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Human Dendritic Cell Activity against *Histoplasma capsulatum* Is Mediated via Phagolysosomal Fusion

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Histoplasma capsulatum **is a fungal pathogen that requires the induction of cell-mediated immunity (CMI) for host survival. We have demonstrated that human dendritic cells (DC) phagocytose *H. capsulatum* yeasts and, unlike human macrophages (Mø) that are permissive for intracellular growth, DC killed and degraded the fungus. In the present study, we sought to determine whether the mechanism(s) by which DC kill *Histoplasma* is via lysosomal hydrolases, via the production of toxic oxygen metabolites, or both. Phagosome-lysosome fusion (PL-fusion) was quantified by using fluorescein isothiocyanate-dextran and phase and fluorescence microscopy and by electron microscopy with horseradish peroxidase colloidal gold to label lysosomes. Unlike Mϕ, *Histoplasma*-infected DC exhibited marked PL-fusion. The addition of suramin to *Histoplasma*-infected DC inhibited PL-fusion and DC fungicidal activity. Incubation of *Histoplasma*-infected DC at 18°C also concomitantly reduced PL-fusion and decreased the capacity of DC to kill and degrade *H. capsulatum* yeasts. Further, culture of *Histoplasma*-infected DC in the presence of bafilomycin, an inhibitor of the vacuolar ATPase, did not block DC anti-*Histoplasma* activity, indicating that phagosome acidification was not required for lysosome enzyme activity. In contrast, culture of *Histoplasma*-infected DC in the presence of inhibitors of the respiratory burst or inhibitors of NO synthase had little to no effect on DC fungicidal activity. These data suggest that the major mechanism by which human DC mediate anti-*Histoplasma* activity is through the exposure of yeasts to DC lysosomal hydrolases. Thus, DC can override one of the strategies used by *H. capsulatum* yeasts to survive intracellularly within Mø.**

Histoplasmosis is the most common systemic mycosis in the United States, as well as the most widespread fungal respiratory infection in the world. It has been estimated that approximately 500,000 new *Histoplasma capsulatum* infections, the causative agent of histoplasmosis, occur each year in the United States alone (1). Although *H. capsulatum* is distributed worldwide, it is endemic in the Mississippi and Ohio River valleys. *H. capsulatum* can be found in the environment within rich soil and in areas that contain bird or bat guano such as caves, chicken houses, and old buildings (6).

The interaction between macrophages (Mø) and *H. capsulatum* is a critical event in the pathogenesis of histoplasmosis as Mø initially provide an environment for replication and dissemination, as well as function as the final effector cell for destruction of *H. capsulatum*. *Histoplasma* yeasts have adapted to survive within human Mø by inhibiting phagosome-lysosome fusion (PL-fusion) (39) and controlling intraphagosomal pH (9, 48). The fungus also appears to be inherently resistant to toxic oxygen metabolites of the respiratory burst (3, 38, 43).

As the induction of cell-mediated immunity is the hallmark of resistance in histoplasmosis, and since dendritic cells (DC) are the most potent antigen-presenting cell in the host, we recently sought to define a role for DC in host defense against *H. capsulatum*. We found that immature human DC were able to recognize and phagocytose *Histoplasma* yeasts but that recognition of *Histoplasma* was via the fibronectin receptor, VLA-5 (11), and not CD18, as was found for human Mø (3, 34). Most interestingly, and in contrast to Mø, DC killed and degraded *H. capsulatum* yeasts. Further, DC processed *Histoplasma* antigens and stimulated proliferation of $CD3^+$ T cells (11).

Since DC were very efficient at killing and digesting Hc yeasts, we sought to determine the mechanism of DC fungicidal activity. The results presented herein demonstrate that, unlike human Mø in which PL-fusion is limited, *H. capsulatum*-infected DC undergo pronounced PL-fusion, suggesting that DC lysosomal hydrolases are sufficient to kill and degrade *H. capsulatum* yeasts.

MATERIALS AND METHODS

Reagents. N^G-monomethyl-L-arginine (L-NMMA) was purchased from Calbiochem, La Jolla, CA. Superoxide dismutase (SOD), catalase, and fluorescein isothiocyanate (FITC)-dextran were purchased from Sigma, St. Louis, MO. Diphenylene iodonium bisulphate (DPI) was a gift from Andrew Cross, The Scripps Research Institute, La Jolla, CA. Bafilomycin A1 was the generous gift of K. Altendorf (Universitat Osnabruck, Osnabruck, Germany). Electron microscopy reagents were purchased from Electron Microscopy Sciences, Inc., Fort Washington, PA.

Preparation of human DC and Mø. Monocytes were isolated by sequential centrifugation on Ficoll-Hypaque and Percoll gradients (Amersham Pharmacia LKB, Piscataway, NJ) from buffy coats obtained from the Hoxworth Blood Center, Cincinnati, OH, or from blood drawn from normal adult donors in our laboratory (34). To obtain DC, monocytes were cultured in six-well tissue culture plates (Corning-Costar, Cambridge, MA) at 6.5×10^5/ml in RPMI 1640 containing 200 mM L-glutamine, 50 μM 2-mercaptoethanol (Sigma), 10% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, MD), 50 ng of kanamycin (Sigma)/ml, 1% nonessential amino acids (BioWhittaker, Walkersville, MD), and 1% pyruvate (BioWhittaker). Human granulocyte-macrophage colony stimulating factor (115 ng/ml; Peprotech, Inc., Rocky Hill, NJ) and human interleu-

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kin-4 (50 ng/ml; Peprotech, Inc.) were also added to each well, and DC were studied after 5 to 8 days of culture (11).

Mø were obtained by culture of monocytes at 10^6/ml in Teflon beakers with RPMI 1640 containing 15% pooled human serum, 10 μg of gentamicin/ml, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (Sigma) (34). Mø were studied after 5 to 7 days in culture.

Yeasts. *H. capsulatum* strain G217B was maintained as described previously (34). Yeasts were grown in Histoplasma Macrophage Medium (54) at 37°C with orbital shaking at 150 rpm. For phagocytosis, intracellular growth, and PL-fusion studies, 48-h log-phase yeasts were harvested by centrifugation, washed three times in Hanks balanced salt solution containing 0.25% bovine serum albumin (HBSA), and resuspended to 50 ml in HBSA. Large aggregates were removed by centrifugation at 200 × *g* for 5 min at 4°C. The top 10 ml was removed, and the single-cell suspension obtained was standardized to the appropriate concentration according to the assay protocol. Heat-killed yeasts were prepared by heating the yeasts at 65°C for 1 h.

Quantitation of intracellular growth of *H. capsulatum* yeasts in DC and Mø. Intracellular growth of *H. capsulatum* yeasts in DC and Mø was quantified by the incorporation of [^{3}H]leucine as described previously (35). DC (10^5) were preincubated for 30 min in 96-well tissue culture plates (Corning-Costar) at 37°C with either SOD (60 μg/ml), catalase (100 μg/ml), DPI (10 μm), or L-NMMA (100 μm) or RPMI 1640 as a control. DC/Mø were preincubated for 24 h in the experiments with suramin. A total of 10^4 viable *H. capsulatum* yeasts were then added to each well, followed by further incubation for 24 to 48 h. After the incubation period, the plates were centrifuged, and the supernatants carefully aspirated through a 27-gauge needle. Then, 50 μl (1.0 μCi) of [^{3}H]leucine (specific activity, 153 Ci/nmol; Dupont/New England Nuclear) in sterile water and 5 μl of a 10× yeast nitrogen broth (Difco Laboratories) were added to each well. After further incubation for 24 h at 37°C, 50 μl of L-leucine (10 mg/ml) and 50 μl of sodium hypochlorite were added to each well, and the contents of the wells were harvested onto glass fiber filters by using an automated harvester (Skatron, Sterling, VA). The filters were placed into scintillation vials, scintillation cocktail was added, and the vials were counted in a Beckman LS 6500 liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA). The results are expressed as the means ± the standard errors of the mean (SEM) of the counts per minute incorporated by the remaining viable yeasts. All experiments were performed in triplicate and three to seven experiments were performed with DC/Mϕ obtained from different donors.

Quantitation of PL-fusion. Two different techniques were used to quantify PL-fusion. In the first, DC and Mø (2×10^5) were adhered to glass coverslips for 2 h at 37°C. The cells then were incubated with FITC-dextran (1 mg/ml) for 4 h at 37°C. The cells then were washed and cultured overnight at 37°C in the presence or absence of suramin (200 μg/ml). The medium was removed, and *H. capsulatum* yeasts (5×10^5) were added to the DC/Mø for 1 h to allow for phagocytosis. Uningested yeasts were removed by washing, and the cells were incubated a further 2 h. The cells were then fixed in 3.75% paraformaldehyde for 20 min. The paraformaldehyde was removed and the cells placed in Dulbecco phosphate-buffered saline containing 5% glucose. Coverslips were removed and mounted cell side down on glass slides in 90% glycerol in phosphate-buffered saline and sealed with clear nail polish. PL-fusion was quantified by counting 100 yeast-containing phagosomes and determining the percentage of phagosomes that contained a rim of green fluorescence around the yeast. The data are expressed as the mean ± the SEM of the percentage of PL-fusion.

In the second assay, DC or Mø (10^6) were incubated with 18-nm colloidal gold stabilized with horseradish peroxidase (HRP:Au18) in polypropylene tubes for 2 h at 37°C in a water bath with orbital shaking at 150 rpm. The cells were washed two times with HBSA and cultured for an additional 2 h at 37°C to ensure that the HRP:Au18 entered the lysosomal compartments. After the second incubation, 5×10^6 viable or heat-killed *H. capsulatum* yeasts were added, and phagocytosis was allowed to proceed for 3 h at 37°C. The cells then were washed twice in HBSA, fixed immediately, and then processed for electron microscopy (18, 39, 40). After polymerization of the samples, ultrathin sections were cut with a diamond knife (Diatome U.S., Ft. Washington, PA) on a Reichert-Jung Ultracut E ultramicrotome (Cambridge Instruments, Buffalo, NY). Samples were picked up on 300 mesh copper grids, stained with uranyl acetate and lead citrate for contrast, and viewed in a JEOL-100CX electron microscope operating at 80 kV.

DC and Mø PL-fusion was quantified by counting the number of 18-nm gold particles in phagosomes containing *H. capsulatum* yeasts (39). The data are expressed as the means ± the SEM of the number of gold particles/yeast-containing phagosome.

In some experiments, DC pulsed with HRP:Au18 at 37°C were cooled to 18°C over a period of 1 h. Simultaneously, an aliquot of *Histoplasma* yeasts were equilibrated to 18°C. Experimental (18°C) and control (37°C) DC were incubated with *H. capsulatum* yeasts for 4, 6, 8, 12, and 24 h at the appropriate temperatures. After each incubation period, samples were transferred to an ice water bath to halt PL-fusion. The DC were then washed twice in cold HBSA, fixed, and processed for electron microscopy. The data are presented as the percentage of phagosomes containing one or more gold particles, the mean gold particles/phagosome, and the percent dead yeasts.

Statistics. Statistical analysis of the data was performed by using Sigma Stat (Jandel Scientific, San Rafael, CA). Depending on the distribution, data were analyzed by parametric or nonparametric statistics. The results were considered significant at $P < 0.05$.

RESULTS

PL-fusion is mediated by *Histoplasma*-infected DC. In vitro studies in human Mø (3, 38, 43) and in murine Mø (16, 52) demonstrate that *H. capsulatum* yeasts are inherently resistant to toxic oxygen metabolites of the respiratory burst. Thus, although the respiratory burst may be stimulated during phagocytosis, *H. capsulatum* yeasts readily replicate in these cells. In addition, PL-fusion in *Histoplasma*-infected human Mø is significantly reduced compared to Mø containing nonpathogenic *Saccharomyces cerevisiae* yeasts (33, 39). Further, induction of PL-fusion in human Mø by adherence to type 1 collagen matrices leads to killing of *H. capsulatum* (39). Since inhibition of PL-fusion appears to be a key part of the strategy that *H. capsulatum* yeasts use to survive within human Mø, we hypothesized that human DC might kill *H. capsulatum* through efficient fusion of their lysosomes with yeast-containing phagosomes.

PL-fusion was quantified by labeling DC and Mø lysosomes with FITC-dextran. After phagocytosis, PL-fusion was visualized by the presence of green rim fluorescence around the yeast. DC and Mø phagocytosed nearly identical numbers of yeasts (data not shown). DC that ingested either viable or heat-killed *H. capsulatum* yeasts demonstrated PL-fusion in ≥80% of yeast-containing phagosomes (Fig. 1A). In contrast, human Mø exhibited minimal PL-fusion upon phagocytosis of viable yeasts, but more than 95% PL-fusion upon ingestion of heat-killed yeasts (Fig. 1A), confirming our previous studies (33, 39).

In a separate set of experiments, DC and Mø were labeled with HRP:Au18, infected with viable or heat-killed *H. capsulatum* yeasts for 3 h, and then were fixed and processed for electron microscopy. HRP targets the lysosomal compartment (12) and can be visualized by the electron-dense gold particles. Figure 2A shows an uninfected DC that is preloaded with HRP:Au18. Gold particles can be visualized within DC lysosomes. Analysis of multiple electron micrographs revealed the process of PL-fusion in *Histoplasma*-infected DC (Fig. 2B to D). Figure 2B shows a *Histoplasma*-containing phagosome that is adjacent to a gold containing lysosome. In Fig. 2C, the lysosome can be seen fusing with the phagosome. Additional adjacent gold-containing lysosomes also are present. Finally, Fig. 2D shows a phagosome in which PL-fusion already has occurred, as evidenced by the electron-dense gold particles present within the phagosomal membrane.

PL-fusion was quantified by counting the mean number of gold particles present per yeast containing phagosome. DC that ingested viable *H. capsulatum* yeasts demonstrated considerable PL-fusion (eight gold particles/phagosome), whereas Mø exhibited little PL-fusion (two gold particles/phagosome) (Fig. 1B). PL-fusion in Mø containing heat-killed yeasts was

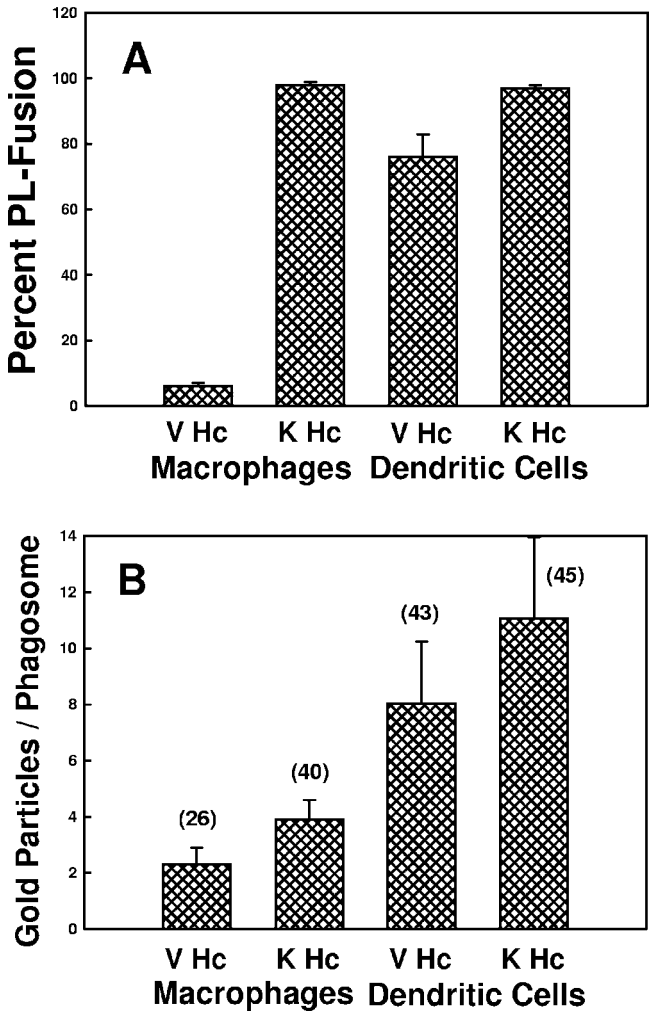

FIG. 1. Quantitation of PL-fusion in DC by light and electron microscopy. (A) DC and Mø were incubated with FITC-dextran for 4 h, washed, and then cultured for 24 h. Viable (V) and heat-killed (K) *H. capsulatum* yeasts were then added, and phagocytosis was allowed to proceed for 1 h. Unbound yeasts were removed by washing, and the cells were incubated an additional 2 h. The monolayers then were fixed in paraformaldehyde, and PL-fusion was quantified as described in Materials and Methods. The data are the means ± the SEM of the percent PL-fusion in six experiments performed in duplicate. (B) DC and Mø were preincubated with 18-nm colloidal gold stabilized with horseradish peroxidase (HRP:Au18) for 2 h. Viable (V) or killed (K) *H. capsulatum* yeasts were then added to the DC, followed by incubation for 3 h. The DC were washed and processed for electron microscopy. PL-fusion was quantified by counting the number of 18-nm gold particles per yeast containing phagosome. The data are the means ± the SEM of *n* phagosomes. The number of phagosomes counted for each experimental condition is given in parentheses above the bars in the graph.

about twice that observed with viable yeasts. DC that ingested heat-killed yeasts had about 11 gold particles/phagosome (Fig. 1B).

Inhibition of PL-fusion reduces the ability of DC to restrict the growth of *H. capsulatum* yeasts. We next wanted to demonstrate that PL-fusion was required for DC fungistatic or fungicidal activity. DC and Mø were cultured overnight in the absence or presence of various concentrations suramin and then incubated for an additional 24 h with *H. capsulatum* yeasts. Intracellular growth was quantified by the incorporation of [^{3}H]leucine as described previously (35). Compared to Mø, the intracellular replication of *H. capsulatum* yeasts in DC was inhibited by 96% (Mø, 43,724 cpm; DC, 1,888 cpm), confirming our original observations (11). The inclusion of suramin enhanced the replication of yeasts in DC in a concentration-dependent fashion (Fig. 3). In contrast, suramin had no effect on the growth of *H. capsulatum* in Mø (data not shown). Further, suramin significantly reduced DC PL-fusion from 89 to 34% (Fig. 4). Suramin did not affect the numbers of yeasts ingested by DC. Thus, a reduction in DC PL-fusion was concomitant with a decrease in the ability of DC to restrict the growth of *H. capsulatum* yeasts.

We next sought to confirm these observations by using an entirely different approach. Incubation at 18°C has been shown by numerous investigators to inhibit the fusion of lysosomes and other membranous organelles such as endosomes and phagosomes (8, 19, 25–29, 41, 42, 49, 51, 53). At 18°C lysosomal membrane lipids are in their gel stage and unable to fuse with endosomes and phagosomes (8, 19). Therefore, we quantified the capacity of DC to kill and degrade *H. capsulatum* yeasts under temperature conditions that inhibited PL-fusion.

To confirm that incubation at 18°C would inhibit DC PL-fusion, DC were preloaded with HRP:Au18, and the cells were divided so that experimental samples were incubated at 18°C and control samples were incubated at 37°C. Both sets of DC then were infected with viable *H. capsulatum* yeasts for 4, 6, 8, 12, and 24 h. After the various incubation periods, the cells were processed for electron microscopy. The ability of temperature to inhibit PL-fusion in *Histoplasma*-infected DC was quantified by determining the percentage of phagosomes that contained one or more gold particles and the mean number of gold particles per yeast containing phagosome. Approximately 90% of the phagosomes in control samples (37°C) contained gold particles at all time points analyzed except for 4 h, at which time 80% of the phagosomes contained gold (Fig. 5). Most importantly there was an increase in the number of gold particles per phagosome over time (Fig. 5). In contrast, in DC incubated at 18°C, the percentage of phagosomes containing gold particles was considerably lower than control samples (Fig. 5), as was the mean number of gold particles per *Histoplasma*-infected phagosome. Thus, at 18°C there was significantly reduced PL-fusion in *Histoplasma*-infected DC.

To demonstrate that PL-fusion was required for DC to kill and degrade *H. capsulatum* yeasts, electron micrographs from both experimental and control groups were analyzed for the percentage of dead *Histoplasma* yeasts. *H. capsulatum* yeasts were designated as dead or in the process of dying by (i) thickening of the plasma membrane (PM), (ii) precipitate formation at the junction of PM and the cell wall, (iii) disaggregation of the yeast cell wall, and (iv) mitochondrial swelling and loss of ultrastructural integrity. As shown in Fig. 6, incubation of *Histoplasma*-infected DC at 18°C strikingly decreased the percentage of killed yeasts in DC phagosomes. No killed yeasts were observed at 4 h in DC incubated at 18°C, whereas 27% of yeasts were already in various states of disintegration in DC incubated at 37°C. Furthermore, the maximum percentage of killed yeasts in DC incubated at 18°C was only 19%, whereas in DC incubated at 37°C 69% of yeasts were dead and de-

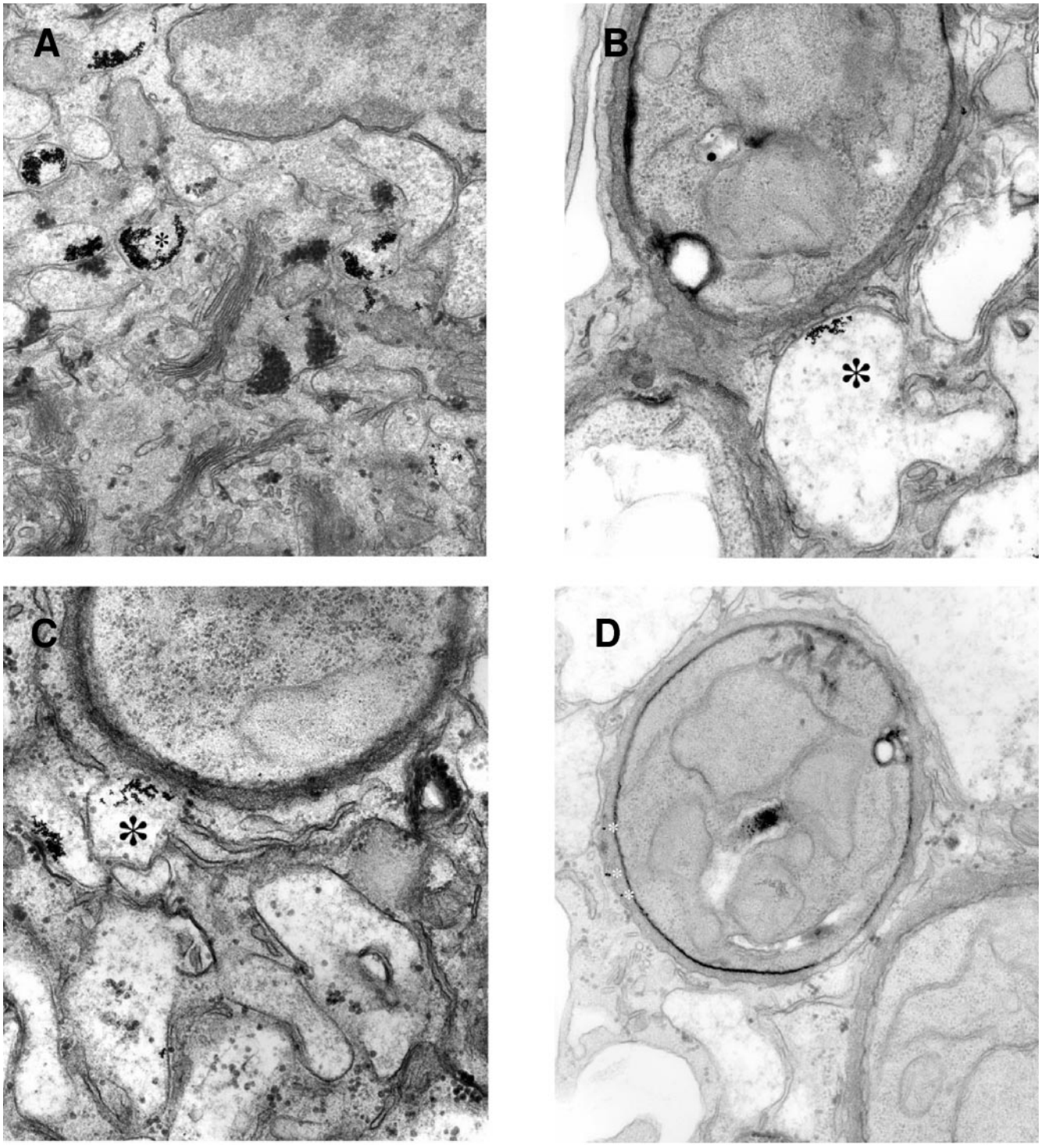

FIG. 2. Electron microscopy of PL-fusion in *H. capsulatum*-infected DC. The procedure was as described in the legend to Fig. 1. (A) Uninfected DC loaded with HRP:Au18; (B) a gold-labeled lysosome can be seen adjacent to a yeast-containing phagosome; (C) the lysosome is seen fusing with the phagosome; (D) phagosome in which fusion with a lysosome has already occurred. Note the electron-dense gold particles within the phagosomal membrane. Magnification, ×16,000.

graded. Again, a decrease in PL-fusion correlated with a decrease in the number of killed yeasts, suggesting that DC mediate killing of *H. capsulatum* yeasts through PL-fusion.

Respiratory burst inhibitors and inhibitors of nitric oxide production do not modulate the capacity of DC to inhibit the intracellular growth of *H. capsulatum* yeasts. Although we considered it unlikely, we next sought to determine whether toxic oxygen metabolites or reactive nitrogen intermediates might play a role in the fungicidal activity of DC. DC were preincubated with SOD, catalase, DPI, and L-NMMA for 30 min at

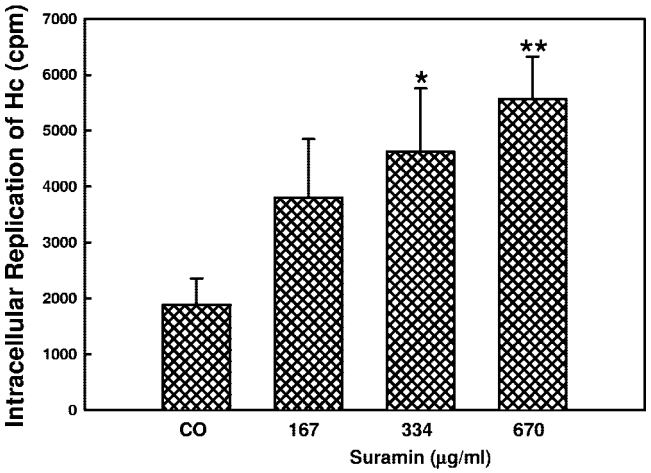

FIG. 3. Suramin enhances the replication of *H. capsulatum* yeasts in DC. DC were cultured for 24 h with various concentrations of suramin. *H. capsulatum* yeasts were then added for an additional 24 h. Intracellular growth was quantified by the incorporation of [^{3}H]leucine as described in Materials and Methods. The data are the means ± the SEM of eight experiments performed in triplicate. The mean counts in the Mø control were 43,724. ✻, $P < 0.05$ compared to the DC control (CO); ✻✻, $P = 0.001$ compared to the DC control.

37°C. Viable *H. capsulatum* yeasts then were added, and the cultures were further incubated for 48 h. SOD, catalase, and L-NMMA had no effect on the capacity of DC to inhibit the intracellular growth of *H. capsulatum yeasts* (Fig. 7). DPI, an inhibitor of NO synthase, caused a modest increase in the growth of *Histoplasma* within DC, but this increase was not statistically significant. Thus, it did not appear that toxic oxygen radicals or NO were involved in DC anti-*Histoplasma* activity.

Phagosomal acidification is not required for DC to inhibit the replication of *H. capsulatum* yeasts. In experiments to be reported elsewhere, we found that phagosomal acidification is not required for inhibiting the growth of *H. capsulatum* yeasts by human Mø activated by adherence to collagen matrices (39) or treated with chloroquine (36). Since both DC and Mø are derived from blood monocytes, we sought to determine

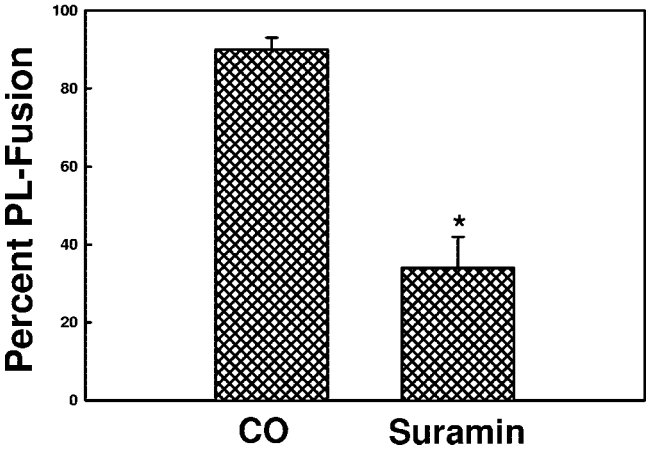

FIG. 4. Suramin inhibits PL-fusion in *H. capsulatum*-infected DC. DC were labeled with FITC-dextran for 4 h at 37°C, washed and then cultured for 24 h in the absence or presence of suramin (200 μg/ml). *H. capsulatum* yeasts were then added, and phagocytosis was allowed to proceed for 1 h. The monolayers were washed, incubated in media a further 2 h, and then fixed. PL-fusion was quantified as described in Materials and Methods. The data are the means ± the SEM of the percent PL-fusion from six experiments performed in duplicate. ✻, $P = 0.002$ compared to the control.

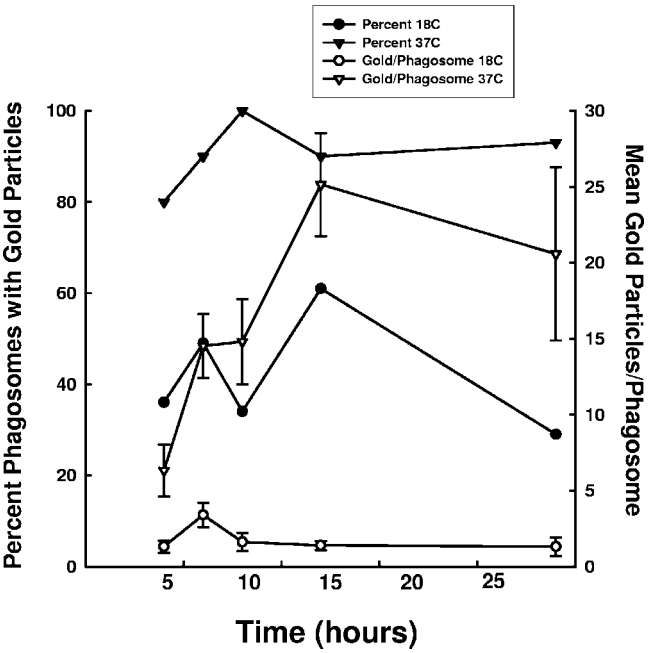

FIG. 5. Incubation at 18°C reduces PL-fusion in DC. DC were loaded with HRP:Au18 and then equilibrated at either 37 or 18°C for 1 h. Previously, temperature-equilibrated *Histoplasma* yeasts were added to the DC and further incubated for various periods of time at either control (37°C) or experimental (18°C) temperatures. After each incubation period, the DC were washed and then processed for electron microscopy. The data are expressed as the means ± the SEM of the number of gold particles per yeast containing phagosome and the percentage of phagosomes containing gold particles. At 18°C, 57, 39, 36, 27, and 17 yeast-containing phagosomes were counted at 4, 6, 8, 12, and 24 h, respectively. At 37°C, 15, 31, 16, 13, and 14 yeast-containing phagosomes were counted at 4, 6, 8, 12, and 24 h, respectively.

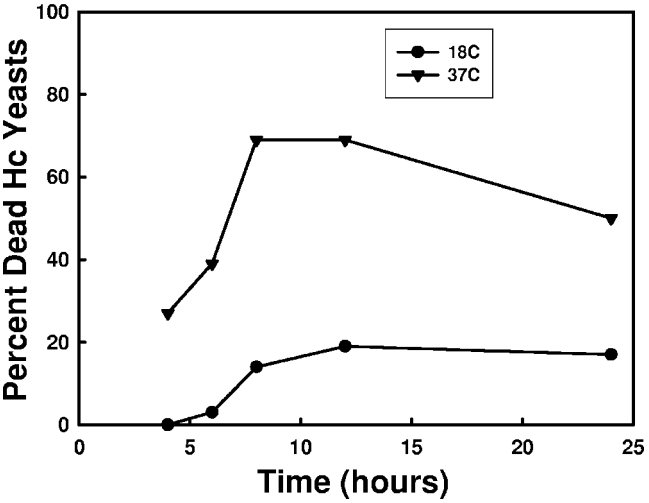

FIG. 6. Incubation at 18°C decreases the percentage of dead *Histoplasma* yeasts in DC. The experiment is the same as described in Fig. 4, except that the data are presented as the percentage of dead *Histoplasma* yeasts within gold-containing phagosomes.

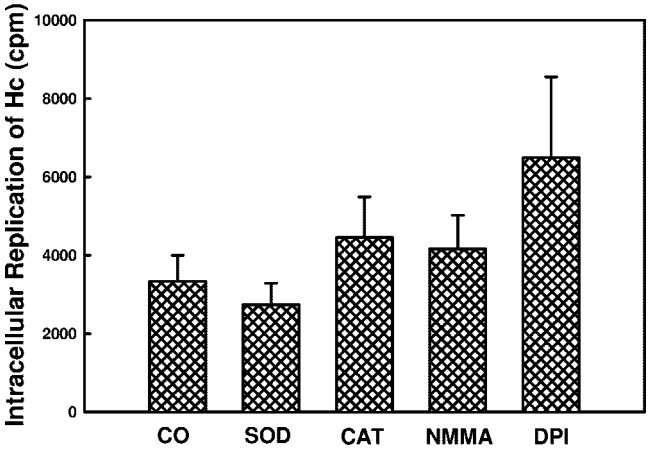

FIG. 7. Respiratory burst inhibitors and inhibitors of nitric oxide production do not alter the ability of DC to control the intracellular growth of *Histoplasma* yeasts. DC were preincubated with SOD, catalase, DPI, and L-NMMA for 30 min. Viable *Histoplasma* yeasts were added to the wells, and the cultures were incubated for 48 h. Intracellular replication was quantified as the incorporation of [^{3}H]leucine as described in Materials and Methods. The data are the means ± the SEM of 10 experiments performed in triplicate.

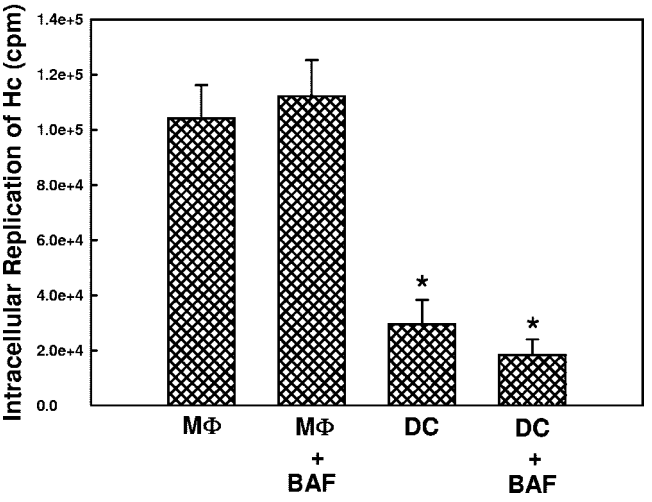

FIG. 8. Phagosomal acidification is not required for DC fungistatic activity against *H. capsulatum*. DC and Mø were infected with *H. capsulatum* yeasts for 24 h in the absence or presence of 25-nm bafilomycin. Intracellular replication was quantified by the incorporation of [^{3}H]leucine as described in Materials and Methods. The data are the mean ± the SEM of six experiments performed in triplicate. ✻, $P <$ 0.001 compared to Mø alone.

whether phagosomal acidification was required for DC fungistatic activity. DC and Mø were infected with *H. capsulatum* yeasts and cultured for 24 h in the absence or presence of 25-nm bafilomycin. Previously, we demonstrated that 25-nm bafilomycin was sufficient to block acidification of RAW 264.7 Mø that had ingested *S. cerevisiae* yeasts (48). The data in Fig. 8 show that the ability of DC to restrict the replication of *H. capsulatum* yeasts compared to Mø was not reversed by bafilomycin. Bafilomycin also did not affect the intracellular growth of yeasts in Mø.

DISCUSSION

H. capsulatum yeasts survive and multiply intracellularly within human Mø with an intracellular generation time of about 20 h (37). In recent studies we demonstrated that human DC also phagocytose *H. capsulatum* yeasts, but the intracellular replication of yeasts was strongly inhibited, as quantified by the incorporation of [^{3}H]leucine (11). Further, by electron microscopy, we observed that most the yeasts were in various stages of being digested, demonstrating that DC not only inhibited intracellular growth but also killed many of the yeasts (11).

There are two major mechanisms by which phagocytes kill pathogenic microorganisms. The first mechanism is through the production of toxic oxygen radicals and/or NO. The second mechanism is through PL-fusion and lysosomal hydrolases. It is important to note that these phagocyte killing mechanisms are not necessarily mutually exclusive, but rather they work together to kill a wide variety of pathogenic microorganisms. Intracellular pathogens of Mø have evolved various stratagems to avoid the killing mechanism(s) to which they are most sensitive.

In the case of *H. capsulatum*, it has become clear that the intracellular survival strategy of the yeasts is to avoid lysosomal hydrolases. In murine Mø, this is accomplished by modulating the intraphagosomal pH to 6.5 (9). At this pH, lysosomal hydrolases that enter the phagosome presumably are inactive (10). In human Mø, *H. capsulatum* avoids lysosomal hydrolases by inhibiting PL-fusion (33, 39). Since avoiding lysosomal enzymes is the strategy for *H. capsulatum* survival in Mø, it was logical to postulate that DC inhibit the growth of and kill *H. capsulatum* yeasts by efficiently mediating PL-fusion.

Indeed, extensive PL-fusion was observed in DC infected with viable *H. capsulatum* yeasts by using two complementary techniques. Minimal PL-fusion was observed in Mø, a finding consistent with our previously published data (33, 39). The amount of PL-fusion observed in *Histoplasma*-infected DC was similar to that demonstrated in Mø infected with the nonpathogenic yeast *S. cerevisiae* (33, 39). The PL-fusion mediated by DC also is similar to that observed in *Histoplasma*-infected Mø adhered to collagen matrices (39). Thus, in a sense, the DC behave like an activated Mø, both with respect to mediating PL-fusion and in the ability to kill and digest the fungus. Finally, it should be noted that all of our experiments were performed with in vitro human monocyte-derived DC, and it is unknown whether these observations will hold true for murine DC or for particular subsets of DC. We have found that murine lung DC are fungistatic for *H. capsulatum* yeasts, but we have not yet addressed the mechanism of this activity (S. L. Newman, unpublished observations).

At present, it is not clear why DC are able to fuse their lysosomes with yeast-containing phagosomes, whereas Mø demonstrate almost no fusion. One possible explanation is that in Mø the signal transduction pathway induced by the phagocytosis of *H. capsulatum* yeasts does not result in PL-fusion, whereas the signal transduction pathway induced in DC does lead to PL-fusion. In this regard, it is noteworthy that Mø and DC utilize different receptors and recognize different ligands on the surface of the yeast to effect phagocytosis. Thus, Mø recognition of *H. capsulatum* is via the CD18 integrin family (3,

34), whereas DC recognize the fungus via the fibronectin receptor, VLA-5 (11). Further, Mø CD18 receptors recognize heat shock protein 60 (HSP 60) on *H. capsulatum* yeasts, whereas DC VLA-5 recognizes a completely different surface molecule(s) (23).

However, it is unlikely that differential signal transduction alone accounts for the disparate responses of DC and Mø. Indeed, phagocytosis of heat-killed *H. capsulatum* by Mø leads to normal PL-fusion, and the yeasts are completely digested (40). Further, it is clear that the yeasts must synthesize new protein to effect inhibition of PL-fusion in Mø (40). Thus, whatever products *H. capsulatum* might produce to block PL-fusion in Mø, these same metabolites do not work in DC. In contrast to this scenario, signal transduction does appear to play a role in the ability of *Toxoplasma gondii* to block PL-fusion in Mø. Thus, blocks in Mø PL-fusion can be overcome simply by opsonizing the live parasites with specific antibody prior to phagocytosis (2, 17, 44). However, *H. capsulatum* yeasts opsonized in immune serum replicate in Mø at the same rate as unopsonized yeasts (37).

To confirm that PL-fusion was required for DC to inhibit the growth of and kill *H. capsulatum* yeasts, we incubated *Histoplasma*-infected DC under conditions in which PL-fusion was inhibited. Suramin is a polybasic anion that binds strongly to plasma proteins. It enters cells by endocytosis and becomes concentrated in their lysosomes, where it inhibits many enzymes, including some of the lysosomal proteases, and suramin has been used previously in several studies to interfere with PL-fusion in Mø (7, 14, 15, 50). In the present study, treatment of DC with suramin concomitantly decreased the capacity of DC to restrict the replication of *H. capsulatum* yeasts and reduced the amount of PL-fusion.

In a separate set of experiments, culture at 18°C was used to block DC PL-fusion. This strategy took advantage of the fact that certain membrane transport processes and enzyme activities, for example, can be shown to cease when the bilayer viscosity is experimentally increased beyond a threshold level (47). The fluidity of a lipid bilayer depends both on its composition and temperature (45). The temperature at which the bilayer is liquid (i.e., free to diffuse) is called the sol stage. At lower temperatures the bilayer becomes rigid and crystalline and is called the gel stage. The temperature driven change from the sol stage to the gel stage is called phase transition. Numerous studies have demonstrated that at temperatures $\leq$18°C lysosomal membranes are in the gel stage (8, 19, 20). Thus, phase transition for lysosomes occurs at 18°C and, therefore, PL-fusion is inhibited.

Culture at 18°C has been used to investigate the intracellular trafficking of various organisms such as opsonized yeasts (19), Semliki Forest virus (26, 27), and influenza virus (28). In addition, the transport of other substances such as *Pseudomonas* exotoxin A (25, 29), diphtheria toxin (41), soluble HRP (49), and asialoglycoproteins (8, 53) have all been analyzed using this technique.

In the present study, incubation of DC at 18°C significantly reduced the amount of fusion that occurred at all time points examined. Thus, there was a decrease in both the percentage of phagosomes containing HRP:Au18 and a decrease in the number of gold particles per phagosome. Concurrent with the decrease in PL-fusion there was a decrease in the percentage of *H. capsulatum* yeasts that were killed. Hence, the maximum amount of dead yeasts observed at any time point in DC incubated at 18°C was 19%, which is 72% lower than the corresponding time in DC incubated at 37°C.

We recognize that neither of the two techniques used to block PL-fusion is specific for that process only. Incubation at 18°C also would be expected to slow the general metabolism of the DC which is optimal at 37°C. Indeed, even the activity of the lysosomal hydrolases is optimal at 37°C. In addition, it also is possible that suramin might be inhibiting some antifungal compound within DC. However, taken together, the data from both sets of experiments support the idea that PL-fusion is the primary mechanism by which DC mediate killing of *H. capsulatum* yeasts, particularly when one considers the fact that *H. capsulatum* yeasts are resistant to toxic oxygen metabolites.

The data also demonstrate that DC anti-*Histoplasma* activity does not require the acidification of the yeast-containing phagosome. This finding is in agreement with our studies that reveal that the pH of the *Histoplasma* phagosome in human Mø is 6.5 for both viable and killed yeasts and that, under conditions in which human Mø kill *H. capsulatum*, further acidification is not required (Newman, unpublished). Thus, these studies suggest that, unlike murine Mø, the lysosomal hydrolases of human Mø and DC are fully competent at a relatively neutral pH.

Although the importance of PL-fusion in DC-mediated killing of *H. capsulatum* yeasts is clear, there does not appear to be a role for toxic oxygen metabolites. Thus, the respiratory burst inhibitors tested did not significantly reverse the capacity of DC to inhibit the growth of *H. capsulatum*. These results are consistent with previous studies that have suggested that *Histoplasma* yeasts are impervious to the effects of toxic oxygen metabolites generated by human and murine Mø (3, 16, 34, 52). The data also are consistent with the fact that all of the very potent fungistatic activity exhibited by human neutrophils against *H. capsulatum* yeasts is mediated by the azurophil granules, and the production of toxic oxygen metabolites is not required for neutrophil fungistatic activity (38).

NO also does not appear to play a role in the capacity of DC to kill *H. capsulatum*, since the NO synthase inhibitors DPI and L-NMMA had only a modest effect. NO is involved in the killing of several intracellular parasites by murine peritoneal Mø (13, 32) and also plays a role in inhibition of the intracellular growth of *H. capsulatum* yeasts in gamma interferon-lipopolysaccharide-activated murine splenic Mø (22) and gamma interferon-activated RAW 264.7 Mø (21, 31). The present study is in agreement with the reports of others that indicate that NO does not appear to play a role in the antimicrobial activity of human Mø against *Toxoplasma gondii*, *Leishmania donovani*, *Chlamydia psittaci* (30), or *Cryptococcus neoformans* (4).

Initial reports on DC described them as nonphagocytic and containing a paucity of lysosomes (46). However, over the past several years it has become clear that "immature" DC can ingest a variety of pathogens and serve as a link between innate and cell-mediated immunity (5, 24). The data presented here reinforce this concept by demonstrating that the lysosomal enzymes of DC are capable of killing and degrading a fungal pathogen, *H. capsulatum*. Thus, it is clear that, regardless of

lysosomal number or appearance, DC lysosomes contain potent fungicidal enzymes.

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