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J Immunol published online 30 January 2015 http://www.jimmunol.org/content/early/2015/01/30/jimmunol.1402376

Supplementary Material http://www.jimmunol.org/content/suppl/2015/01/30/jimmunol.1402376

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Cryptococcus neoformans—Induced Macrophage Lysosome Damage Crucially Contributes to Fungal Virulence

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Upon ingestion by macrophages, Cryptococcus neoformans can survive and replicate intracellularly unless the macrophages become classically activated. The mechanism enabling intracellular replication is not fully understood; neither are the mechanisms that allow classical activation to counteract replication. C. neoformans—induced lysosome damage was observed in infected murine bone marrow–derived macrophages, increased with time, and required yeast viability. To demonstrate lysosome damage in the infected host, we developed a novel flow cytometric method for measuring lysosome damage. Increased lysosome damage was found in C. neoformans–containing lung cells compared with C. neoformans–free cells. Among C. neoformans–containing myeloid cells, recently recruited cells displayed lower damage than resident cells, consistent with the protective role of recruited macrophages. The magnitude of lysosome damage correlated with increased C. neoformans replication. Experimental induction of lysosome damage increased C. neoformans replication. Activation of macrophages with IFN-γ abolished macrophage lysosome damage and enabled increased killing of C. neoformans. We conclude that induction of lysosome damage is an important C. neoformans survival strategy and that classical activation of host macrophages counters replication by preventing damage. Thus, therapeutic strategies that decrease lysosomal damage, or increase resistance to such damage, could be valuable in treating cryptococcal infections. The Journal of Immunology, 2015, 194: 000–000.

C yptococcus neoformans is an opportunistic fungal pathogen of global significance. C. neoformans infection occurs with incidence of 1 million new cases each year (1) and is associated with a high-level mortality (2). Especially in immune-compromised populations, uncontrolled fungal pneumonia is followed by highly lethal dissemination to the CNS (1–4).

Resident alveolar macrophages are among the first host cells to contact C. neoformans, and the pathogen is frequently found within lung macrophages (5). As the infection escalates, monocytes are recruited and mature into inflammatory “exudate” macrophages (6). Based on external signals, especially secreted cytokines, macrophages become classically or alternatively activated. Naive and alternatively activated macrophages are insufficient to control C. neoformans infection, allowing intracellular growth of the yeast (7–10). In contrast, classically activated macrophages can kill C. neoformans in vivo (6, 8, 11, 12), and classically activated exudate macrophages are a particularly important effector cell in this system (6). Classical macrophage activation and subsequent clearance of C. neoformans requires the support of adaptive immunity (13–20).

Following ingestion by macrophages and other phagocytes, C. neoformans organisms enter host lysosomes (21–26). Lysosomes are membrane-limited organelles, the contents of which are maintained at about pH 4.75 in macrophages by the vacuolar ATPase (27, 28), in contrast to the neutral pH of the surrounding cytosol. Lysosomes contain a variety of homeostatic and microbicidal hydrolases and numerous specialized microbicidal activities, including critical constituents of the phagocyte oxidase system, which catalyzes the production of microbicidal reactive oxygen species into the lysosomal lumen. In vitro, purified lysosomal components from dendritic cells are toxic to C. neoformans (23, 26). Despite these strong lysosomal defenses, naive macrophages containing C. neoformans become quickly overgrown by C. neoformans in vitro. Also, individuals who succumb to C. neoformans infection as well as C. neoformans–sensitive murine strains do not display primary deficiencies in macrophage populations or intrinsic problems with lysosomes. Thus, delivery of C. neoformans to the macrophage phagolysosomal compartment is insufficient to control cryptococcal infection without strong classical activation of macrophages (13–20), suggesting that C. neoformans possess some robust ability to counter the microbicidal properties of host lysosomes.

How C. neoformans survive and replicate when faced with the toxic environment of the phagolysosome is unknown. One survival mechanism employed by other pathogens faced with a sim-
ilar challenge, such as Candida, is to damage the lysosome phospholipid membrane (29). Although it is unknown if lysosome damage importantly contributes to intracellular virulence of *C. neoformans* within macrophages, previous studies provided some evidence of *C. neoformans*-associated effects on phagosomal membrane integrity and postulated phagolysosomal damage (24, 30, 31). However, relative frequency and the extent of *C. neoformans*-mediated phagolysosomal damage have not yet been quantitatively measured in *C. neoformans*-infected live macrophage. Furthermore, it is unknown if such damage would be an important mechanism for intracellular survival of *C. neoformans* within macrophages. We hypothesized that *C. neoformans* actively damages host macrophage phagolysosomes to facilitate its intracellular growth within macrophages and that classical activation of macrophages limits *C. neoformans*-mediated phagolysosomal damage as a mechanism to inhibit *C. neoformans* growth and to promote *C. neoformans* killing by macrophages during protective immune responses.

In this study, we demonstrate that intracellular *C. neoformans* causes progressive and substantial lysosome damage during growth within murine macrophages in vitro and in vivo and that IFN-γ stimulation of macrophages limits damage, reduces *C. neoformans* growth, and increases intralysosomal *C. neoformans* killing. This study used recently developed live-cell microscopy techniques to provide quantitative and sensitive measurement of lysosome damage and a novel flow cytometric strategy to evaluate damage occurring in the lungs of *C. neoformans*-infected animals. These data provide evidence that lysosome damage is a crucial mechanism employed by *C. neoformans* to promote its intracellular virulence and that inducible lysosome remittance is an important mechanism that contributes to *C. neoformans* control within activated macrophages.

Materials and Methods

**Materials**

We purchased the following tissue culture reagents from Life Technologies (Carlsbad, CA): RPMI 1640, phenol red-free RPMI 1640, DMEM, FCS, GlutaMAX, sodium pyruvate, nonessential amino acids, penicillin/ streptomycin, HBSS, nigericin, valinomycin, fluorescein dextran (average m.w. 3000 Da), Texas Red dextran (average m.w. 3000 Da), and SYTOX red. IFN-γ, TNF-α, and M-CSF were purchased from PeproTech (Rocky Hill, NJ). IL-4 as well as allopurinol-cyt-C–conjugated anti-CD11c (clone N418), PerCP-Cy5.5–conjugated anti-CD45 (clone 30F11), and PE-Cy7–conjugated anti–Gr-1 (clone RB6-8C5) were from BioLegend (San Diego, CA). Sabouraud dextrose broth and agarose were purchased from BD Biosciences (San Jose, CA). Normal mouse serum was from Innovative Research (Novi, MI). Fluconazole was purchased from Sigma-Aldrich (St. Louis, MO). Uvitex-2B was purchased from Polyscience (Warrington, PA). Glass-bottom culture dishes (catalog number P35G-1.5-14-C) were from MatTek (Ashland, MA). Dextran doubly conjugated with fluorescein plus sulforhodamine101 (average m.w. ~10,000 Da) was a custom product purchased from TdB Consultancy (Upsala, Sweden).

**Mice**

BALB/C (The Jackson Laboratory, Bar Harbor, ME) and C57BL/6 (Taconic Farms, Hudson, NY) were housed at the VA Ann Arbor animal care facility and used at ~5–7 wk of age.

**Animal ethics statement**

Animal care and use were performed in strict accordance with an animal use protocol reviewed and approved by the Veterans Affairs Institutional Animal Care and Use Committee (permit number 0512-025) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All surgery was performed under ketamine and xylazine anesthesia, and every effort was made to minimize suffering.

**C. neoformans culture**

*C. neoformans* strain H99 (ATCC 208821; American Type Culture Collection) was cultured as previously described (6, 12, 32). Briefly, cultures started from aliquots frozen in 10% glycerol were incubated at 37°C for 4 d in Sabouraud dextrose broth. For use in vitro and in vivo, *C. neoformans* were washed in sterile saline. For some experiments, *C. neoformans* were heat-killed by boiling for 4 h. For all in vivo infections and some in vitro infections, *C. neoformans* were labeled with Uvitex-2B (as in Ref. 33) in PBS at 1 × 10⁷ *C. neoformans*/ml using 100 μg/ml Uvitex-2B.

**Bone marrow–derived macrophage culture**

Macrophages were differentiated from bone marrow as previously described (32, 34–36). Briefly, marrow was flushed from the mouse long bones and dispersed into a single-cell suspension. These cells were cultured for 7 d in DMEM supplemented with 20% FCS and 50 ng/ml M-CSF. Cultures were nourished with additional M-CSF–containing media on the third day of culture. All in vitro experiments were performed in RPMI 1640 containing 10% FCS and 5 ng/ml M-CSF.

**Loading macrophage lysosomes**

For microscopy, suspended macrophages were plated onto coverslips placed in glass-bottom culture dishes at 8 × 10⁵ cells/coverslip. After allowing several hours for the cells to attach, the culture media was replaced with media containing 150 μg/ml FITC–dextran (Fd). Following overnight incubation, cells were rinsed thoroughly with warmed HBSS and cultured in media without dye-dextran for at least 3 h to insure that all internalized dye had trafficked into the macrophage lysosomes.

**In vitro infection of macrophage**

Saline-rinsed *C. neoformans* were opsonized by 45-min incubation with 10 μg/ml *C. neoformans*–specific F12D2 IgG1 and 20% normal mouse serum. The F12D2 IgG1 is a subclass-switch variant of the original F12D2 IgG3. The production and properties of the mAB F12D2 have been described (37). *C. neoformans* were then diluted in culture media to 0.05–0.1 multiplicity of infection (MOI), such that macrophages started experiments with, at most, one *C. neoformans* per macrophage. Cultured macrophages were infected by replacing culture media with *C. neoformans*–containing media. Macrophages and *C. neoformans* were co cultured for 1 h, and then extracellular *C. neoformans* were rinsed away using warm HBSS. Next, *C. neoformans*–infected macrophages were incubated in RPMI 1640 culture media containing 20 μg/ml fluconazole to inhibit replication of any remaining extracellular *C. neoformans*.

**Microscopy**

Measurement of lysosome damage was performed as previously described (34, 35). At indicated times following infection, coverslip dishes were rinsed with culture media replaced with HBSS. Dishes were then mounted onto the heated stage of the microscope. The microscope used for all imaging save for the experiments involving experimental induction of damage was an Olympus IX70 inverted epifluorescence microscope (Olympus, Center Valley, PA) using 100X oil immersion objective (numerical aperture 1.30) and equipped with a X-cite 120 metal halide light source for fluorescence (EXFO, Mississauga, ON, Canada), a temperature-controlled stage, and a CoolSNAP HQ2 monochrome camera (cooled CCD, 1392 × 1040, 14-bit).

To measure lysosome damage, we acquired three images: the first using a 436-nm excitation filter, another using a 492-nm excitation filter (both for measurement of damage), and a third using a 405-nm excitation filter, another using a 436-nm excitation filter (in both cases using a 492-nm emission filter). Following overnight incubation, cells were rinsed thoroughly with warmed HBSS and cultured in media without dye-dextran for at least 3 h to insure that all internalized dye had trafficked into the macrophage lysosomes.

**Image analysis**

Image analysis was performed using Metamorph software as previously described (34, 35). Molecular Devices, Downington, PA). Briefly, background signals were subtracted from all images, and signal distortions from uneven illumination were corrected by dividing all acquired images by a normalized image acquired from a control sample with an even level of illumination. We determined intracellular pH based on the differential emission of fluorescein, which is largely pH independent in the 436-nm excitation channel but highly pH-dependent on the pH 492-nm excitation channel. Ratio images (492-nm signal/436-nm signal) were translated to pH maps based on microscope calibration. To calibrate the microscope, 492/436-nm ratio images were acquired as above from macrophages identically labeled with Fdx in pH clamping buffer (130 mM KCl, 1 mM MgCl₂, 15 mM HEPES, and 15 mM MES). Clamping buffer contained 10 μM nigericin and 10 μM valinomycin, which fix intracellular pH. Buffers were used at pH 9 to 4 using 0.5-pH unit increments. Average 492/436-nm ratio values from cells at each pH were plotted, a four-variable
sigoidal standard curve was constructed using SigmaPlot software (San Jose, CA), and pH maps were generated using this calibration data to convert 492/436-nm ratio values to pH. Regions showing pH 5.5 indicated lysosome release, and the percent of Fdx release was calculated by dividing the Fdx fluorescence originating in regions depicting lysosome release by total cellular Fdx fluorescence. We have previously shown pH 5.5 to be a valid empirical cutoff for these type of data (34, 35).

Enumeration of live versus dead C. neoformans

We observed differing morphological characteristics in C. neoformans particles contained in Fdx-loaded lysosomes depending on C. neoformans viability. Live yeast excluded Fdx, and thus the Fdx fluorescence appeared as annular halos on the perimeter of individual Fdx-dim yeast cells (Fig. 4A, top panel). In contrast, heat-killed C. neoformans absorbed lysosomal Fdx appearing as Fdx bright-filled bodies (Fig. 4A, bottom panel). These properties were subsequently used to differentiate between viable and killed C. neoformans experimentally based on fluorescence patterns in Fdx-containing macrophage lysosomes. Using this difference in Fdx staining pattern, we enumerated live versus dead C. neoformans inside imaged macrophages using Metamorph software. Although the vast majority of internalized yeast were live and excluded Fdx (Supplemental Fig. 1B, 1C), a detectable portion did not, consistent with a loss of membrane integrity and yeast death (Supplemental Fig. 1B, 1C). Sensitivity or other factors may account for the varying labeling among the observed yeast cells (Supplemental Fig. 1C), indicated lysosomal breakdown of C. neoformans, consistent with previous reports in other cell types (5, 23, 26).

In vitro C. neoformans growth

These experiments were performed as previously published (32). To determine the ability of C. neoformans to grow in macrophages, bone marrow–derived macrophages (BMM) were added to the wells of 24-well culture plates at 1 × 10⁵ cells/well. Cells were stimulated overnight with cytokines (20 ng/ml IL-4, 20 ng/ml TNF-α, and/or 100 ng/ml IFN-γ) as indicated. C. neoformans cultures were opsonized and added at 2.5 × 10⁵ C. neoformans/well. After 24 h, supernatants were removed and reserved and then the intracellular fraction of C. neoformans was liberated by lysing the macrophages in sterile water. We determined the number of C. neoformans CFUs by serial dilution of samples plated on Sabouraud dextrose agar plates, which were counted after 48 h. Data from supernatants and cellular fractions were summed during data analysis.

Experimental induction of lysosome damage

A second microscope was used for these experiments. This instrument was a Nikon TIE (Nikon, Tokyo, Japan) inverted epifluorescence microscope using 40× (numerical aperture 0.95) and 4× (numerical aperture 0.20) air immersion objectives and a motorized stage. Fluorescence illumination was generated by a luminator Spectra X light engine (Beaverton, OR) and images acquired using a CoolSNAP HQ2 monochrome camera. Samples were maintained at 37°C using an OKO laboratories (Olivetti, Italy) stage incubator.

Our photo-induced damage protocol was based on previously published methods (34, 38). BMM (2 × 10⁵) were seeded onto the coverslip of 25 µl cell-containing RPMI 1640. This resulted in a spot of macrophages attached to the coverslip ~4 mm in diameter. These cells were loaded with 150 µg/ml Fdx and 75 µg/ml Texas red dextran, rinsed, chased, and infected with Uvix-2B–labeled H99 C. neoformans as above. Because imaging and photo exposure were conducted in medium, phenol red-free RPMI was used for all of these experiments. Two hours postinfection, excess C. neoformans were rinsed away and then the media replaced with fresh RPMI 1640 without serum. Next, coverslips were mounted on the microscope stage. Using a 4× objective, the cell-covered area is located and exposed to bright orange/red light for 5 min using a 572/35-nm bandpass filter. The field size of the 4× objective allowed the vast majority of macrophages in the 4-mm spot to be simultaneously illuminated. Alternatively, control coverslips were incubated in the heated stage incubator for an identical time period without the photo exposure. Following exposure or mock exposure, cells were imaged using the 40× objective in a 7 × 7 grid pattern with 0.5 mm between each image; this pattern surveyed most of the cell-covered area on the coverslip. Microscope control and imaging was controlled using Nikon-Elements Software (Nikon). FCS was then added to each coverslip to 10% and cells incubated at 37°C with 5% CO2. After 24 h, CFU count for C. neoformans was determined as above. Image processing was conducted using Metamorph software as above. Note, as these cells were grown on coverslips, the coverslip containing their intracellularly engulfed yeast, the lysosomal pH was slightly higher than otherwise observed in control cells in other experiments; therefore, the empirical cutoff for lysosome damage was raised from 5.5 to 5.75 for these experiments.

In vivo infection of mice

Intratracheal administration of C. neoformans was performed as previously described (6, 12, 39, 40) with some modifications for the administration of FITC and sulforhodamine 101 double-labeled dextran (F/SR101dx). C. neoformans cultures were rinsed in sterile saline, stained with Uvix-2B, and counted. These cells were diluted into sterile saline containing 12 mg/ml sterile filtered F/SR101dx.

For the administration into mice, animals were anesthetized with ketamine and xylazine at 100 mg/kg and 6.8 mg/kg of bodyweight, respectively, and immobilized on a surgical board. Under sterile technique, the trachea was exposed and inoculated with 1 × 10⁶ C. neoformans cells mixed with F/SR101dx or labeled macrophages in 40 µl sterile saline using a 30-gauge needle. Control animals were administered F/SR101dx without C. neoformans. The surgical wound was closed using cyanoacrylate adhesive, and the mice were allowed to recover under thermal support. Mice were monitored daily for signs of distress until the completion of the experiment.

Bronchoalveolar lavage

Mice were sacrificed 48 h postinfection according to approved protocols. Lung airway cells were harvested by bronchoalveolar lavage (BAL) as previously described (40). Briefly, the trachea was exposed and polyethylene tubing affixed using surgical twine. The lungs were lavaged four times with 1 ml/lavage with PBS containing 5 mM EDTA for a total volume of ~4 ml. Lavage cells were filtered through a nylon screen of 100 µM pore size and subsequently concentrated by centrifugation. Resuspended cells were then enumerated using a hemocytometer.

Flow cytometry

BAL samples were analyzed on a BD Biosciences LSRII flow cytometer system (BD Biosciences); instrument settings, including lasers and filters, have been described recently (41). Cells from BAL were analyzed by flow cytometry using two strategies. In the first strategy, designed to measure lysosome damage as quickly ex vivo as possible, cells were stained with SYTOX red for 15 min at room temperature in the dark and then immediately analyzed by flow cytometry. The second strategy aimed to identify myeloid cell sets, so samples were stained using the following Abs: APC-Cy7-conjugated anti-CD11c, PerCP-Cy5.5-conjugated anti-CD45, and PE-Cy7-conjugated anti-Gr-1, then stained with SYTOX red, and analyzed by flow cytometry. Compensation and spectral unmixing was applied through analysis of cells stained with Abs against CD45 conjugated to the above fluorophores. Flow cytometric data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Pairwise comparisons depicted in Figs. 4C, 6B, and 6C are unpaired Student t tests between the indicated populations. All other bar graphs were analyzed by one-way ANOVA followed by Student-Newman-Keuls method. ANOVA analysis showed significant differences between groups (p < 0.005 for all experiments). Significant differences between individual groups by Student-Newman-Keuls method are as indicated in bar graph legends. Correlation data were analyzed by Pearson product-moment correlation coefficient analysis. All statistical analysis was conducted using SigmaPlot software (Systat Software, Chicago, IL).

Results

Ingested C. neoformans replicates and induces lysosome damage in macrophages

Our first goal was to determine whether C. neoformans induces significant lysosome damage. To measure the extent and kinetics of lysosome damage induced by C. neoformans, we used a ratiometric fluorescence microscopic method developed by our group (34, 35). Cultured BMM were loaded overnight with Fdx and then chased for several hours with fresh media, such that all Fdx was contained in lysosomes (27, 36, 42). The Fdx-loaded cells were infected with IgG-opsonized C. neoformans at a low MOI, such that macrophages started the experiment with one C. neoformans per macrophage. Following 1 h of infection, excess yeast were rinsed away, and infected macrophages were imaged either immediately (1 h postinfection) or 24, 48, and 72 h postinfection. Although C. neoformans–phagosome–lysosome fusion was complete at 1 h postinfection (Fig. 1A), there was no detectable...
lysosome damage at this time, as evidenced by containment of Fdx in exclusively acidic (red) compartments (Fig. 1A–C). In contrast, 24 h later, significant lysosome damage was detectable in a subset of C. neoformans–infected macrophages, indicated by Fdx that has been released into the neutral (cyan and blue) cytoplas (Fig. 1A–C). Levels of lysosome damage increased over time as progressive increases in lysosomal Fdx released into the cytoplasm were observed 48 and 72 h postinfection (Fig. 1A–C). No lysosome damage was observed in uninfected macrophages over the entire course of the experiment (Fig. 1A–C; No yeast).

The average number of intracellular yeast in each macrophage also increased over time, consistent with intracellular growth of C. neoformans (Fig. 1D) and concurrent C. neoformans replication and lysosome damage. Although insufficient to overcome fungal growth, some fungicidal activity of macrophage was observed. A 15-fold increase of dye-incorporating dead yeast between 1 and 72 h was observed (see Supplemental Fig. 1 for demonstration of method). These killed C. neoformans cells were a minor subset compared with the numbers live intracellular cryptococci, consistent with C. neoformans replication prevailing over the active killing of the yeast inside macrophages (Fig. 1D).

To further assess the relationship between C. neoformans growth and lysosomal damage, we segregated macrophages 48 h postinfection into groups based on the number of C. neoformans in each macrophage. Macrophages containing higher yeast burden displayed greater lysosome damage than did those with fewer yeast per macrophage (Fig. 1E). Collectively, these data demonstrate that ingested C. neoformans induces lysosome damage in macrophages and that the damage is strongly linked to intracellular growth of C. neoformans.

Alveolar phagocytes are subjected to C. neoformans–induced damage during pulmonary infection

To extend our in vitro observations to the natural course of cryptococcal infection in vivo, we used ratiometric flow cytometry to measure damage in phagocytes from the lungs of mice infected with C. neoformans as described previously (6, 7, 12, 19, 20, 32). For these experiments, dextran conjugated to both pH-sensitive FITC and pH-insensitive F/SR101dx was administered to mice intratracheally, with or without Uvitex-2B–labeled C. neoformans (Fig. 2Ai). This dye is taken up by lung cells and trafficked into lysosomes. The acidic environment of the lysosome depresses the FITC signal, resulting in a low FITC/SR101 ratio in cells with intact lysosomes. Subsequent damage releases the lysosomal F/SR101dx into the pH neutral cytosol, increasing the fluorescence of FITC and thus raising the FITC/SR101 ratio (Fig. 2Ai).

In uninfected mice receiving F/SR101dx, the majority of alveolar cells recovered by BAL displayed high levels of SR101 fluorescence (Fig. 2B, boxed area) and low FITC/SR101 average ratios (Fig. 2C), indicating a low baseline level of phagolysosomal damage and that the cells were largely unperturbed by the cell isolation process. In mice that received both F/SR101dx and Uvitex-2B–stained C. neoformans, we detected F/SR101dx+ cells (Fig. 2B, boxed area) as well as many F/SR101dx+ cells. Among the F/SR101dx+ cells, many displayed subthreshold Uvitex-2B fluorescence and thus did not contain C. neoformans. These cells (Fig. 2E, lower cell population) displayed only background levels of phagolysosomal damage, similar to those from uninfected mice (Fig. 2C, lower cell population). In contrast, cells with high Uvitex-2B fluorescence, indicating the cells contained intracellular C. neoformans (Fig. 2E, upper cell population), showed higher average phagolysosomal damage (Fig. 2F) and increased percentages of phagolysosomal damage-positive cells (Fig. 2G), compared either to cells from uninfected mice or to the uninfected cells from infected mice. Thus, phagolysosomal damage occurs in C. neoformans–infected cells in vivo.

Dextran-positive cells in the alveolar space at 48 h postinfection are mostly alveolar macrophages

Alveolar macrophages are the predominant phagocyte population in the alveolar air spaces (6, 43, 44) and are thought to be the first cells to encounter C. neoformans in the lungs. To establish that the F/SR101dx-containing cells at 48 h postinfection were indeed alveolar macrophages, we analyzed cells recovered by BAL from F/SR101dx-treated, C. neoformans–infected lungs by flow cytometry. The vast majority of CD11c+ BAL leukocytes contained F/SR101dx in both the uninfected mice (96.3 ± 0.6%; mean ± SEM) (Fig. 3A) and C. neoformans–infected mice (88.7 ± 2.1%) (Fig. 3B). The converse was also true, in that virtually all of the F/SR101dx-containing cells were CD11c+ in uninfected mice (90.5 ± 1.8%) and infected mice (91.4 ± 1.2%). The CD11c+, F/SR101dx+ cells were also CD45+ (Fig. 3C, 3D) and mainly GR-1+ (Fig. 3F, 3G). Thus, F/SR101dx effectively labeled alveolar macrophages (CD11c+ CD45+ GR-1+) in the mouse alveolar space, and few other cells acquired significant levels of F/SR101dx.

Phagolysosomal damage occurs in alveolar and recently recruited macrophages

A small Gr-1−, CD11c+, and F/SR101dx+ triple-positive population (Fig. 3D, top panel) was observed in the lungs of infected mice (7.6 ± 2.8% of CD11c+ F/SR101dx+ double-positive cells), whereas no such population (0.1 ± 0.03%) was observed in the lungs of uninfected mice (Fig. 3C, top panel). Gr-1− staining suggests that these were more recently recruited mononuclear phagocytes than the resident Gr-1+ alveolar macrophage population. Phagolysosomal damage was observed in the C. neoformans–containing cells from both the Gr-1+ (Fig. 3F, compare conditions 2 and 3 in Fig. 3H, 3I) and Gr-1+ (Fig. 3G, conditions 4 and 5 in Fig. 3H, 3I). Interestingly, the phagolysosomal damage observed in the Gr-1+ population was less than that observed in the Gr-1− population (Fig. 3H, 3I, compare conditions 2 and 4). Thus, significant C. neoformans–induced phagolysosomal damage was observed in both resident and infiltrating mononuclear phagocytes if they had ingested C. neoformans.

Lysosome damage requires cryptococcal viability

To investigate the mechanisms underlying C. neoformans–mediated lysosome damage, we focused on BMM as an in vitro model of lung macrophages. Our next goal was to determine whether C. neoformans–induced lysosome damage requires C. neoformans viability. To address this, macrophages were administered IgG-opsonized live C. neoformans or IgG-opsonized heat-killed C. neoformans. At 48 h postinfection, live C. neoformans extensively damaged lysosomes, whereas heat-killed C. neoformans triggered only minimal release of Fdx (Fig. 4A, 4B). Because, unlike the heat-killed C. neoformans, live yeast grew over the course of this experiment, we compared matching subsets of macrophages containing similar number of live versus heat-killed C. neoformans. The lysosome damage was profoundly reduced in macrophages containing four to seven heat-killed C. neoformans compared with those with an equivalent number of live yeast (Fig. 4C). These data indicated that live C. neoformans is a much stronger inducer of lysosome damage than the killed organism, and an active C. neoformans process is required for the induction of lysosome damage.

Macrophage lysosome damage correlates with cryptococcal growth

Our data strongly suggest that C. neoformans growth and lysosome damage are linked (Figs. 1–4), implying that lysosome damage
FIGURE 1. *C. neoformans* induces lysosome damage that increases over time and with yeast replication. BMM lysosomes were loaded with Fdx during overnight incubation followed by 3 h of chase in Fdx-free media. BMM were then infected with IgG-opsonized *C. neoformans* strain H99. After 1 h, excess yeast were rinsed away and cultures imaged immediately (1 h postinfection) or at 24, 48, and 72 h postinfection. (A) Representative images showing the phase contrast of macrophages and internalized *C. neoformans* (top panels), Fdx distribution pattern within macrophages (middle panels) and pH maps, pseudocolored such that each color represents a defined range of pH (bottom panels; pH color key is displayed to the right of the images). Scale bar, 10 μm. (B) Average lysosome damage, quantified as the percent of Fdx released from the lysosome into the cytosol. (C) The data from (B) replotted as the percent of cells positive for lysosome damage (release >10%). For (B) and (C), statistical comparisons are between infected and uninfected macrophages at each time point and between indicated time points and live *C. neoformans* (Cn)-containing cells at the 1-h postinfection time point. One symbol was displayed for each time point because both comparisons resulted in similar p values. (D) Mean number of total and dead *C. neoformans* (Cn) per analyzed macrophage. Statistical comparisons are between indicated time points and the 1-h postinfection time point. (E) Mean lysosome damage as a function of number of *C. neoformans* per macrophage at 48 h postinfection time point. Note that lysosome damage, the number of *C. neoformans*, (Figure legend continues)
To explore this hypothesis, C. neoformans was labeled with Uvitex-2B (33, 45, 46) and then used to infect Fdx-loaded macrophages. Before yeast replication, Uvitex-2B staining was bright (Fig. 5A, red arrowheads). C. neoformans replication diluted the dye, yielding dimly stained daughter cells (Fig. 5B, yellow arrows), which could be readily enumerated separately from the brightly stained undivided yeast (Fig. 5B, red arrowheads). These properties were used to quantify replicative and nonreplicative yeast. After 48 h of infection, there were statistically significant correlations between the level of lysosome damage and numbers of divided (Uvitex-2B dim) C. neoformans per macrophage (Fig. 5C; \( p < 0.001 \)) and the numbers of C. neoformans that had both divided and remained viable (Fig. 5D; \( p < 0.001 \)). These correlations indicate that macrophages showing more lysosome damage were more permissive of C. neoformans growth and those that displayed less lysosome damage were better

and the number of killed C. neoformans increased over the course of the experiment. Data are mean ± SEM and combined from two independent experiments (total analyzed macrophages per time point, \( n > 100 \)). **\( p < 0.005 \) Student-Newman-Keuls.
in controlling *C. neoformans* growth, further supporting the notion that lysosome damage is a mechanism by which *C. neoformans* promotes its intracellular survival and replication.

**Experimentally induced lysosome damage enhances cryptococcal growth**

To test whether lysosome damage directly contributes to intracellular replication of *C. neoformans* rather than simply accompanying *C. neoformans* intracellular growth, we experimentally increased lysosome damage and assayed the effect of this damage on *C. neoformans* replication. We used an established photodamage protocol (34, 38) in which macrophage lysosomes were loaded with Texas Red–dextran as a photosensitizer along with Fdx and subsequently the macrophages infected with Uvitex-2B–labeled *C. neoformans*. To experimentally induce *C. neoformans*–independent lysosome damage, the cells were then exposed to...
intense orange/red light, whereas control (mock exposed) cells were incubated on the microscope stage without light for a similar time period. The photo exposure induced high levels of lysosome damage in most of the exposed cells, whereas the mock-exposed cells remained at baseline levels of lysosome damage (Fig. 6A). In fact, the infected mock-exposed cells displayed a near-baseline level of lysosome damage, whereas the infected photo-exposed cells showed significantly increased release of lysosomal contents (Fig. 6B). Importantly, there was increased \textit{C. neoformans} replication in the photo-exposed macrophages compared with the mock-exposed macrophages (Fig. 6C), providing direct evidence that lysosome damage benefits \textit{C. neoformans} and increases the replication of the yeast.

**FIGURE 4.** Induction of lysosome damage requires viable \textit{C. neoformans} (Cn). BMM lysosomes with Fdx were allowed to phagocytose IgG-opsonized live \textit{C. neoformans} or heat-killed \textit{C. neoformans} particles. At 48 h postinfection, lysosome damage and the number of yeast per BMM were determined. (A) Representative images of BMM containing live \textit{C. neoformans} or heat-killed \textit{C. neoformans}. Scale bar, 10 μm. Lysosome damage for all BMM (B) and the subset of BMM (C), which contained four to seven particles. Note that the killed \textit{C. neoformans} appear to be filled with Fdx, whereas the live yeast exclude Fdx. Data are mean ± SEM, combined from two independent experiments [total analyzed macrophages per condition: (B), n = 90; (C), n = 60]. **p < 0.005 Student-Newman-Keuls (B) and unpaired Student t test (C).

**FIGURE 5.** \textit{C. neoformans}–mediated lysosome damage correlates with intracellular survival and replication of \textit{C. neoformans}. Fdx-loaded BMM were infected with Uvitex-2B–stained \textit{C. neoformans} and assayed immediately or after 48 h for lysosome damage and for the number, viability status, and Uvitex-2B fluorescence signal of intracellular yeast. Representative BMM at baseline (A), brightly fluorescent undivided \textit{C. neoformans} (red arrowheads); and at 48 h (B), when replication of the yeast diluted Uvitex-2B, permitting distinction between \textit{C. neoformans} that had divided (thin yellow arrows) and \textit{C. neoformans} that had not divided (red arrowheads). In (A) and (B), scale bars, 10 μm. Relationship between lysosome damage (x-axis) to the number per BMM of: divided \textit{C. neoformans} (Cn; circles) (C) or viable, divided \textit{C. neoformans} (squares) (D). Correlation parameters for the illustrated data sets are listed to the right of each graph. Lysosome damage correlated with intracellular survival and proliferation of \textit{C. neoformans}. Data are combined from five coverslips across two independent experiments, totaling n > 170 BMM.
Stimulation with IFN-γ induces lysosomal renitence, which protects macrophages from C. neoformans–induced lysosomal damage

Classical activation of macrophages limits lysosome damage induced by bacterial pathogens and noninfectious particles via a mechanism termed inducible lysosome renitence (34). The major factor driving classical activation of macrophages, IFN-γ, has been shown to be a key factor in reducing C. neoformans growth by macrophages in vitro (32) and in limiting cryptococcosis in vivo (8, 11, 12, 40). To determine whether IFN-γ–induced lysosome renitence opposes C. neoformans–mediated lysosome damage, macrophages were stimulated overnight either without cytokine or with IL-4, TNF-α, IFN-γ, or IFN-γ plus TNF-α; these treated macrophages were then infected with C. neoformans and assessed for lysosome damage, C. neoformans growth, and killing of C. neoformans at 24 h postinfection. Once again, C. neoformans infection induced high levels of lysosome damage in unstimulated macrophages (Fig. 7A, 7B). Somewhat unexpectedly, lysosomal damage was partially reduced by stimulation with IL-4, but not by TNF-α (Fig. 7A, 7B). In contrast, stimulation with IFN-γ almost entirely eliminated lysosome damage (Fig. 7A, 7B). Furthermore, the reduction of lysosome damage by IFN-γ was significant regardless of whether comparisons involved all images macrophages (percent Fdx release for unstimulated macrophages: 21.48 ± 3.51 versus IFN-γ–stimulated macrophages: 0.97 ± 0.16; p < 0.001 Student-Newman-Keuls) or only macrophages containing similar numbers (three to six yeast per macrophage) of C. neoformans (Fig. 7B). This suggests that IFN-γ stimulation inhibited C. neoformans–induced lysosome damage independent of any IFN-γ effects on C. neoformans growth rate. Addition of TNF-α did not interfere with the IFN-γ–dependent reduction in C. neoformans–mediated lysosome damage, as cells stimulated with both cytokines displayed very low levels of damage (Fig. 7A, 7B). IFN-γ stimulation of macrophages also reduced C. neoformans–induced lysosome damage 48 h postinfection (Fig. 7F).

Stimulatory conditions that prevented lysosome damage–reduced C. neoformans proliferation in macrophages

As lysosome damage increased C. neoformans replication (Fig. 6) and was prevented by classical macrophage activation (Fig. 7A, 7B), we next asked whether limitation of C. neoformans to macrophage lysosomes is a mechanism for constraining C. neoformans growth. Accordingly, we enumerated microbial growth in macrophages stimulated with the various cytokines. Stimulation with IL-4 or TNF-α, which allowed significant lysosome damage (Fig. 7B), did not reduce microbial replication (Fig. 7C, 7E). In contrast, stimulation with IFN-γ, which limited lysosome damage (Fig. 7B), significantly reduced microbial replication (Fig. 7C, 7E). The IFN-γ–dependent reduction in C. neoformans growth was also evident in experiments using macrophages infected with Uvitex-2B–stained C. neoformans, in which both C. neoformans division and the average number of divided C. neoformans per macrophage were reduced by IFN-γ stimulation (Fig. 7G–I).

Consistent with these data, IFN-γ significantly increased macrophage killing of C. neoformans, whereas IL-4 and TNF-α alone did not (Fig. 7D). Interestingly, dual stimulation with IFN-γ and TNF-α further reduced microbial growth beyond IFN-γ alone

**FIGURE 6.** Photo exposure–induced lysosome damage increases replication of C. neoformans (Cn). BMM were loaded with Fdx and Texas Red dextran and then infected with Uvitex-2B–stained C. neoformans. One group of coverslips was photo exposed using bright orange/red light (Photo Exposed), whereas control coverslips were mock exposed (Mock). A grid array of images was then acquired of each coverslip using an automated acquisition sequence and the microscope motorized stage. (A) Representative images of coverslip imaging displaying 7 × 7 grids. Left panel displays phase contrast with Uvitex-2B staining overlaid in green to illustrate the presence of C. neoformans. Middle and right panels display pH maps of a mock photo-exposed coverslip (middle panel) and a photo-exposed coverslip (right panel). Scale bar, 100 μm. (B) Average lysosome damage, quantified as the percent of Fdx released from the lysosome into the cytosol of the C. neoformans–containing BMM. (C) Average CFU 24 h postinfection for coverslips photo exposed compared with mock-exposed coverslips. In (B) and (C), data are combined from n > 5 coverslips combined from three independent experiments. *p < 0.05, **p < 0.005 Student t test.
(Fig. 7C, 7E) and further increased fungal killing by macrophages (Fig. 7D). Collectively, these data indicate that IFN-γ-activated macrophages prevent lysosome damage and thereby enable more efficient containment and killing of the microbes (Fig. 8).

**Discussion**

We provide the first quantitative evidence, to our knowledge, that *C. neoformans* induces lysosome damage in live macrophages. In addition, we demonstrate bona fide macrophage lysosome damage in the infected lung macrophage populations during early murine pulmonary cryptococcosis, a period of infection exhibiting rapid expansion of *C. neoformans*. This damage requires viable *C. neoformans*, and its magnitude increases over time and with fungal growth in the macrophage (Fig. 8A). Our data demonstrate for the first time, to our knowledge, that lysosome damage contributes to the enhanced survival and intracellular growth of *C. neoformans* (Fig. 8B). Combined, these findings imply that initiation of damage to the phagolysosomal membrane by viable *C. neoformans* promotes the microbe’s intracellular growth, which may, in turn, result in more extensive damage. The outcome of this vicious cycle is cumulative lysosome damage, extremely inefficient killing of *C. neoformans*, and robust *C. neoformans* replication. We also show that IFN-γ stimulation can disrupt damage induction by *C. neoformans* and that when *C. neoformans* is contained within an undamaged lysosome by IFN-γ-induced lysosomal renitence, the cycle of intracellular *C. neoformans* growth and progressive damage is disrupted (Fig. 8C). Collectively, these novel results identify lysosomal damage as an important mechanism of intracellular cryptococcal virulence and demonstrate that prevention of that damage by IFN-γ-induced lysosomal renitence is required for maximal fungicidal activity by the macrophage.

Although there was some previous evidence of phagosomal disruption during in vitro cryptococcal infection of macrophage (30), the data presented in thus study are the first, to our knowledge, to quantitatively measure *C. neoformans*-mediated lysosomal damage in vitro and in vivo and to define damage as a live pathogen–induced, progressive biological process. It is unknown whether the observed *C. neoformans*-mediated release of lysosomal contents is the result of exit of *C. neoformans* from the macrophage endocytic compartment (similar to *Listeria mono-
cytogenes) or C. neoformans residence inside a permeabilized but nevertheless existent membrane compartment (similar to Mycobacterium tuberculosis). Although further research is required to determine the frequency of these two outcomes, there is little evidence from previous electron microscopy studies for full C. neoformans escape from lysosomal membranes and residence in cytosol (5, 30). Collectively, our data show that lysosome damage rapidly develops in C. neoformans–infected macrophages both in vitro and in vivo and that it is associated with increased fungal growth and diminished killing by macrophages. To our knowledge, this is the first evidence linking lysosome damage with cryptococcal intracellular growth.

We recently defined inducible lysosome renitence as a novel activity induced by several factors, including IFN-γ, which opposes lysosome damage induced by silica particles, photo exposure, and L. monocytogenes (34). The data presented in this study show that IFN-γ–induced lysosomal renitence functioned to reduce C. neoformans–induced lysosome damage, which corresponded to a reduction in microbial growth and an increase in fungicidal activity (Fig. 7). Thus, induced lysosome renitence is a critical countermeasure for lysosome damage induced by fungi and an important mechanism by which the fungicidal activity of macrophages is potentiated by IFN-γ.

Our data show that increased lysosome damage results in enhanced cryptococcal growth, and IFN-γ stimulation reduces lysosome damage and cryptococcal growth. This presents the dilemma of whether IFN-γ stimulation reduces C. neoformans–mediated lysosome damage first, which in turn causes a reduction in C. neoformans growth (Fig. 8C), or whether IFN-γ stimulation reduces C. neoformans growth independent of lysosomal integrity, which then results in decreased lysosome damage (Fig. 8D). Although we cannot definitively solve this causality dilemma, our previous work showed that IFN-γ stimulation reduces lysosome damage in nonmicrobial models in which replication does not occur (34). Thus, it is likely that IFN-γ stimulation reduces lysosome damage independent of mere inhibition of C. neoformans growth. This conclusion is reinforced by Fig. 7B, in which lysosome damage was reduced by IFN-γ stimulation even when populations displaying equal levels of fungal growth were compared. Additionally, macrophage stimulation with TNF-α alone induces microbicidal effects against other microbes (47, 48) but did not result in induced lysosome renitence nor improved C. neoformans inhibition (Fig. 5). However, when combined with IFN-γ–induced lysosome renitence, TNF-α considerably improved fungal killing. Taken together, we infer that the IFN-γ–dependent reduction in C. neoformans growth and increase in C. neoformans killing are crucially dependent on restriction of the microbe within the lysosomal compartment and are thus dependent on inducible lysosome renitence.

Although C. neoformans–induced lysosome damage and C. neoformans exocytosis (49, 50) both involve evasion of macrophage lysosomes, we view these as biologically distinct mechanisms. Several published reports have hinted that phagosomal disruption is preferentially followed by C. neoformans exocytosis. First, drugs that increased phagosomal pH also increased C. neoformans exocytosis (33). Exocytosis was preceded by release of a dye dextran from the C. neoformans–containing phagosome (31). However, in that work, there was no indication that the phagosomal dye was released into the cytosol as opposed to extracellular space. IFN-γ does not alter the frequency of cryptococcal expulsion (10); however, IFN-γ limited lysosome damage and preserved lysosomal pH (Fig. 7). Finally, the majority of exocytosis occurs during the first few hours of macrophage infection, albeit tested at higher MOI (51), which is in contrast to the observed kinetics of phagolysosome damage, in which maximal damage occurred several days postinfection (Fig. 1). Collectively, these data overwhelmingly support that C. neoformans exocytosis and C. neoformans–mediated lysosome damage are chronologically and biologically independent.

The data presented in this study show that C. neoformans–induced phagolysosome damage significantly enhances C. neoformans virulence. In contrast, the effects of C. neoformans–mediated phagolysosome damage on host macrophages as well as the C. neoformans processes underlying the induction of lysosomal damage both remain unknown. In the accompanying article (52), Coelho et al. show that intracellular C. neoformans infection of macrophages results in macrophage programmed cell death. These combined data sets open the possibility that C. neoformans–induced phagolysosome damage may enhance programmed cell death in macrophages. Intriguingly, lysosome damage induced by ground silica has been shown to induce inflammation and pyroptosis in activated macrophages (35, 53), consistent with the observed activation of caspase-1 in cells infected with fungal pathogens (54–57). Lysosome damage can also have a role in apoptosis as released lysosomal cathepsins can cleave apoptosis proteins to initiate programmed cell death in cultured fibroblasts (58–66). Although C. neoformans–induced lysosome damage may trigger programmed cell death, it is also possible that C. neoformans–induced programmed cell death induces or enhances lysosome damage. Reactive oxygen species from depolarized mitochondria can damage lysosomes (67). The activation of Bax and Bim can also lead to lysosomal destabilization (68–71). Thus, the
C. neoformans–mediated cell death observed in the accompanying article (52) could be downstream, upstream, or coincided with C. neoformans–mediated lysosome damage, underscoring the need for future studies.

The effector cell population for C. neoformans clearance in vivo has recently been defined by our group as fungicidal exudate macrophages (6). Significantly less lysosome damage was observed in the exudate macrophages than in resident alveolar macrophages on an individual cell basis (Fig. 3). Because lysosome damage supports C. neoformans growth (Figs. 5, 6) and inversely correlates with fungal killing (Figs. 5, 7), these data suggest that improved resistance to lysosome damage contributes to the proficiency of exudate macrophages as antifungal effector cells. Exudate macrophages were more prone to classical activation than alveolar macrophages (6, 72). Overall, these data provide new insight into possible mechanisms enabling exudate macrophages to contribute to anticytotoxicity immunity.

In summary, our data provide the first complete evidence, to our knowledge, of a fungal pathogen using lysosome damage as a virulence mechanism to overcome host defenses and to promote fungal persistence. It is also the first demonstration, to our knowledge, of lysosomal damage triggered by a pathogen in vivo. We further show that the host adaptive immune response counteracts this through IFN-γ–mediated lysosomal remittance, especially within newly recruited exudate macrophages. The presence or absence of inducible lysosome remittance, which depends on the availability of extrinsic cytokines, expands our mechanistic understanding of cryptococcal opportunism in the context of macrophage–pathogen interaction. Although further research is required, our data strongly imply that resistance to lysosome damage is a major mechanism that enables classically activated macrophages to combat infection by C. neoformans and other invasive fungi. Thus, therapeutic strategies that oppose C. neoformans’s ability to damage lysosomes or that bolster macrophage defenses against phagolysosomal damage might effectively complement fungicidal agents in the treatment of cryptococcosis and possibly other invasive fungal infections in immunocompromised patients.

Acknowledgments

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Supplemental Figure 1. Demonstration of fungicidal activity against intracellular *C. neoformans*.
Fdx-loaded *Cn*-infected BMM were analyzed for *Cn* viability and degradation, based on exclusion of Fdx from the yeast cell interior. A-C are representative images of BMM containing (A) live *Cn*; (B) dead *Cn*; & (C) dead and partially degraded *Cn*. To the lower right of each image in A-C is a digitally enlarged inset of each phagolysosome (bounded by a 7 μm square green box in the original panel). Note that to highlight the organelle morphology a higher fluorescence intensity threshold was utilized for the enlarged pH map images.