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Macrophage Mitochondrial and Stress Response to Ingestion of Cryptococcus neoformans

Carolina Coelho,*‡§ Ana Camila Oliveira Souza,§ Lorena da Silveira Derengowski,§ Carlos de Leon-Rodriguez,* Bo Wang,*‡ Rosiris Leon-Rivera,‖‡# Anamelia Lorenzetti Bocca,§ Teresa Gonçalves,‡ and Arturo Casadevall*

Human infection with Cryptococcus neoformans, a common fungal pathogen, follows deposition of yeast spores in the lung alveoli. The subsequent host–pathogen interaction can result in eradication, latency, or extrapulmonary dissemination. Successful control of C. neoformans infection is dependent on host macrophages, but macrophages display little ability to kill C. neoformans in vitro. Recently, we reported that ingestion of C. neoformans by mouse macrophages induces early cell cycle progression followed by mitotic arrest, an event that almost certainly reflects host cell damage. The goal of the present work was to understand macrophage pathways affected by C. neoformans toxicity. Infection of macrophages by C. neoformans was associated with alterations in protein translation rate and activation of several stress pathways, such as hypoxia-inducing factor-1α, receptor-interacting protein 1, and apoptosis-inducing factor. Concomitantly we observed mitochondrial depolarization in infected macrophages, an observation that was replicated in vivo. We also observed differences in the stress pathways activated, depending on macrophage cell type, consistent with the nonspecific nature of C. neoformans virulence known to infect phylogenetically distant hosts. Our results indicate that C. neoformans infection impairs multiple host cellular functions and undermines the health of these critical phagocytic cells, which can potentially interfere with their ability to clear this fungal pathogen. The Journal of Immunology, 2015, 194: 2345–2357.

The interaction of the pathogenic fungus Cryptococcus neoformans with macrophages is thought to be a critical event in the course of cryptococcal infection (1–8). However, host macrophages show little fungicidal activity in vitro (7, 9) and instead allow C. neoformans to reside in a mature acidic phagolysosome, where it replicates. C. neoformans is believed to use macrophages for extrapulmonary dissemination in a Trojan horse strategy (10). Moreover, the ability for replication within the phagosome is correlated with increased virulence (1, 11, 12), originating the notion that C. neoformans is a facultative intracellular pathogen.

Survival of C. neoformans in the phagolysosome has been attributed to various fungal characteristics (13, 14), of which the most prominent is a large polysaccharide capsule, but many others are essential for infection, such as melanin and phospholipase B1. Although ingestion of C. neoformans by macrophages is followed by many hours in which the host cell is viable, several studies have reported damage to host cellular processes, including: increased phagosome permeability (1), inhibition of cyclin D1 (15), and DNA instability (16), followed by mitotic arrest (17). Furthermore, intracellular residence of C. neoformans decreases Ag presentation, T cell proliferation, and cytokine production by macrophages (18, 19). Additional evidence of host cell damage is apparent when large residual vacuoles are observed in macrophages from which C. neoformans has exited by nonlytic exocytosis (20). However, the

The online version of this article contains supplemental material.

Abbreviations used in this article: AIF, apoptosis-inducing factor; BMDM, bone marrow–derived macrophage; CCCP-I, carbonyl cyanide m-chlorophenyl hydrazine; HIF-1α, hypoxia-inducing factor–1α; HK, heat killed; Jc-1, 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide; LDH, lactate dehydrogenase; Δψm, mitochondrial potential; PDGFBB, platelet-derived growth factor BB; RIP, receptor-interacting protein; ROS, reactive oxygen species; RPM, ribopuromycyl-tetraethylbenzimidazolylcarbocyanine iodide; Treg, regulatory T cell; TRIM, triggering receptor expressed on myeloid cells 1.

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mechanisms by which *C. neoformans* damages cells have not been investigated in detail.

Intracellular pathogens have evolved strategies to manipulate host machinery for their survival (21). Interference with signal transducer activity, manipulation of the lysosomal compartment, and host cell survival versus death are a few examples of commonly targeted processes. For example, both Francisella and *Mycobacterium* possess virulence factors that decrease caspase-1 activation, therefore decreasing production of caspase-1–derived inflammatory IL-1β (22). Cell death pathways rely on mitochondrial mediators for, at least, a portion of the pathway, and therefore many survival versus death decisions are integrated in the mitochondria. In addition, mitochondria are no longer regarded solely as the cell’s powerhouse but also are recognized as playing a role in immune function, producing reactive oxygen species (ROS) (23) for activation of the inflammasome (24). Consequently, viral, bacterial, and protozoan pathogens have myriad factors that manipulate host cell mitochondria (25, 26), but comparable information is not yet available for fungal pathogens.

Current views of *C. neoformans* intracellular pathogenesis posit a passive resistance of fungi to host attack, but little has been done to explore active fungal attack on the host. Survival of the host cell after nonlytic egress and the absence of widespread host cell death in *C. neoformans–macrophage* studies have encouraged the view that host cells suffer little or no damage from this organism. In this work, we have investigated macrophage injury after *C. neoformans* infection. Our results indicate that *C. neoformans* phagocytosis results in modifications of critical cellular functions, including impaired mitochondrial function, activation of caspase-1 and cellular stress pathways, and altered protein synthesis rate. The accumulation of cellular damage associated with *C. neoformans* intracellular residence could promote and potentiate *C. neoformans* survival in macrophages and contribute to cryptococcal virulence.

**Materials and Methods**

**Fungal strains**

*C. neoformans* var. *grubii* strain H99 (serotype A), acapsular mutant cap59, and original wild-type K99 were a kind gift of J. Heitman (Durham, NC). Yeast cells for infection were grown for 2 d in Sabouraud dextrose broth (Difco, Carlsbad, CA) at 37°C.

**Macrophage and macrophage-like cells**

Three types of macrophages were used for most experiments: the macrophage-like murine cell line J774.16 (27), bone marrow–derived macrophages (BMDM), and peritoneal macrophages. J774.16 macrophages were kept in DMEM complete media consisting of DMEM (CellGro), 10% NCTC-109 Life Technologies medium (LifeTechnologies), 10% heat-inactivated FBS (Atlanta Biologicals), and 1% nonessential amino acids (CellGro). BMDM were obtained by extracting bone marrow from hind leg bones of 6– to 8-wk-old BALB/c female mice (National Cancer Institute) and maturing them in vitro for 6–8 d in DMEM media with 20% L-929 cell–conditioned media, 10% FBS, 2 mM l-glutamine (CellGro), 1% nonessential amino acids (CellGro), 1% HEPES buffer (CellGro), and 2-ME (Life Technologies). Peritoneal macrophages were extracted by injecting 10 ml ice-cold PBS into the peritoneal cavity of the mice, and then cultured and infected in the same conditions as J774.16 cells. The peritoneal macrophage population was defined as adherent CD11b+ cells. In all assays, macrophages were plated to achieve a density of 1 × 10^6 cells per milliliter at the time of *C. neoformans* infection. *C. neoformans* organisms were added at a multiplicity of infection of 1.2 (unless otherwise noted) along with capsular mAb 18B7 (28) at 10 μg/ml. For some experiments, *C. neoformans* was heat killed (HK) by incubation at 60°C for 60 min or with oxidative damage by incubating yeast pellet with 30% (w/v) H_2O_2 for 30 min. All animal experiments were conducted according to ethical guidelines, with the approval of the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

**Killing and caspase activation assays**

For fungal killing assays, the cells were detached by vigorous pipetting and diluted in sterile water onto Saboraud agar plates. CFU were counted after 2 d at 30°C. Specific caspase activity was detected using a fluorochrome inhibitor of caspases (Immunochromathy Technologies) assay. Briefly, caspase-1–labeled recombinant peptide was inhibited 1 h before the determination of phagocytosis and stained with 0.1 μg/ml Hoechst for detection of necrotic/late apoptotic cells. Cells were analyzed immediately by laser scanning cytometry in the iCys Research Imaging Cytometer (CompusCyte Corporation) (17). Caspase activation was measured by imaging 20–40 fields in each well with the ×40 objective at 0.5 μm resolu-

**ATP, lactate dehydrogenase, and glucose measurements**

Total ATP content and lactate dehydrogenase (LDH) release can be used to estimate cell numbers and cellular viability. ATP measurements were performed by adding Triton X-100 at 0.25% and 2 mM EDTA (Sigma-Aldrich) to the wells containing infected cells. Next, 25 μl of this extract was incubated with Enliten ATP assay system (Promega), and counts per second were measured in a standard luminometer. We confirmed in each assay the extraction of ATP solely from mammalian cells and no extraction of *C. neoformans–derived* ATP, as indicated by the manufacturer's instructions. Briefly, for LDH 10 μl macrophage supernatants was added to 100 μl enz-ymatic mix, and absorbance was read after 30 min at 450 nm. For glucose 1:100 dilutions of cell supernatants were mixed with the assay mix, and the glucose content was determined by comparison with glucose standards.

**Immunoblot analysis**

Cytosolic protein extracts were obtained by resuspending the cell pellet in 250 mM sucrose, 50 mM Tris-HCl pH 7.4, and 5 mM MgCl2, and total cell extracts were obtained in RIPA buffer. Both were supplemented with OneStop Mini Protease Inhibitor Cocktail (Roche Applied Science), followed by 20 strokes with a Dounce homogenizer and centrifugation at 3000 rpm for 10 min for cytosolic extracts or 14,000 rpm for 30 min for total cell extracts. Western blot was performed in a NuPAGE SDS-PAGE (LifeTechnologies) system for the following primary Abs: rabbit anti–apoptosis-inducing factor (APF) and mouse anti–receptor-interacting protein (RIP) 1 (1:2000–1:200; from BD Biosciences), cleaved PARP D-214 (Cell Signaling), mouse anti–cytochrome c (1:200) (clone TH8.2C12; Abcam), and MFN1 (H-65) and MFN2 (H-68; Santa Cruz Biotechnology). β-actin Ab coupled to peroxidase (1:5000; Santa Cruz Biotechnology) was used as a loading control.

**Gene expression**

Macrophores were plated to achieve a density of 8 × 10^5 cells at the time of infection on 10-cm^2 dishes and infected as before. RNA was extracted using a RNeasy Mini Kit (QIAGEN) and stored at −80°C until analysis. Gene expression analysis was performed at the Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine. Briefly, a total of 10 μg RNA was converted to cDNA using the Nugen Pico SL (Nugen) and labeled using Nugen Encore Biotin kit. Target cRNA was hybridized to the murine genome Affymetrix Mouse Gene 1.0 ST (Affymetrix). Biological replicates were performed four times, and each experimental set was analyzed separately and then averaged for data analysis. All microarray datasets (CEL files) were normalized using the ExonRNA algorithm. Data variance was stabilized and transformed on a logarithmic scale, and quality control was performed. Genes were considered differentially expressed if p < 0.05 and fold change was >1.5. Genes satisfying these conditions were imputed into Ingenuity Pathway Analysis (Ingenuity Systems; www.ingenuity.com) for analysis of upstream regulators and Gene Ontology. Gene expression analysis was confirmed by RT quantitative PCR for selected genes (list of primers is given in Supplemental Table I).

**Ribopurinolyseation and immunofluorescence**

Cells were infected as described above, and 15 min before the end of the experiment media were supplemented with 91 μM puromycin and 208 μM emetine. One well was left without puromycin and used as negative control (C−). Cells were fixed with 3% paraformaldehyde, protease inhibitors mixture Omniplete Mini (Roche), 0.015% digitonin, and 10 U/ml RNase OUT in 50mM Tris-HCl buffer for 15 min at room temperature, blocked in a solution of 0.05% saponin, 1 μg/ml Fc block Ab (BD Biosciences), 10 mM glycine, and 5% FBS in PBS for 10 min, followed by the addition
of 1:100 of 2D10-conjugated to Alexa 488 or Alexa 647 fluorophores, a kind gift of J. W. Yewdell (National Institute of Allergy and Infectious Diseases). Cells were counterstained with Hoechst at 0.1 μg/ml; and fluorescence was quantified in an iCys Cumpuyte LSC. For mitochondrial morphology, cells were plated in optical quality dishes (Maktek Corporation); stained for ribopuromycylation (RPM) as before or with Alexa 488–conjugated cytchrome c Ab, clone 6H2.B4 (BDPharmingen); and imaged at 0.2-μm steps with the Inverted Olympus IX71 coupled to a Photometrics CoolSnap HQ charge-coupled device camera and analyzed using Velocity 3D (PerkinElmer).

Mitochondrial potential and ROS measurements

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was added 15 min before the termination of the assay, according to the manufacturer’s instructions (Immunochemistry Technologies). JC-1 dye accumulates as a red aggregate in healthy mitochondria and as a green monomer in depolarized mitochondria. ROS were measured with CellROX Deep Red Reagent (LifeTechnologies) for 30 min at a 5-μM concentration. Tetrachemylrhodamine (TMRE) and MitoTracker green (Molecular Probes) were added to the cells for 45 min before termination of the experiment at 200 and 25 μM, respectively. In vivo experiments, mice were infected with 1 × 10^6 C. neoformans i.p. or sterile PBS (vehicle) for the indicated time, and 30 min before sacrifice each mouse received 30 μl of JC-1 dye diluted in PBS, which was also injected i.p. Peritoneal lavage cells were immunostained with CD45-PerCP-Cy5.5 (eBiosciences) and F4/80–Alexa 647 (Life Technologies) and Uvitec for exclusion of extracellular yeasts. Fluorescent signal from viable cells, as measured by DAPI exclusion, was detected with a Becton Dickinson LSRII instrument (BD Biosciences).

Transmission electron microscopy

Macrophages were infected with C. neoformans for 24 h and fixed with 2% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate at room temperature for 2 h, followed by overnight incubation in 4% formaldehyde, 1% glutaraldehyde in PBS. The samples were subjected to postfixation for 90 min in 2% osmium, serially dehydrated in ethanol, and embedded in Spurrs resin. From 0.1 to 0.2 μm sections were cut on a Reichert U4 ultratome UCT and stained with 0.5% uranyl acetate and 0.5% lead citrate. Samples were viewed in a JEOL 1200EX transmission electron microscope at 80 kV.

Statistical analysis and plotting

Graphs, statistical analysis, and figures were assembled in Prism version 6.00 for Mac OS X (GraphPad Software, San Diego, CA). Fig. 4 was generated using String database (29).

Results

Gene expression changes upon infection of J774.16 macrophage-like cells following opsonic ingestion of C. neoformans

We investigated gene expression changes occurring in the J774.16 macrophage-like cell line at 2 and 24 h after ingestion of yeast cells (Fig. 1 and full gene list in Supplemental Table II). Initial gene expression changes could be observed already at 2 h post infection, with much larger changes becoming apparent at 24 h of infection. Ingestion of live C. neoformans by macrophages differentially modulated the expression of 110 genes, whereas infection with HK C. neoformans affected 61 genes. Very few genes were differentially modulated by both live and dead yeast cells: Srrt was downregulated at 24 h, and Cc12, Cc17, Gas5, Glip1, Gprc5a, Hmgb1, Phlda1, Id2, Sh3psd2a, Mup2, Rhob, and Snhg1 levels were altered after 2 h. When macrophages were infected with live C. neoformans, three genes remained upregulated throughout the course of infection: Empl, Serpinel1, and Id2 (confirmed by RT-PCR in Supplemental Fig. 1). Ingenuity Pathway Analysis of gene expression changes showed that after 2 h of infection the macrophages upregulated pathways involved in cell death and survival, cellular movement, proliferation, and cell-to-cell signaling. Predicted upstream regulators activated at this time are platelet-derived growth factor BB (PDGFBB), TNSF11 or RANKL, cycle to NF-κB ligand, and triggering receptor expressed on myeloid cells 1 (TREM1). PDGFBB is a mitogenic factor, TNSF11 is an antiapoptotic cell survival factor (30), and TREM1 was recently described as a hypoxia-responsive factor (31) that is involved in the response to Aspergillus fumigatus (32). Analysis of genes affected at 24 h revealed activation of such cellular functions as amino acid metabolism, posttranslational modifications, small-molecule biochemistry, cell growth and proliferation, and cell death and survival. Predicted upstream regulators activated at 24 h were IL-4, IL-5, hypoxia-inducible factor–1α (HIF-1α), and CD38. Both IL-4 and IL-5 are Th2-associated cytokines, and both have been found in Cryptococcus-infected mouse lungs (33). HIF-1α is a major regulator of response to hypoxia that was associated with fungal pathogenesis (34), and loss of this enzyme impairs immune responses to Listeria monocytogenes (35). In summary, gene expression changes in J774.16 cells upon C. neoformans infection were consistent with those reported in vivo and indicated simultaneous activation of immune and stress responses.

C. neoformans infection of murine macrophages alters protein translation rate in host cells

To further explore possible mechanisms that could eventually lead to C. neoformans–induced cell stress, we investigated protein synthesis, activation of cellular death pathways, and mitochondrial function in C. neoformans–infected macrophages. Consequently, we measured macrophage protein synthesis rate using immunoaffinity fluorescence to detect puromycin binding to nascent mRNA chains (36). Quantification of ribosome-bound puromycin revealed a decrease in the rate of translation in J774.16 macrophage-like cell line infected with C. neoformans (Fig. 2A). This observation agrees with the gene expression data suggesting a modulation of postranslational modifications. However, when we replicated the experiments in primary macrophages extracted from the peritoneal cavity, we observed an increase in rate of translation upon C. neoformans infection (Fig. 2B). None of the cells showed alterations in the cellular distribution of the active ribosomes as seen by fluorescence microscopy (Fig. 2C). Thus, although C. neoformans infection can modulate the overall rate of protein synthesis in macrophages, we did not observe a uniform pattern of increased or decreased protein synthesis in the infected macrophage subsets.

C. neoformans infection of murine macrophages and J774.16 macrophage-like cell line results in major changes in macrophage metabolism and only partial restriction of C. neoformans growth in vitro

C. neoformans infection of murine macrophages results in restriction of fungal growth and some degree of fungal killing for the first 24 h of infection (Fig. 3A). Live cell microscopy has shown that during the first 24 h of infection C. neoformans and macrophage cells coexist in vitro with >90% of macrophages remaining viable (C. de Leon-Rodriguez, unpublished observations). We found that commonly used methods to measure cell viability by colorimetry (tetrazolium dye reduction and nuclear dye incorporation) were unreliable because we could not separate yeast and macrophage contributions to measurements. Consequently, we used total ATP levels as a measure of macrophage number based on selective extraction of ATP from macrophages and not from yeast cells. With this approach we confirmed that ATP levels in infected cells decreases to 60–80% when compared with uninfected cells in the first 24 h (Fig. 3B), indicating that C. neoformans infection is accompanied by severe biochemical changes in the surviving cells, a small degree of macrophage cell death, or both of these processes.
FIGURE 1. Gene expression changes upon infection of macrophage-like J774.16 cells following opsonic ingestion of C. neoformans. Gene expression changes in J774.16 macrophage-like cells owing to their opsonic ingestion of live C. neoformans or HK C. neoformans were analyzed. (A) Venn diagrams of gene expression changes occurring in uninfected J774.16 cells (Ctrl) compared with changes occurring in Live or HK C. neoformans–infected cells. (B) STRING database illustration of reported associations between the genes that were found differentially regulated after 24 h infection with live C. neoformans. Orange boxes show empirical gene functional clusters. Different line colors represent types of evidence found (see legend) for the association between proteins (nodes).
C. neoformans infection can activate programmed cell death pathways in murine macrophages and macrophage-like J774.16 cells

To further explore whether C. neoformans–induced intracellular stress could result in accelerated macrophage death, we assessed activation of programmed cell death effector molecules, such as caspases, AIF, and RIP1. The J774.16 macrophage-like cell line showed a trend to activate caspase-1 and caspase-3 throughout infection, but this result did not reach statistical significance (Fig. 4), whereas BMDM activated caspase-1,-3, and -8. Furthermore, we determined that killing of yeast cells by J774.16 or BMDM macrophages in vitro was not affected by pan-caspase inhibition but was decreased by inhibition of NO formation (Supplemental Fig. 2). When we assessed protein expression of apoptosis pathway intermediates, J774.16 macrophage-like cells increased expression of RIP and AIF early in the course of infection (Fig. 4B, 4C), whereas BMDM showed activation of AIF and released cytochrome c into the cytosol. Furthermore, there was an increase of J774.16 cells with Annexin V externalization as well as cells with both Annexin V externalization and propidium

FIGURE 2. C. neoformans (Cn) infection of murine macrophages alters protein translation rate in host cells. Murine macrophages were infected with opsonized live C. neoformans or HK C. neoformans (HK Cn) for 24 h, and protein translation was measured by the RPM method as described by David et al. (36). RPM consists of puromycin tagging of nascent ribosomes. The amount of bound puromycin is ascertained by measuring puromycin-bound 2D10 Ab, and it is proportional to the amount of actively translating ribosomes. Quantification of actively translating ribosomes by RPM for (A) J77.16 macrophage–type cells and (B) peritoneal macrophages. Experiments were repeated three times for each cell type. Shown are the mean and SEM of all experiments. *p < 0.05, ****p < 0.001 for two-way ANOVA with Bonferroni multicomparison correction. (C) Representative images of RPM staining in peritoneal macrophages. Original magnification ×63; scale bar, 20 μm. Experiments were repeated three times for a minimum of 30 cells per condition per experiment, and representative images are shown.
iodide permeability (Fig. 4D). In addition, J774.16 cells increased LDH levels in supernatants (Fig. 4E), providing a clue that *C. neoformans* infection in J774.16 cells caused a type of necrotic death. No detectable LDH release was found in BMDM (data not shown), which together with the activation of caspase-3 indicates classical features of apoptosis in BMDM. In addition, we measured glucose concentration in supernatants and found no difference of infected macrophages relative to noninfected cells, ruling out glucose depletion due to fungal growth as a mechanism for macrophage toxicity (Fig. 4F). We then proceeded to test primary peritoneal macrophages, observing that peritoneal macrophages activated caspase-3 and increased their LDH levels in the supernatant. Results are summarized in Table I. Thus, although only a small subset of macrophages died upon the first 24 h of *C. neoformans* infection, we showed that *C. neoformans* induced cellular stress pathways and necrotic and apoptotic features in a variety of infected macrophages; however, we also observe that the type of stress and death pathway activated was dependent on macrophage type.

Murine macrophages and J774.16 macrophage-like cells depolarize mitochondria postinfection with *C. neoformans*

Given that mitochondria are critical for many aspects of cellular homeostasis, we studied mitochondrial potential (ΔΨm) alterations (37). The ΔΨm can be measured through incorporation of fluorescent dyes for which brightness of TMRE is proportional to ΔΨm. For the dye Jc-1, the color of cells will shift from green to red as mitochondria hyperpolarize. Therefore, the ratio of red/green will allow for semiquantitative measurement of ΔΨm (37). As experimental controls, we added carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP-1) to depolarize mitochondria or rotenone to hyperpolarize mitochondria. All macrophages depolarized their mitochondria at 24 h of infection (Fig. 5A–C). We note that for BMDM ΔΨm was decreased to a much lower extent than for the other cell types (Fig. 5B), and because this cell type was also resistant to CCCP-1 depolarization, we suggest the existence of a cell type–specific resistance to depolarization. Transmission electron microscopy revealed no alterations in mitochondrial ultrastructure (Fig. 5D), but depolarization of mitochondria was accompanied by alterations in the mitochondrial network, with increased fission in infected cells (Fig. 5E). This finding suggests that mitochondria are not directly damaged by infection but, rather, have their activity modulated by prolonged *C. neoformans* residence. Our attempts to modulate ΔΨm in macrophages were unsuccessful because mitochondrial inhibitory drugs were highly toxic to *C. neoformans* cells (data not shown). We investigated whether *Acanthamoeba castellanii* could modulate their mitochondria upon ingestion of *C. neoformans* but found no difference in ΔΨm in these unicellular organisms (data not shown).

Mitochondrial alterations do not correlate with oxidative burst but are mediated by NO

We evaluated whether mitochondrial modulation was related to ROS (24) and NO levels (38). We found that neither J774.16 nor
FIGURE 4. Programmed cell death pathways are activated in murine macrophages and the macrophage-like J774.16 cell line infected with *C. neoformans* (Cn). Cell death in murine macrophages infected with *C. neoformans* was characterized by caspase activation, measured through binding of a fluorescent caspase-specific peptide and immunoblot quantification of molecules involved in cell death pathways. Cell death was quantified by measuring externalization of Annexin V and by release of LDH into the extracellular media. (A) Measurement of caspase activation after *C. neoformans* infection of J774.16 cells (left), BMDM macrophages (center), and peritoneal macrophages (right). Experiments were repeated three to five times for J774.16 macrophages and BMDM with duplicate wells and twice for peritoneal macrophages. (B) Quantification of protein expression for RIP, AIF, cleaved PARP, and release of cytochrome c from the cytosol post infection of J774.16 cells and BMDM. Expression levels were normalized for β-actin content. (C) Representative immunoblots for J774.16 cells. (D) Quantification of Annexin V+ and propidium iodide (PI)+ cells after *C. neoformans* infection of J774.16 cells (left) and peritoneal cells (right). (E) LDH release for J774.16 cells (left) and peritoneal cells (right). (F) Glucose quantification. (Figure legend continues)
C. neoformans peritoneal observed in vitro was also observed in vivo. In a model of infection, we proceeded to investigate if the mitochondrial depolarization observed in leukocytes and macrophages at 24 h post infection in comparison with mock-infected cells (Fig. 6B). Peritoneal macrophages were capable of releasing ROS in response to these conditions triggered ROS production in J774.16 macrophage-like cells and to peritoneal macrophages (data not shown), depolarization was decreased at 24 h of infection (Fig. 6D), suggesting a role for NO in mitochondrial depolarization. However, ROS production by mitochondria occurs upon exposure to zymosan, a preparation of Saccharomyces cerevisiae cell wall, and H2O2-killed C. neoformans, but none of these conditions triggered ROS production in J774.16 macrophage-like cells (Fig. 6B). Peritoneal macrophages were capable of releasing ROS in response to C. neoformans (data not shown) and when exposed to zymosan or H2O2-killed C. neoformans (Fig. 6C). However, ROS production by mitochondria occurs upon mitochondrial hyperpolarization, and, therefore, in the particular case of C. neoformans infection in peritoneal macrophages, it is likely that Δψm modulation is disconnected from ROS production. When S-ethyl-isothiourea, an inhibitor of NO, was added to H99-infected J774.16 macrophage-like cells and to peritoneal macrophages (data not shown), depolarization was decreased at 24 h of infection (Fig. 6D), suggesting a role for NO in mitochondrial depolarization.

Intraperitoneal infection of mice with C. neoformans causes a decrease in mitochondrial polarization in peritoneal total leukocytes and macrophages

We proceeded to investigate if the mitochondrial depolarization observed in vitro was also observed in vivo. In a model of peritoneal C. neoformans infection, we observed that peritoneal lavage cells, both total leukocytes (CD45+) and macrophages (F4/80+), were depolarized at 24 h post infection in comparison with mock-infected mice (Fig. 7), validating our in vitro findings in a murine model of infection. Overall, our results show multiple alterations in mitochondrial function upon C. neoformans infection, namely, gene expression changes, alterations of protein translation rates, and alteration in mitochondrial dynamics (summarized in Table I).

### Table I. Summary of findings

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<td>a</td>
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<tr>
<td>Protein translation</td>
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<td></td>
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</table>

Italicics represent conclusions from the data, not results of the experiment.

*Not pertinent for conclusions of study.

†Not performed owing to technical limitations.

↓↓, downregulated; ↓↓↓, strongly downregulated; PCD, programmed cell death.

BMDM produced significant amounts of ROS after phagocytosis of C. neoformans (Fig. 6A, data not shown for BMDM). To discard the possibility that the observed lack of ROS production was due to a quenching effect of C. neoformans, we exposed macrophage cells to zymosan, a preparation of Saccharomyces cerevisiae cell wall, and H2O2-killed C. neoformans, but none of these conditions triggered ROS production in J774.16 macrophage-like cells (Fig. 6B). Peritoneal macrophages were capable of releasing ROS in response to C. neoformans (data not shown) and when exposed to zymosan or H2O2-killed C. neoformans (Fig. 6C). However, ROS production by mitochondria occurs upon mitochondrial hyperpolarization, and, therefore, in the particular case of C. neoformans infection in peritoneal macrophages, it is likely that Δψm modulation is disconnected from ROS production. When S-ethyl-isothiourea, an inhibitor of NO, was added to H99-infected J774.16 macrophage-like cells and to peritoneal macrophages (data not shown), depolarization was decreased at 24 h of infection (Fig. 6D), suggesting a role for NO in mitochondrial depolarization.

**Discussion**

Considerable evidence now exists that intracellular residence of C. neoformans is associated with damage to macrophages, as evidenced by phagosomal leakiness (1), giant vacuole formation (20), and cell cycle arrest (17). Because progressive accumulation of cellular damage can result in cell death, we set out to investigate the mechanisms of macrophage cell damage after C. neoformans phagocytosis, with a specific focus on mitochondrial function, a major integrator of cellular decisions such as survival, oxidative burst, and immune responses (24). Our initial studies focused on determining whether host cell gene expression changes could potentially explain the results previously reported. The transcript level of dozens of genes was affected, many of those genes related to a stress response, and these changes were consistent with phenotypic observations following C. neoformans infection of macrophages. For example, previous work has shown that initial proliferation of macrophages infected with C. neoformans is followed by cell cycle arrest (17, 39). Gene expression data from this study provide information consistent with and supportive of the cell cycle changes observed. PDGFBB, which belongs to the same family as CSFR1 and is a mitogenic factor for endothelial cells (40), could be a mediator of the initial proliferation, whereas activation of HIF-1α (41) and cyclin G2 (42) at later times might contribute to cyclin D1 inhibition (15) and the subsequent cell cycle arrest upon C. neoformans infection (17). The protein synthesis machinery is a target for viral (43), bacterial, and parasitic pathogens (44, 45). Translation interference by fungal pathogens was suggested for Candida albicans and in C. neoformans infection (discussed in Ref. 45), but had not been shown for fungal infections thus far.

Next we demonstrated that C. neoformans intracellular residency affected cell stress, survival, and death responses. We observed a low mortality of infected macrophages, and no pathway predominated to trigger wholesale cell death, as determined by the

in cell supernatants for J774.16 cells. Experiments were repeated three times for each cell type. Data were normalized to percent of uninfected macrophages (No Cn). Shown are mean and SEM of all experiments. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 for two-way ANOVA with Bonferroni multicomparison correction.
mixed pattern of activation of death pathways. It is possible that simultaneous interferences in multiple pathways, despite being small in magnitude, could result in significant effects in macrophage health and ability to clear the pathogen when combined. 

*C. neoformans* resides and replicates within host macrophages, but the host cells do not typically display features of necrotic or apoptotic deaths. Of interest, Th1 activation of macrophages decreases the amount of lysosomal damage to the host cells, and therefore macrophage damage can be modulated by the cytokine milieu [as shown in an accompanying article (46)], providing further evidence that equilibrium between host and pathogen is modulated by the cytokine milieu (33, 47). The absence of a predominant death pathway, combined with the inability of macrophages to eliminate *C. neoformans*, phagosome leakiness, and cell cycle arrest, may be caused by multiple subtle alterations in cellular function, including mitochondrial function. Our results are consistent with the capacity of *C. neoformans* to cause latent infection in granulomas, with long-term survival of both host and pathogen (3, 8), whereas concomitantly *C. neoformans* residency in macrophages causes sufficient dysfunction to explain the suboptimal stimulation of immune responses reported previously (18, 19). Finally, our data are consistent with the scenario that macrophage death is likely to occur in a small subset of cells, in which the cumulative damage induced by *C. neoformans* exceeds a certain threshold, and depending on the type of cellular response, this cell death can become either apoptotic or necrotic.

**FIGURE 5.** Murine macrophages and macrophage-like J774.16 cells depolarize mitochondria post infection with *C. neoformans* (Cn). Murine macrophages and macrophage-like J774.16 cells were infected with opsonized *C. neoformans*, and their Δψm was measured at several time intervals. CCCP-1, which causes rapid depolarization of mitochondria by uncoupling the proton gradient, was used as a depolarization control. Representative histograms for (A) J774.16, (B) BMDM, and (C) peritoneal macrophages. Numbers represent percent of cells with polarized mitochondria in the experimental condition shown. Shaded gray represents noninfected macrophages (No Cn), and each experimental condition is shown by the red line. Δψm was measured by flow cytometry analysis of the Jc-1 red/green ratio or by TMRE dye accumulation. Ratio of red/green fluorescent signal reflected mitochondrial polarization for Jc-1. TMRE accumulation is proportional to mitochondrial polarization. Experiments were repeated two to three times for each macrophage cell type, and a representative experiment is shown. (D) Mitochondrial morphology was studied by electron microscopy. No alteration of mitochondria morphology, such as nonexistent or swollen cristae, was observed. Scale bar, 500 nm; bottom row is magnification of mitochondria on top row. (E) Peritoneal macrophages infected with *C. neoformans* show a fragmented mitochondrial network, shown by cytochrome c immunostaining (green). Images were counterstained with Hoechst nuclear dye (blue). Original magnification ×63; scale bar, 50 μm. Experiments were repeated three times, and representative images are shown.
**C. neoformans** infection caused a decrease in \( \Delta \psi_m \) in all macrophage cell types tested, an observation previously reported for bacterial pathogens *Shigella flexneri* (48) and *Mycobacterium tuberculosis* (49). Mitochondrial depolarization occurred in every macrophage type tested and in a mouse model of infection, suggesting that this is a conserved response to *C. neoformans* infection. The necessity for mitochondrial modulation derives both from regulation of fuel and energy requirements and from the emerging role of mitochondria as integrators of cellular decisions of death, survival, and immune activation (24). For example, mitochondrial depolarization reflects a metabolic switch essential for T lymphocyte (50) and dendritic cell activation, allowing immune cells to survive in hypoxic environments and undergo rapid proliferation (38). Similarly, mitochondrial signaling is necessary for macrophage activation and might even contribute directly to microbicidal abilities (23). In our model, such a metabolic switch in vitro could be triggered by TREM1 (51), HIF-1\( \alpha \), or CD38 (52, 53), as suggested by the gene expression data, because each of them can regulate mitochondrial activity and metabolism. It was suggested that a possible role of mitochondria is to produce ROS for activation of the inflammasome (24); however, our data do not support a role for mitochondrial ROS in *C. neoformans* infection. Instead, our data suggest a role for NO in mitochondrial modulation (38, 54) where prolonged inhibition of NO attenuates mitochondrial depolarization. An added complexity to the mitochondrial equation is that *C. neoformans* requires both the glycolytic pathway and the mitochondria for full virulence (55, 56), and therefore mitochondrial functions are crucial for both sides of host–pathogen interaction (57).

Macrophage heterogeneity based on different anatomical sources (58) or culture conditions (59) is well established, a fact that has been attributed to adaptations to their local microenvironment. Given this heterogeneity, we evaluated three macrophage types for their interaction with *C. neoformans* and observed that each responded differently. Our study did not investigate alveolar macrophages owing to concerns about the relevance of this cell in host defense. Although alveolar macrophages are presumably the first to come into contact with *C. neoformans*, their role in host defense in mice is unclear because their depletion reduces vulnerability to infection (7). In fact, alveolar macrophages are in a minority in the infected lungs, as they become rapidly outnumbered by bone marrow (monocyte)-derived exudate macrophages during the inflammatory response to infection. Consequently, exudate macrophages are the major subset of mononuclear effector cells in the lungs (60), and therefore our studies focused on macrophage types that resemble exudate macrophages. Finally,
one must consider that virulence is a complex equation (61) and acknowledge that a limitation of this work is that we studied only one *C. neoformans* strain and that there may be strain-specific factors that could produce strain-dependent outcomes in *C. neoformans*–macrophage interactions (12). Given the observed heterogeneity in responses of the host to the pathogen, we propose two scenarios to explain these results. Toxicity might have started with disruption of one cellular process that then cascaded to affect multiple cell processes. For example, mitochondrial alterations can potentiate ER stress (62) or, alternatively, mitochondrial alterations could be caused by deregulation of cyclin D1 (63). In any case, our data provide multiple explanations for the previously reported cell cycle arrest upon *C. neoformans* ingestion (17). According to this view, the differences observed in activation of programmed cell death pathways would reflect a heterogeneous response to *C. neoformans*–inflicted stress owing to cell type anatomical origin, and the differences between J774.16 and the other macrophages could reflect differences between an immortalized cell line and primary cells. Alternatively, one might hypothesize that *C. neoformans* causes damage to various cellular systems, which in turn results in multiple hits to the host cell. The absence of a specific damage pathway is intriguing until one considers that *C. neoformans* can infect a great variety of hosts, including mammals, worms, insects, plants, and amoebae, and that its virulence for mammals may be accidental (64). In this regard, *C. neoformans* is very different from pathogenic microbes with narrow host ranges, such as certain viruses that depend on one host, which have evolved precise mechanisms to manipulate host biology and immune responses. In contrast, our results show that *C. neoformans* inflicts damage to the cell by multiple mechanisms, and when this distinction is considered, such a cell pathogenic strategy can be understood in the sense that it allows survival in many different cell types. The fact that in contrast to macrophages *C. neoformans*–infected amoebae did not manifest mitochondrial depolarization is consistent with this view, which posits different damage to different hosts. These scenarios are not mutually exclusive.

In summary, our results establish that macrophages activate several immune defense mechanisms upon *C. neoformans* infection but are unable to effectively clear fungal infection in vitro. Residency of fungal cells in macrophages was associated with a progressive deterioration in host cellular functions. Specifically, *C. neoformans* ingestion activated several stress pathways, affected protein translation, and caused mitochondrial depolarization. Our results provide evidence for subtle cytopathic effects of *C. neoformans* on macrophages that with time could progress to eventual cell death, as evidenced by the small percentage of macrophage deaths at 24 h. This damage could help the persistence of *C. neoformans* within macrophages, the inability to clear *C. neoformans* infection, and inefficient immune responses, translating into chronic infections. We established that mitochondrial modulation is a significant factor in fungal infection of macrophages, as shown for bacterial infections (38, 65). The multiple-hit intracellular survival strategy, illustrated in this article, provides a paradigm for understanding how pathogens, such as *C. neoformans*, are able to infect such evolutionarily distant hosts, ranging from Kingdom Protozoa to Kingdom Animalia.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure 1. Gene expression data by qRT-qPCR.
RNA from all samples was pooled, cDNA was transcribed and qRT-PCR measurements made. Shown is mean RQ (2^ΔΔCT) and upper range of possible RQ values for pooled RNA of all samples.
Supplemental Figure 2. Outcome of Cn-macrophage interaction in vitro can be affected by manipulating NO but not caspases.

ATP levels or killing of Cn was not affected by addition of z-VAD-FMK, a pan-caspase inhibitor or by addition of antioxidants n-acetyl-cysteine (NAC) or addition of β-mercaptoethanol (BME). SEITU, an NO inhibitor, decreases macrophage fungicidal activity but has no effect on Cn alone while rotenone and antimycin, drugs targeting mitochondrial electron transport chain, were toxic to Cn alone without affecting macrophage killing of Cn. A) ATP quantification for Cn infected J774.16, at 18 h infection and, B) for BMDM. C) Killing of Cn by J774.16 macrophages after 18 h of infection and, D) by BMDM. E) Cn alone or Cn phagocytosed by macrophages (Cn+ J774.16) mitochondrial drugs 5 μM rotenone and 7 μM antimycin or F) 100 μM of SEITU, an inhibitor of NO. Cn was plated and Colony Forming Units (CFU) counted. Experiments were repeated twice for each cell type, in triplicates for CFU and quintuplicates for ATP. Shown is mean and SD of triplicate wells of one representative experiment. Two-way ANOVA with Bonferroni correction was used to calculate adjusted p-value. n.s. = p > 0.05, * = p-value < 0.05.
**Supplemental Table 1**

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Supplemental Table 1. Primers for RT-qPCR used in this study.