor inhibits cathepsin B and L only, and calpain is a general inhibitor of cathepsin activity. However, because our assays use only purified cathepsin B and not the entire DC lysosomal extract, the specificity of the inhibitors is not a factor. Surprisingly, our results showed that the addition of any cathepsin B inhibitor, at the concentrations used, enhanced the antifungal activity of cathepsin B rather than abrogating its activity (Figure 4A). To determine whether this observation was a result of the failure of the inhibitors to prohibit enzymatic activity of cathepsin B, we measured the percent inhibition of the cathepsin B enzymatic activity with each inhibitor (Figure 4B). We found that each inhibitor did significantly inhibit the enzymatic activity of cathepsin B. The calpain inhibitor showed 47.34% inhibition (52.66% activity) at 10 μg/ml, the cathepsin B&L inhibitor showed 55.67% inhibition (44.33% activity) at 50 μM and 95.83% inhibition (4.13% activity) at 100 μM, and CA-074 showed 95.69% inhibition (4.31% activity) at 20 μM, suggesting that cathepsin B may have non-enzymatic activity against C. neoformans.

Microscopy of C. neoformans treated with cathepsin B and cathepsin B + inhibitors show cryptococcal structural changes and cell wall rupture. Due to the killing observed following incubation with cathepsin B and its inhibitors, we were interested in determining their effects on the fungus during in vitro incubation. To assess this, scanning electron microscopy (SEM) (Figure 5A) or transmission electron microscopy (TEM) (Figure 5B) was performed on C. neoformans after growth in the presence of phosphate buffer, cathepsin B, calpain inhibitor, CA-074, or cathepsin B + an inhibitor for 12 h. We chose to examine the cells after 12 hours of incubation in order to observe the effects of cathepsin B and either calpain inhibitor or CA-074 on live cells, since 24 h of incubation results in complete killing of C. neoformans. SEM of C. neoformans strain H99 showed structural changes in the organism following incubation with cathepsin B and cathepsin B + calpain inhibitor or CA-074 compared to untreated conditions (Figure 5A). TEM of C. neoformans strain H99 showed a crescent morphology indicative of structural changes and possible cell death due to cathepsin B and cathepsin B + calpain inhibitor or CA-074 treatment compared to untreated cells or cells treated with calpain inhibitor or CA-074 alone (Figure 5B). In addition, cells showing structural changes also show ruptured cell walls but intact cell membranes (Figure 5B). Quantification of EM data showed altered cryptococcal cell morphology (crescent-shaped) in 10% of untreated cells and in 17% and 15% of calpain or CA-074 treated cells, respectively. Further, cells treated with cathepsin B, calpain inhibitor + cathepsin B, or CA-074 + cathepsin B had statistically higher percentages of altered cell morphology (27%, 27%, and 25%, respectively) compared to cells treated with either untreated cells or compared to cells treated with inhibitors alone (P<0.0001). In support of these data, confocal microscopy using live BMDCs showed that C. neoformans (green) trafficking into DC lysosomal compartments (red) also adopts a similar crescent-shaped morphology (Figure 5C).

Cathepsin B and inhibitor treatment result in osmotic lysis of Cryptococcus neoformans. Our results in Figure 5 showed that incubation with cathepsin B and calpain inhibitor lead to a compromised cell wall of C. neoformans. We hypothesized that the cell wall ruptures induced by cathepsin B and inhibitor treatment enhanced osmotic lysis of the cryptococcal cells, resulting in death. To examine this, C. neoformans strain H99 yeast cells were incubated in phosphate buffer alone or in phosphate buffer with 50 μg/ml cathepsin B and 10 μg/ml calpain inhibitor for 24 h at 37°C, following which the CFU in the wells were determined by diluting and plating on YPD agar or osmotically supportive YPD agar containing 1.4 M sorbitol. By plating on the osmotically supportive YPD agar, we almost completely abrogated the killing of C. neoformans (Figure 6). An alternative approach, electron microscopy (EM) was performed following incubation in YPD + 1.4 M sorbitol broth using cathepsin B with the specific inhibitor CA-074. Both TEM and SEM confirmed that the addition of sorbitol reversed the crescent-shaped appearance of the organism (Figure 6B and 6C). Therefore, we conclude that the cathepsin B plus inhibitor treatment leads to osmotic lysis of the fungus.

Discussion
While many studies have shown that neutrophils and macrophages are able to kill fungal organisms, little is known about the mechanisms of DC killing of fungal organisms. Studies with other pathogenic fungi have shown that there are different outcomes based on which immune cell type encounters the organism. For Histoplasma capsulatum, macrophages are permissive for fungal growth, while...
Figure 3 | Confirmation of the presence of Cathepsin B in BMDC lysosomal extracts. Lysosomal extracts prepared from BMDCs were examined for the presence of cathepsin B. For ELISA analysis, fractions were taken during each step of the lysosomal purification process to examine the amount of cathepsin B present. Recombinant mouse cathepsin B in the pro-form was used as the positive control for Western blot analysis and for the ELISA standard. Cathepsin B was detected by separating the lysosomal extracts through SDS-page then transferring to a PVDF membrane and probing with anti-human/mouse cathepsin B. Recombinant mouse cathepsin B in the pro-form is 39 kDa, and active cathepsin B is 25-26 kDa. A). Western blot analysis of cathepsin B shows the presence of cathepsin B in two different samples of BMDC lysosomal extract. The blot is representative of three separate experiments. B) ELISA shows that 4000 pg cathepsin B per mg protein is present in BMDC lysosomal extracts. Aliquots of the different stages in the lysosomal purification process showed that cathepsin B is found primarily in the lysosomal fraction. Data shown are means ± standard error of the mean (SEM) of three independent experiments.

Figure 4 | Inhibition of cathepsin B enzymatic activity enhances the antifungal effects against C. neoformans. C. neoformans strain H99 yeast cells were incubated in phosphate buffer alone, in phosphate buffer with either 50 μg/ml cathepsin B, 10 μg/ml calpain inhibitor, 50 or 100 μM cathepsin B & L inhibitor, 20 μM CA-074, or cathepsin B plus an inhibitor for 24 h at 37 °C, following which the numbers of CFU in the wells were determined. A) Data shown are means ± standard errors of the means (SEM) of the cumulative results of 3 independent experiments. An asterisk * indicates a significant difference compared to the results for H99 in phosphate buffer alone (p < 0.05) and τ indicates a significant difference compared to the initial the inocula (white bar) (p < 0.05). B) The percent cathepsin B enzymatic activity following incubation with each different inhibitor was measured using the Cathepsin B Activity Assay Kit. Data shown are means ± standard errors of the means (SEM) of the cumulative results of 3 independent experiments. Asterisks * show significant differences in activity compared to cathepsin B alone (p < 0.05).
DCs kill the organism\(^1\). While DCs and macrophages can both recognize \textit{H. capsulatum}, different receptors involved in recognition can lead to different fates for the fungus\(^4\). Macrophages recognize \textit{H. capsulatum} heat-shock protein 60 by the CD18 integrin, which leads to uptake into an environment in which the fungus is able to live and replicate\(^4\). In contrast, DCs are able to kill \textit{H. capsulatum}, and even though they possess the CD18 integrin on their surface, DCs recognize cyclophilin A on \textit{H. capsulatum} using the fibronectin receptor VLA-5\(^3\). DC killing of \textit{H. capsulatum} is due to phagolysosomal fusion and DC lysosomal hydrolases\(^1\).\(^4\). Human DCs are also able to phagocytose, kill, and process \textit{Candida albicans} for antigen presentation\(^1\). The DCs were able to kill \textit{Candida} as efficiently as macrophages through non-oxidative mechanisms\(^8\). An understanding of the mechanisms of DC lysosomal killing is an important area of research due to the ability of \textit{Cryptococcus} to live and replicate inside macrophages during a pulmonary infection\(^4\).

DCs must be able to kill, process, and present antigen to initiate the adaptive immune response. Thus, it is important to understand how DCs are able to kill \textit{C. neoformans} and other fungi. DCs have been shown to phagocytose \textit{C. neoformans}, where it can be degraded in the lysosomal compartment and processed for antigen presentation\(^2\). They have been shown to kill \textit{C. neoformans} by both oxidative and non-oxidative mechanisms\(^2\). Our current studies focused on the non-oxidative mechanisms, such as the lysosomal cathepsin enzymes. We chose to focus on the cathepsins for these studies because they are known lysosomal enzymes that are important in degradation of antigens destined for presentation by DCs. Due to the role of DCs as antigen presenting cells (APCs), it is reasonable that

Figure 5 | Microscopy of \textit{C. neoformans} treated with cathepsin B and cathepsin B + inhibitors show cryptococcal structural changes. \textit{C. neoformans} was grown in the presence of cathepsin B, calpain inhibitor, CA-074, or cathepsin B with inhibitors for 12 h, fixed with 4% formaldehyde/1% glutaraldehyde, prepared for electron microscopy, and examined by SEM or TEM. A) SEM of \textit{C. neoformans} strain H99 shows structural changes (arrows) in the organism following incubation with cathepsin B and cathepsin B + inhibitors, but not in untreated conditions. B) TEM of \textit{C. neoformans} shows structural changes in cells treated with cathepsin B and cathepsin B + an inhibitor, but not in untreated cells or in those treated with only calpain inhibitor or CA-074. In addition, cells showing structural changes (arrows) also show ruptured cell walls but intact cell membranes. Data shown are representative of 30 fields per condition imaged. Magnification is 5,000 X for SEM images and 25,000 X for TEM images. C) Purified BMDCs were incubated with \textit{C. neoformans} yeast cells at a 2:1 ratio with 1 µg/ml of Oregon green-labeled 3C2 opsonizing antibody and 50 nM LysoTracker red. Samples were incubated at 37°C for 1 hour prior to imaging. A representative confocal image of \textit{C. neoformans} organisms (green) shown in a LysoTracker red-positive compartment (red) with a similar crescent-shaped morphology as seen through electron microscopy. Confocal image is representative of 3 experiments performed, 30 fields imaged per experiment.
killing the phagocytosed organism would be advantageous, as it would facilitate antigen presentation. Furthermore, due to cathepsin B’s role in digesting peptides for MHC class II antigen presentation and our findings that cathepsin B has anti-fungal activity against *C. neoformans* strain H99, it is likely that this enzyme has dual roles in both killing and in antigen presentation in DCs following phagocytosis of the organism.

While our data show that cathepsin B can inhibit the growth of *C. neoformans*, we recognize that there are other lysosomal components likely participating in the killing of *C. neoformans*, and cathepsin B does not act alone. Current studies are focused on fractionation of DC lysosomal extracts to identify additional lysosomal components involved in antifungal activity. We also have preliminary data to suggest that DC lysosomal extract has antifungal activity against other pathogenic fungi including *Candida albicans* and *Coccidioides immitis* (unpublished observations), and we will continue to explore the mechanisms of antifungal activity. In this study, we found that the purified lysosomal cathepsin B has inhibitory effects on the growth of *C. neoformans*. The inhibition is concentration-dependent, and we have demonstrated that cathepsin B is found in our lysosomal extracts. Inhibition of purified cathepsin B activity with the three different inhibitors resulted in enhanced activity. While the inhibitors varied in their specificity for cathepsin B, using general cathepsin inhibitors was not an issue due to the fact that we only used purified cathepsin B for our assays. Our data suggest that DC lysosomal cathepsins play a role in the antifungal activity of lysosomal extracts against *C. neoformans*, and interestingly, the inhibition of the enzymatic activity of cathepsin B resulted in enhanced antifungal activity. Currently, we do not know the mechanism of this enhanced effect, and studies are underway to explore this phenomenon.

One possible mechanism for the action of cathepsin B with its inhibitors in killing *Cryptococcus* is that they may act as antimicrobial peptides, due to the cell wall ruptures observed by TEM (Figure 5). Antimicrobial peptides (AMPs) are part of the innate immune system and are found primarily in the skin and the mucosal surfaces. They can also be found in certain immune cells such as neutrophils. The majority of AMPs work by causing a disruption in the cell wall of the organism, which correlates with our data presented in these
studies. They accomplish this by forming pores in the cell wall or by coating the organism, and when a critical density is reached this causes significant curvature strain that ultimately leads to cell wall rupture (reviewed in ). AMPs have been shown to be important in fungi, . There has been increased interest in AMPs recently in the host defense against bacteria, viruses, parasites, and other cells were harvested on day 8 following plating. DCs were purified by positive selection of cathepsin B leads to enhanced killing of the organism. We demonstrated that treatment with cathepsin B and its inhibitors lead to structural changes and rupture of the fungal cell wall, similar to the structural changes and rupture that have been described in studies. Strains were resuspended in crude lysosomal extract and plated at a concentration of 2 × 10^5 cells/ml in complete medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg of streptomycin/ml, and 50 mM 2-mercaptoethanol (complete medium). Mice. Female BALB/c (H-2d) mice were purchased from the National Cancer Institute/Charles River Laboratories and were housed at The University of Texas at San Antonio Small Animal Laboratory Vivarium. Mice were handled according to approved guidelines, and the study and experimental design was approved by The University of Texas at San Antonio Institutional Animal Care and Use Committee (IACUC).

Culture of Cryptococcus. Cryptococcus neoformans serotype A strain H99, Cryptococcus gattii serotype B strain R265 (gift of Joseph Heitman, Duke University Medical Center, Durham, NC), C. gattii serotype C strain WA87 (gift of Brian Wicks, University of Texas Health Science Center; San Antonio, TX), and C. neoformans serotype D strain R4249 were recovered from 15% glycerol stocks stored at −80°C and maintained on a yeast-extract-peptone-dextrose (YPD) media (Difco). Yeast cells were grown for 18 h at 30°C with shaking in YPD broth. Cells were centrifuged and washed three times with sterile phosphate-buffered saline (PBS). Viable yeast cells were quantified using Trypan blue dye exclusion in a hemacytometer.

BMDCs. BMDC culture was performed as previously described . Briefly, bone marrow cells were flushed from the femurs and tibiae of mice. Cells were centrifuged and plated at a concentration of 2 × 10^5 cells/ml in complete medium supplemented with 20 ng/ml recombinant murine GM-CSF (Peprotech, Rocky Hill, NJ). Cells were incubated at 37°C, 5% CO2. Half of the medium was replaced every three days, and cells were harvested on day 8 for cytokine analysis. BMDCs were purified by positive selection using magnetically labeled CD11c antibodies according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA).

Generation of crude lysosomal extract from BMDCs. Crude BMDC lysosomal extracts were obtained as previously described . The lysosomal isolation kit (Sigma-Aldrich, St. Louis, MO) was used. Briefly, lysed DCs were centrifuged at 1000 x g for 10 minutes to remove intact cells and cellular debris. The supernatant was removed and centrifuged at 20,000 x g for 20 minutes to pellet lysosomes. The pellet containing lysosomes was resuspended and sonicated for 20 seconds at 40% power on a Model 300 VT Ultrasonic Homogenizer (Bio-Logics, Inc., Manassas, VA). The resulting crude lysosome extract was stored at −80°C until use.

In vitro examination of cryptococci in BMDCs. Purified BMDCs were incubated with C. neoformans yeast cells at a 2:1 ratio with 1 μg/ml of Oregon green-labeled 3C2 panning antibody (gift of Thomas Kozel, University of Nevada, Reno, NV) and 50 nM Lysotracker red (Molecular Probes). Samples were incubated at 37°C for 1 hour prior to imaging. Confocal imaging was performed on a Leica TCS S2P inverted confocal microscope (Leica Microsystems, Wetzlar, Germany), and Leica confocal software (LCS) was used for the acquisition of images. The images were viewed using a 63x, 1.40-number-aperture oil-immersion Leica lens and were digitally increased in size fourfold using LCS.

Incubation of lysosomal extracts with Cryptococcus. Cryptococcal killing assays were performed as previously described . Strains were resuspended in 10 mM phosphate buffer with 2% RPMI, pH 5.5 (phosphate buffer). Fungi were added to a 96-well plate in a volume of 50 μl (2.5 × 10^6/ml). Phosphate buffer was added to control wells, and 50 μl of lysosomal extract were added to test wells, for a total volume of 100 μl/well in duplicate wells. Plates were incubated for 24 hours at 37°C, 5% CO2. Cryptococci were titrated in sterile PBS and plated on YPD agar to determine colony forming units (CFU). Killing was defined as CFU significantly below the inoculum, and inhibition was defined as CFU significantly below that of growth in phosphate buffer alone.

Incubation of purified lysosomal enzymes with Cryptococcus. Purified cathepsins B, D, and L (Enzo Life Sciences, Inc., Farmingdale, NY) at 10 μg/ml (physiological concentration) and 50 μg/ml (excess concentration) were incubated with C. neoformans as previously described for lysosomal extract experiments. All enzymes were commercially manufactured and 4-nitroaniline. Plates were incubated for 24 hours at 37°C, 5% CO2. Following incubation, organisms were plated for CFU. Cathepsin B Western blot. For Western blotting, 15 μl of the lysosomal extracts (10 μg protein) or 15 μl (1500 pg) of recombinant mouse cathepsin B (R & D Systems, Minneapolis, MN) were mixed with 5 μl of 4X Laemmli buffer and boiled for 5 minutes. Recombinant mouse cathepsin B in the pro-form (39 kDa) was used as a positive control. Active cathepsin B is approximately 25–26 kDa. For SDS-PAGE, 5 μl of Precision Plus Protein Dual Color Standards (BIO-RAD) and 20 μl of each lysosomal extract and purified cathepsin B (positive control) were loaded into a 10% MINI-PROTEAN TGX gel (BIO-RAD). The gel was run for 40 minutes at 200 V, transferred onto a PVDF membrane (Thermo Scientific, Rockford, IL) and blocked with 5% skim milk in Tris-buffered saline with 1% Tween 20 (TBS-T) at room temperature. After 1 h, the membrane was incubated with 1 μg/ml anti-human/mouse cathepsin B (R & D Systems) in 5% milk/TBS-T for 2 hours at room temperature. The membrane was washed three times for 5 min and then incubated with anti-rat IgG HRP (R & D Systems) at a 1:1000 dilution in 5% milk/ TBS-T for 1 h at room temperature. The membrane was washed three times for 5 min with TBS-T and then two times for 10 min with TBS followed by incubation with SuperSignal West Dura Extended Duration substrate (Thermo Scientific) for 5 minutes. The membrane was exposed to CL-XPosure Film (Thermo Scientific) and developed using an AFP Imaging Mini-Medical series developer (Medlink Imaging, Teterboro, NJ).

Cathepsin B ELISA. Total cathepsin B in lysosomal extracts was quantified by ELISA. The capture and detection antibodies and recombinant mouse cathepsin B (used for the standard) were purchased from R & D Systems (Minneapolis, MN). Microtiter plates were coated with 1 μg/ml of anti-human/mouse cathepsin B diluted in PBS and incubated overnight at 4°C. After washing three times with PBS-Tween buffer (1X PBS and 0.05% Tween 20), the wells were blocked with 10% skim milk at room temperature for 1 h. After washing, the plates were washed three times and the samples were added. After 2 h incubation at room temperature, the plates were washed three times. Then, biotinylated anti-mouse cathepsin B diluted to 1 μg/ml in reagent diluent (1% goat serum in 1X PBS) was added. After incubation for 1.5 h at room temperature, the plates were washed and streptavidin-HRP (R & D Systems) was added. After 25 minutes, the plates were washed 5 times, and 1X TMB substrate (3,3′,5′-Tribromoindoline, San Diego, CA) was added and incubated in the dark for 15–20 minutes. The reaction was stopped by adding 2N H2SO4. Samples were read at 405 nm on a BioTek EL808 automated plate reader (BioTek, Winooski, VT) and analyzed with Gen 5 software (BioTek).

Cathepsin B inhibition. Cathepsin inhibitor (Sigma-Aldrich Co), cathepsin B & L inhibitor (BioVision, Inc., Mountain View, CA), or CA-074 (Enzo Life Sciences) was added to C. neoformans at 10 μg/ml, 50 or 100 μM, or 20 μM, respectively, and incubated with or without purified cathepsin B at 50 μg/ml, following the same procedure indicated above. Following 24 h incubation, organisms from wells were diluted in sterile PBS and plated on YPD agar to determine CFU/ml. For osmotic stress experiments, cathepsin B and cathepsin inhibitor were incubated with C. neoformans strain H99 for 24 hours at 37°C, 5% CO2, and then either plated on YPD agar or on osmotically supportive YPD containing 1.4 M sorbitol. Percent cathepsin
**Electron microscopy.** Cryptococcus neoformans strain H99 (serotype A) was incubated with cathepsin B and/or inhibitors as described above for 12 hours. For osmotic stress EM experiments, cells were incubated for 24 h then transferred to YPD or YPD + 1.4 M sorbitol broth for 8 h at 30°C. Cells were fixed with 4% formaldehyde/1% glutaraldehyde and processed for scanning electron microscopy (SEM) or transmission electron microscopy (TEM) by the electron microscopy staff. Images were examined at 2500X and 5000X for SEM and 25000X and 40000X for TEM.

**Statistical analysis.** One-way ANOVA with the Tukey’s multiple comparison test was used to compare CFU using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Significant differences were defined as p < 0.05.

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**Author Contributions:**

CRH and KLW wrote the main manuscript text, CRH, HB and KLW prepared all figures, CRH, HB and KLW conducted the experiments. HB, CRH, KLW and F.L.W analyzed the data. All authors reviewed the manuscript.
