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Extracellular Vesicles from *Cryptococcus neoformans* Modulate Macrophage Functions▽†

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Cryptococcus neoformans **and distantly related fungal species release extracellular vesicles that traverse the cell wall and contain a varied assortment of components, some of which have been associated with virulence. Previous studies have suggested that these extracellular vesicles are produced *in vitro* and during animal infection, but the role of vesicular secretion during the interaction of fungi with host cells remains unknown. In this report, we demonstrate by fluorescence microscopy that mammalian macrophages can incorporate extracellular vesicles produced by *C. neoformans*. Incubation of cryptococcal vesicles with murine macrophages resulted in increased levels of extracellular tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), and transforming growth factor β (TGF-β). Vesicle preparations also resulted in a dose-dependent stimulation of nitric oxide production by phagocytes, suggesting that vesicle components stimulate macrophages to produce antimicrobial compounds. Treated macrophages were more effective at killing *C. neoformans* yeast. Our results indicate that the extracellular vesicles of *C. neoformans* can stimulate macrophage function, apparently activating these phagocytic cells to enhance their antimicrobial activity. These results establish that cryptococcal vesicles are biologically active.**

Cryptococcus neoformans is an encapsulated yeast that causes disease in diverse species, including humans. Infection is most commonly acquired by inhalation of environmental propagules. *C. neoformans* rarely causes disease in immunocompetent individuals, but patients with immunological disorders can develop disseminated and neural cryptococcosis (63).

Extracellular microbial products have been amply demonstrated to modulate the interaction between host cells and pathogens. Many virulence factors and immunogens are released in their soluble forms by fungal cells to the extracellular space (4, 9, 16, 19, 37, 49, 53, 60, 62, 65, 67). *C. neoformans*, for instance, constitutively secretes large amounts of its capsular polysaccharide glucuronoxylomannan (GXM) (61). Disease progress is associated with detection of GXM, which is a potent modulator of the immune response (reviewed in reference 81). Other secreted virulence-related factors include galactoxylomannan (GalXM) (14), phospholipases (16), and urease (12, 62). In addition to acting as virulence factors, culture supernatant components are immunogenic, conferring protection against *C. neoformans* infection (51, 53).

Phagocytes are particularly important effector cells in the control of systemic mycoses (54). The interaction of *C. neoformans* with phagocytes, including macrophages, monocytes, dendritic cells, and neutrophils, has been widely studied (23, 32, 43, 46, 50, 59, 68, 77). Cryptococcal GXM is antiphagocytic (34) and a powerful immunomodulator (45, 79). *C. neoformans* capsule size directly correlates with the efficacy of phagocytosis *in vitro* (6, 15, 82). Phagocytosis of *C. neoformans* can result in either fungal killing (24, 30) or survival (2, 3, 39–41, 71, 80). Killing of *C. neoformans* apparently involves the production of oxidative species (24), while the mechanisms of fungal escape include phagosome extrusion, cell-to-cell spread, and phagosomal permeabilization (2, 3, 40, 41, 71). Capsular polysaccharides and melanin are known to modulate the interaction of *C. neoformans* with phagocytes in favor of the fungus (27, 39, 47, 48, 71, 72, 74, 76), but the role of other structures in the outcome of yeast phagocytosis is virtually unknown.

A number of recent studies have shown that GXM, GalXM, pigments, proteins, and lipids are trafficked in vesicles that traverse the cell wall (7, 14, 20, 56, 57, 62, 64, 65). Extracellular vesicles are also produced by the pathogens *Candida albicans*, *C. parapsilosis*, *Sporothrix schenckii*, and *Histoplasma capsulatum*, as well as by the model yeast *Saccharomyces cerevisiae* (1), suggesting that extracellular vesicle secretion is a general property of fungal cells. Secreted vesicles are heterogeneous. For instance, vesicles secreted by *C. neoformans* were classified into four different groups based on morphology and electron density (64). Additionally, vesicle diameter ranges from 30 to 400 nm, with the majority having dimensions of 100 to 150 nm (20, 64, 65). The combined use of serology, biochemistry, proteomics, and lipidomics led to the identification of 2 polysaccharides, phospholipids, 4 neutral lipids, and 76 proteins as extracellular vesicle components secreted by *C. neoformans*, which

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means that at least 81 different molecules are released to the extracellular milieu by vesicular secretion (14, 57, 64). It is likely that this number is an underestimate resulting from the difficulty of proteomic studies in vesicles from highly encapsulated cryptococcal cells, since a higher number of vesicular proteins were characterized in other fungi. For example, in *H. capsulatum*, proteomics and lipidomics of extracellular vesicles revealed an even more complex composition, including 283 proteins and 17 different phospholipids (1).

In this study, we evaluated the influence of extracellular vesicles on the fate of *C. neoformans* after phagocytosis by mouse macrophages. Our results show that fungal vesicles are biologically active and stimulate macrophages. Moreover, our results demonstrate that vesicles from an acapsular mutant strain were more effective in eliciting macrophage activation and augmenting fungal killing than vesicles from encapsulated strains. Taken together, our findings suggest that fungal secretory vesicles have the potential to influence the interaction of *C. neoformans* with host cells.

MATERIALS AND METHODS

***C. neoformans* growth conditions.** For vesicle isolation, *C. neoformans* strains HEC3393 (serotype A, human isolate), B3501 (serotype D), and Cap 67 (serotype D acapsular strain, generated in a B3501 background [28]) were cultivated for 72 h in a minimal medium composed of dextrose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 M). Fungal cells were cultivated at room temperature with continuous shaking. For macrophage infection assays, fungal cells were cultivated in Sabouraud liquid medium for 48 h at room temperature with continuous shaking. All media were prepared with apyrogenic water, and glassware was rendered sterile and pyrogen free by heating to 190°C for 4 h (18).

Macrophage cultures. The RAW 264.7 murine macrophage cell line (obtained from the American Type Culture Collection [ATCC]) was cultivated in complete Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mg ml^{-1} gentamicin, minimal essential medium (MEM) nonessential amino acids (catalog no. 11360; Gibco-Invitrogen), 10 mM HEPES, and 50 mM 2-beta-mercaptoethanol. The cells were maintained at 37°C in a 7.5% CO_2 atmosphere. The macrophage cultures and the experiments described below were all prepared under lipopolysaccharide-free conditions.

Vesicle isolation. Isolation of extracellular vesicles was done using the protocol described by Rodrigues et al. (65). After growth until the stationary phase ($\sim 10^7$ to 10^8 cells/ml), two liters of cell-free culture supernatants was obtained by sequential centrifugation at 5,000 and 15,000 × *g* (15 min at 4°C). The supernatants were concentrated by approximately 20-fold using an Amicon ultrafiltration system (cutoff, 100 kDa). The concentrate was again sequentially centrifuged at 4,000 and 15,000 × *g* (15 min at 4°C). The remaining supernatant was then ultracentrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was discarded, and the pellet was washed by five sequential suspension and centrifugation steps, each consisting of 100,000 × *g* for 1 h at 4°C with 0.1 M Tris-buffered saline. To remove extravesicular GXM contamination, vesicles were subjected to passage through a column packed with cyanogen bromide-activated Sepharose coupled to a monoclonal antibody to GXM, as described previously (65). Fractions that were not bound to the antibody-containing column were again centrifuged at 100,000 × *g*. The presence of extracellular vesicles in the pellets produced by centrifugation at 100,000 × *g* was checked by chemical methods, as previously described (65). The quantification of vesicle fractions was performed based on the presence of sterols in their membranes by the use of a quantitative fluorimetric Amplex Red sterol assay kit (Molecular Probes). In different assays, average values of 6 to 10 μg of sterol were detected as the final product of 2-liter cultures. The total sterol obtained after ultracentrifugation suggests a recovery of 0.5 fg per yeast cell. The apparent low efficiency during vesicle isolation was expected, since they could naturally disrupt in the supernatant. Vesicle suspensions were then adjusted to 30 to 50 μg of sterol/ml of phosphate-buffered saline (PBS) for use in the subsequent experiments.

Visualization of incorporation of vesicular components by macrophages. RAW 264.7 cells were plated onto the wells of a 24-well plate covered with glass coverslips (5×10^5 cells per well). Vesicle samples were stained with the lipophilic fluorophore dialkylcarbocyanine iodide (DiI) (catalog no. V22885; Invitrogen) as described previously (55). Briefly, vesicle suspensions were normalized to a sterol concentration corresponding to 2 μg/ml and supplemented with a DiI solution (final concentration, 3 μM). After 30 min at room temperature, the vesicle suspension was ultracentrifuged at 100,000 × *g* for 1 h and washed three times as described above. DiI-labeled vesicles were suspended in PBS to form 2 μg/ml sterol solutions and then incubated with the macrophages for 30 min at 37°C in a 7.5% CO_2 atmosphere. Controls included culture medium incubated with DiI alone and washed with PBS under the same conditions. Vesicle-treated macrophages were then fixed with 4% paraformaldehyde in PBS for 5 min and blocked with PBS–5% bovine serum albumin (BSA) for 1 h at room temperature. Plasma membranes and nuclei were labeled with cholera toxin subunit B (CtxB) (1 μg/ml) and 4′,6-diamidino-2-phenylindole (DAPI) (10 μg/ml), respectively, as described previously (75). Samples were then placed in mounting medium (50% glycerol–50 mM *N*-propyl gallate–PBS) over glass slides and visualized with a Leica AOBS laser scanning confocal microscope (Mannheim, Germany) with a 63× oil immersion optic. *z*-axis series and single scan images were obtained. The *x*- and *z*-axis sections were finally reconstructed using ImageJ software (NIH [http://rsb.info.nih.gov/ij/]). Alternatively, cells incubated with DiI-stained vesicles were sequentially blocked in PBS containing 5% BSA and incubated for 1 h at room temperature with pooled serum samples from 10 individuals diagnosed with cryptococcosis. As a negative control, cells were incubated with normal human serum samples from healthy volunteers with no previous diagnosis of any systemic mycosis. Pooled serum samples were used at a 1:100 dilution in PBS–1% BSA. After washing, cells were incubated with Alexa Fluor 488-labeled anti-human immunoglobulin antibody (Invitrogen). Samples were then washed, placed in mounting medium as described above, and visualized with an Axioplan 2 fluorescence microscope (Zeiss, Germany). Images were acquired using a Color View SX digital camera and processed with the analySIS (Soft Image System) software system.

Cell viability. To evaluate whether the exposure of macrophage-like cells to vesicle samples would lead to cell lysis, cell supernatants were assayed for the presence of lactate dehydrogenase (LDH) activity. LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes. Membrane damage results in LDH release to culture supernatants. RAW 264.7 monolayers (1×10^6 cells/well) were stimulated for 16 h with vesicle suspension (2 μg/ml sterol). After this period, aliquots of the supernatant were collected and supplemented with NADH and pyruvate to final concentrations of 0.3 and 4.7 mM, respectively, followed by 10 min of incubation at room temperature. Based on the property that NADH, and not NAD, absorbs strongly at 340 nm, the decrease in absorbance (A_{340}) was measured in a spectrophotometer. Positive and negative controls consisted, respectively, of a RAW 264.7 Triton X-100 (10%) lysate and supernatants of nontreated cells after 16 h of cultivation. All experiments were performed in triplicate sets and statistically analyzed by Student's *t* test.

Production of cytokines and nitric oxide (NO) after exposure of macrophages to vesicle samples. RAW monolayers were washed twice in serum-free DMEM, placed in medium supplemented with various concentrations of fungal vesicles, and incubated for 16 h at 37°C (7.5% CO_2 atmosphere). In these experiments, we used 40 ng of vesicular sterol to activate 10^5 macrophages, suggesting that 0.4 pg of sterol is enough to activate a macrophage. As a positive control, macrophages were stimulated with 1 μg/ml lipopolysaccharide (LPS). Supernatants were then collected and assayed for interleukin-10 (IL-10), transforming growth factor β (TGF-β), and tumor necrosis factor alpha (TNF-α) content by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems). Alternatively, NO levels were measured in supernatants as previously described (25). N6-(1-Iminoethyl)-L-lysine dihydrochloride (L-NIL) (Cayman Chemical), the specific inhibitor of the inducible nitric oxide synthase (iNOS), was used to confirm that the enzyme was specifically induced during the described activation process. For these experiments, cells were stimulated with vesicles during 16 h in the presence of 3 μM L-NIL and cultured with 1% (vol/vol) Nutridoma-SP (Boehringer Mannheim) instead of FCS. To exclude the possibility that cellular responses were affected by the presence of contaminant LPS, control experiments were performed in the presence of purified polymyxin B, an antibiotic that binds LPS and neutralizes its effects. Fungal vesicles and LPS were incubated with macrophage-like cells in the presence of polymyxin B, and nitric oxide production was evaluated. All experiments were performed in triplicate sets and statistically analyzed by using one-way analysis of variance (ANOVA) followed by a Bonferroni posttest.

Determination of internalization of *C. neoformans* by flow cytometry and fluorescence microscopy. To determine internalization levels of *C. neoformans* during interaction with RAW 264.7 cells, we adapted the method of Chaka et al. (10). Briefly, yeast cells were incubated with fluorescein isothicyanate (FITC) at 0.5 mg/ml in PBS (25°C) for 10 min. After extensive washing with PBS, *C.*

neoformans yeast cells were incubated with macrophage-like cells for 3 h at a 5:1 fungus/host cell ratio, followed by extensive washing with PBS for removal of nonadherent fungi. The fluorescence intensity of the macrophage-like cells was therefore a function of association with FITC-labeled *C. neoformans*. The infected cells were then detached from tissue culture plates by pipetting or scraping, fixed with 4% paraformaldehyde, and analyzed in a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using winMDI 2.9 software. The data were statistically analyzed using the one-way ANOVA test followed by the Bonferroni posttest. To visualize the overall aspect of the cells, RAW cells infected with FITC-labeled yeast cells were washed with PBS, sealed under a coverslip, and visualized by epifluorescence. Samples were observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired using a Color View SX digital camera and processed with the analySIS software system (Soft Image System).

Antifungal activity of RAW 264.7 stimulated with vesicles. Macrophage monolayers were stimulated for 16 h with vesicles at a final concentration corresponding to 0.4 μg/ml sterol. The supernatant was removed and the monolayer washed with serum-free DMEM. Aliquots of fungal cells suspended in DMEM were added to the macrophage monolayers at a 5:1 yeast/macrophage ratio. After incubation for 3 h at 37°C (7.5% CO_2 atmosphere), the samples were washed three times with PBS to remove nonadherent fungi; fresh medium was added, and incubation was continued for an additional 2 or 5 h (for a total of 5 or 8 h of infection) under the conditions described above. After incubation, cells were again washed and then lysed with sterile cold water. Macrophage lysates containing fungal cells were immediately plated onto Sabouraud agar plates for CFU determination. Controls consisted of supernatants of nonstimulated macrophages cultivated under the same conditions. All experiments were performed in triplicate sets and statistically analyzed by one-way ANOVA followed by the Bonferroni posttest.

RESULTS

Vesicles secreted by *C. neoformans* are internalized by macrophages. Fungal vesicles are believed to be released to the extracellular space during *in vitro* macrophage infection and animal cryptococcosis (65). However, it is unknown whether vesicular components are incorporated by host cells and whether these cells respond to vesicles. In this context, we first evaluated whether incubation of macrophages with vesicle fractions isolated from cryptococcal supernatants would result in their delivery into host cells. To follow internalization of extracellular vesicles, we stained isolated vesicles with the red-fluorescent lipophilic compound dialkylcarbocyanine iodide (DiI). Coincubation of RAW 264.7 cells performed using DiI-labeled vesicle preparations and confocal microscopy analysis of RAW 264.7 revealed that they were internalized by the macrophage-like cells (Fig. 1A). Vesicles that were smaller than 200 nm were not visualized, a result which was possibly related to the resolution limit of fluorescence microscopy. The absence of colocalization with CtxB indicates that vesicles do not fuse with plasma membranes. Three-dimensional reconstruction of these cells is shown in movie S1 in the supplemental material.

We demonstrated previously that vesicles secreted by *C. neoformans* also transport antigenic proteins (64). We therefore used serum samples from cryptococcosis patients to track vesicle-associated antigenic components. In fact, components showing serologic reactivity colocalized with vesicle compartments labeled with DiI within macrophages (Fig. 1B). Serum samples from healthy individuals did not react with vesicle-treated RAW cells (data not shown).

Macrophage exposure to the vesicles for 16 h did not lead to cell lysis (Fig. 1C), showing that, under the experimental conditions used in this study, vesicular compounds do not cause acute toxicity to these cells.

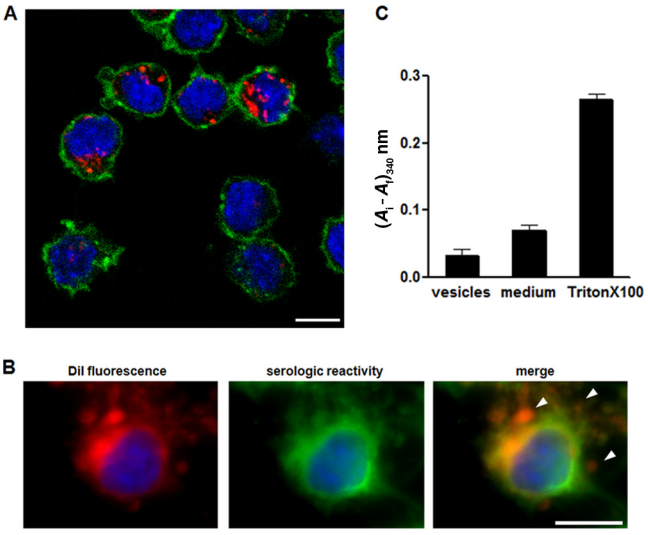

FIG. 1. Incorporation of vesicular components by macrophages. DiI-stained vesicles (red fluorescence) were incubated with RAW 264.7 macrophages, followed by staining of the nucleus with DAPI (blue fluorescence). (A) A plasma membrane was stained with FITC-labeled cholera toxin subunit B (green fluorescence). (B) Macrophages were incubated with serum samples from patients with cryptococcosis followed by Alexa Fluor 488 anti-human IgG antibody. The merged image at the right (third panel) demonstrates that DiI-stained compartments colocalize, at least partially, with antigenic vesicular components (arrowheads). Scale bars, 10 μm. (C) Macrophage viability is apparently not affected by the vesicles, as demonstrated by the measurement of LDH activity in supernatants of macrophages treated with vesicles (at a 2 μg/ml sterol concentration) or incubated in regular medium. As a positive control for enzyme activity, the macrophages were incubated with Triton X-100. $(A_i - A_f)_{340}$ nm indicates the difference between the initial and final absorbance readings.

Secreted vesicles modulate NO and cytokine production by macrophages. NO has a well-established role in the protective responses that occur during *C. neoformans* infection (66). In this context, we evaluated whether vesicles produced by encapsulated and acapsular *C. neoformans* strains would modulate the production of NO by RAW macrophages. Vesicle fractions from both wild-type parental and mutant strains induced dose-dependent production of NO (Fig. 2A). However, larger amounts of NO were produced upon stimulation with vesicles from the acapsular mutant. In support of these data, NO production was diminished when we treated macrophages with vesicles produced by the acapsular mutant in the presence of serotype A and D GXMs in comparison to macrophages subjected to treatment with native vesicles from acapsular cells (Fig. 2B). Together with the fact that vesicles from the two encapsulated strains induced similar levels of NO (Fig. 2B), these data suggest that different serotypes of GXM reduce vesicle-induced NO production. The polysaccharide concentration used in this experiment was determined based on the GXM concentration usually found in vesicles (30 μg/ml; data not shown). LPS was used as a positive control (data not shown).

Under our experimental conditions, NO production was dependent on the activity of induced nitric oxide synthase (iNOS), since NO production was drastically reduced by the presence of L-NIL (Fig. 2B). High levels of NO were detected after exposure to vesicles in the presence of polymyxin B. In

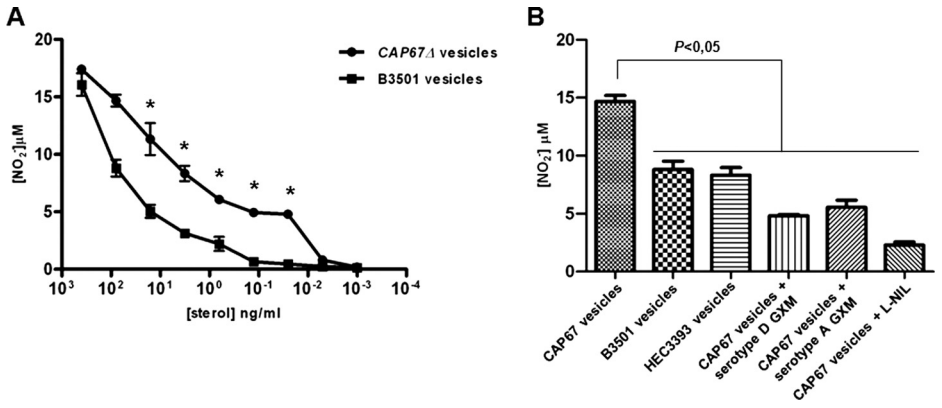

FIG. 2. Vesicle-stimulated macrophages produce nitric oxide. (A) Dose-dependent production of NO was observed for vesicle fractions from both acapsular and encapsulated fungi. The asterisks indicate $P < 0.05$. Vesicle concentrations are expressed as a function of the sterol content in their membranes. (B) Macrophages were incubated overnight with vesicles from different strains at a final concentration of 0.4 μg/ml of sterol. GXM and L-NIL were added in final concentrations of 30 μg/ml and 3 μM, respectively. The levels of nitric oxide production in the culture medium (no vesicles) were below the detection limit of the technique. Bars represent averages of three measurements; brackets denote standard deviations. Data shown are representative of the results of three independent experiments.

contrast, the effect of LPS was completely inhibited by the presence of polymyxin B under these conditions (data not shown).

The response of macrophage-like cells to stimulation with vesicles was also determined by evaluation of their ability to produce cytokines. The cytokines analyzed were TNF-α, IL-10, and TGF-β. TNF-α was analyzed because of its capacity to induce the fungicidal activity of mouse peritoneal macrophages against *C. neoformans* (31), while IL-10 and TGF-β are positively modulated by the vesicular component GXM (42, 74). To evaluate the influence of known modulatory effects of GXM on macrophages (74), we compared the macrophage responses to vesicles isolated from an encapsulated *C. neoformans* isolate (HEC3393) with the responses to those isolated from an acapsular mutant (CAP67). Vesicle fractions from both strains induced a significant increase in TNF-α production compared to those from nonstimulated cells (Fig. 3). It is noteworthy that CAP67 vesicles induced an even higher production of TNF-α in comparison to HEC3393 vesicles (Fig. 3), possibly because these fractions lack GXM, which causes potent depressive effects on immune function (44, 45, 70, 73). IL-10 and TGF-β levels were increased following exposure to either vesicle fraction (Fig. 3). Although levels of TGF-β production by macrophages were similar in the two systems, HEC3393 vesicles induced significantly higher production of IL-10 than CAP67 vesicles. These data indicate that macrophages differentially respond to stimulation by vesicles of *C. neoformans* depending on vesicle composition.

Phagocytic and microbicidal activity of macrophages is stimulated by secreted vesicles. We evaluated the ability of vesicle-primed macrophages to phagocytose nonopsonized encapsulated *C. neoformans* cells. Control and vesicle-stimulated macrophages were challenged with FITC-labeled *C. neoformans*. Association indices were then determined by flow cytometry. We observed that 62% of macrophages activated with CAP67 vesicles displayed fluorescence (Fig. 4A; M1 marker)

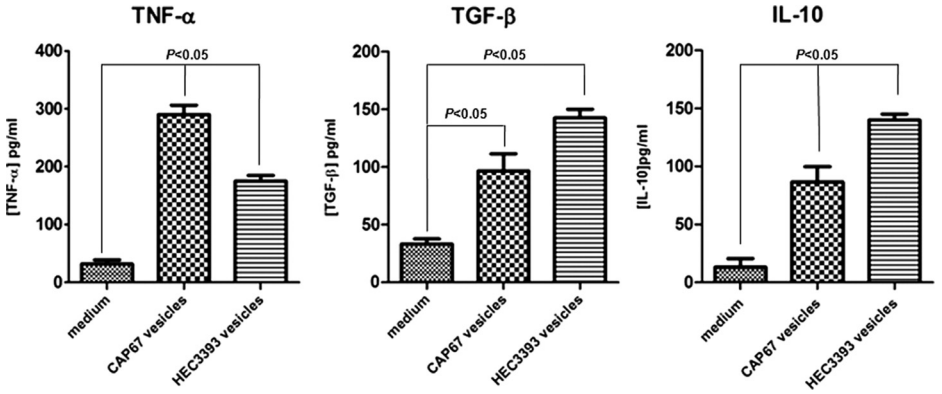

FIG. 3. Profile of cytokine production by macrophages in response to stimulation with vesicle fractions. Macrophages were stimulated with vesicles isolated from culture supernatants from encapsulated (HEC3393) and acapsular (CAP67) *C. neoformans* cells. The sterol concentration corresponded to 0.4 μg/ml in all fractions. The basal production of each cytokine in the medium alone is also shown (first bars). TNF-α, TGF-β, and IL-10 concentrations in the supernatant were determined by capture ELISA. Statistical significance values are highlighted; $P < 0.05$. Bars represent averages of three measurements; brackets denote standard deviations. Data shown are representative of the results of three independent experiments.

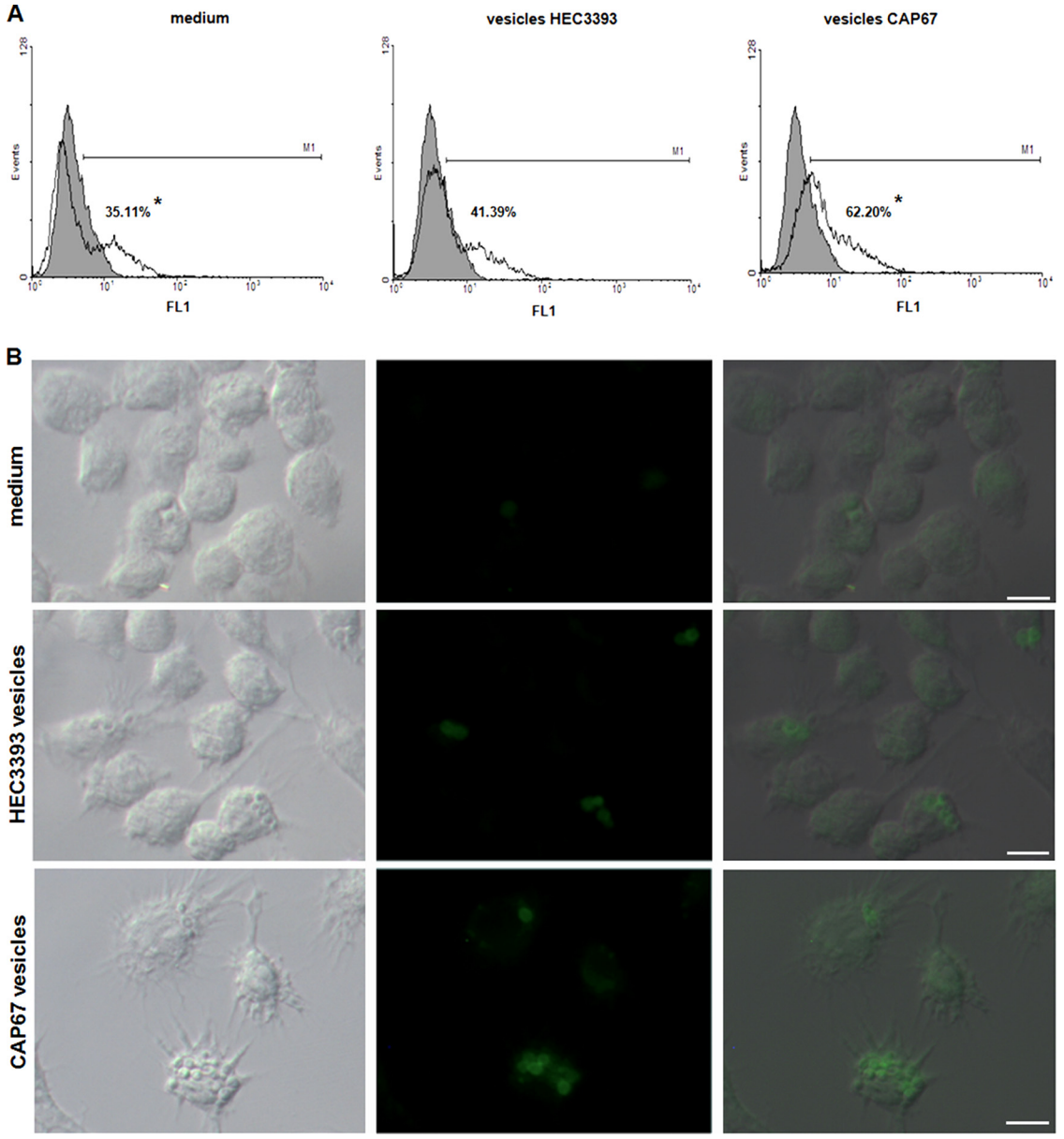

FIG. 4. Macrophages stimulated with vesicles exhibit enhanced phagocytosis. (A) Macrophages were treated overnight with vesicles derived from CAP67 or HEC3393 at a sterol concentration of 1 μM. Gray peaks represent uninfected macrophages; open histograms show the population analysis after interaction with FITC-*C. neoformans* within the M1 region, and the corresponding macrophage fluorescence increase is indicated numerically. These data are representative of the results of three individual experiments. The percentages of positive fluorescence (M1) are indicated. Asterisks denote $P > 0.05$. (B) Analysis by fluorescence microscopy of the material prepared for flow cytometry. Infected cells were visualized using differential interference contrast microscopy and fluorescence microscopy. Scale bars, 10 μM.

as a consequence of infection with FITC-labeled *C. neoformans*. Interestingly, 41% of the macrophages were positive for FITC staining after exposure to HEC3393 vesicles. In contrast, only 35% of control cells contained yeast cells. Although the percentage of fluorescence in M1 tended to be higher in macrophages treated with HEC3393 vesicles than in control macrophages, there was no statistical significance to the difference. Epifluorescence analysis suggested that yeast internalization was maximized under the conditions used in our experiments. The findings were supported by differential interference contrast (DIC) and fluorescence microscopy results (Fig. 4B). Confirming the results of fluorescence-activated cell sorter (FACS) analysis, macrophages stimulated with CAP67 vesicles had more internalized fungi per macrophage than under any other set of conditions (data not shown).

Since phagocytosis and production of antimicrobial compounds increased after treatment with fungal vesicles, we evaluated the microbicidal activity of macrophages after exposing them to vesicles from encapsulated and acapsular *C. neoformans* strains. Control or vesicle-treated RAW cells were incubated with nonopsonized *C. neoformans*. After 3 h, culture fluids containing unattached fungi were removed and replaced

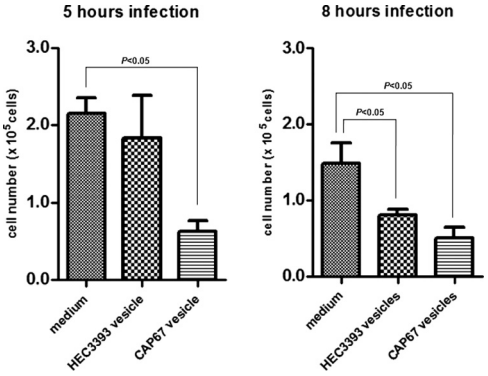

FIG. 5. Fungicidal activity of vesicle-stimulated macrophages. Macrophages treated with vesicles isolated from encapsulated or acapsular *C. neoformans* were incubated with the encapsulated *C. neoformans* strain for different periods for CFU determination of numbers of intracellular *C. neoformans*. CFU values were multiplied using the dilution factor to generate absolute cell numbers. Bars represent averages of three measurements; brackets represent standard deviations. Statistically significant differences are highlighted.

by fresh media. This step was followed by an additional incubation of 2 or 5 h. After these periods, supernatants were removed and cell lysates of control and stimulated macrophages were assayed for the presence of viable cryptococci. Macrophages treated with CAP67 vesicles manifested greater killing activity than HEC3393 vesicle-activated macrophages (Fig. 5). In fact, stimulation of macrophages with vesicles from encapsulated yeast cells resulted in significant microbicidal activity only after 8 h of incubation. Although we cannot ensure that the only difference between the HEC3393 and CAP67 vesicles was the presence of GXM, it is conceivable that the less efficient fungicidal activity observed of macrophages treated with HEC3393 was related to the fact that GXM negatively modulates the macrophage response against *C. neoformans* (45, 73). Albeit a tendency of growth inhibition was visualized on control macrophages after 8 h of incubation, it is important that no statistical significance difference was observed compared to the results obtained after 5 h of incubation.

DISCUSSION

The host response to microbial antigens is a complex process that is influenced by a number of factors, including the concentration of immunogens, the efficacy of exposure of these molecules to effector cells, and the ability of the infectious agent to produce and secrete immunologically active compounds. Consequently, the outcome of the interaction of infectious agents with host cells includes multiple possibilities, such as the persistence of the microbe within host tissues, causing cell damage, and the limitation of microbial colonization, resulting in disease control (8).

It was suggested that fungal cells secrete vesicles during interaction with phagocytes *in vivo* (65). Therefore, one might expect that the concentration of such a complex array of molecules in vesicular structures could directly influence the interaction of fungal pathogens with their hosts. Supporting this hypothesis, Gram-negative bacteria have been shown to constitutively secrete outer membrane vesicles composed of lipopolysaccharide, phospholipids, outer membrane proteins, and soluble periplasmic proteins, most of which are important virulence determinants (5, 36). This secretion mechanism is thought to be employed by bacteria to deliver virulence factors in the host cell (29). Recently, Lee and colleagues demonstrated that Gram-positive bacteria also secrete vesicles, suggesting that this process could be involved with protein transfer to other bacteria and is conserved among different organisms (38). Although the origin of fungal extracellular vesicles studied by our group is very different from that of their bacterial counterparts, we investigated whether cryptococcal vesicles also affect host-pathogen interactions.

In the present work we demonstrate that extracellular secreted vesicles from pathogenic fungi directly modulate host-pathogen interactions and propose a model for these interactions. According to this model, differences in vesicle numbers and composition, as well as in the presence of GXM, could affect the outcome of the *C. neoformans*-macrophage interaction by affecting the state of macrophage activation. Strongly suggestive evidence that vesicles are made in tissue comes from electron microscopy and the observation of a serum antibody response to vesicle components in infected mice (64, 65). Although our work indicates that comparable effects could occur *in vivo*, such studies remain to be done, and currently there are formidable technical hurdles interfering with the demonstration of *in vivo* effects. Nevertheless, our results are likely to have anticipated the observation of extracellular vesicles as important modulators of infection outcome and participation in the activation of immune response, as recently described for mammalian exosomes (69).

C. neoformans produces several exocellular antigens with apparently conflicting functions with regard to pathogenesis. For instance, GXM, the major cryptococcal virulence factor, protects the fungus against phagocytosis and inhibits leukocyte migration (17, 21, 22, 34). However, this polysaccharide, in its capsular form, is also an efficient inducer of the activation of the alternative pathway of the complement system, which results in the deposition of opsonins in the capsule and increased phagocytosis (33, 35). Another example of apparently opposing functions of capsular components of *C. neoformans* involves the soluble forms of GXM and GalXM. Both of these well-known capsular components activate inducible nitric oxide synthase and the consequent production of nitric oxide in macrophages (74). However, a heavily encapsulated cryptococcal strain suppressed the production of this antimicrobial compound in CpG-oligodeoxynucleotide-stimulated macrophages (78), which confirms previous studies showing that *C. neoformans* cells fail to induce nitric oxide synthase in primed murine macrophage-like cells (52). These results reveal the enormous complexity of the events involved in the interaction of *C. neoformans* with phagocytes and the difficulty in predicting which pathogenesis-related structure will have the greatest effect during infection of host cells. The cryptococcal vesicles contain at least 81 different fungal molecules with potentially different functional impacts on immunity (64, 65). Therefore, the response of host cells to vesicle preparations is virtually unpredictable, which led us to experimentally address some of the functions of macrophages after their exposure to vesicle fractions of *C. neoformans*.

As recently reported for bacterial vesicles (5), our results indicate that macrophages are able to internalize vesicular components. Exposure of macrophages to vesicle preparations resulted in enhanced levels of IL-10 and TGF-β, anti-inflammatory cytokines whose production is positively modulated by the vesicular component GXM (11, 42). In agreement with the role of GXM in the upregulation of IL-10, CAP67 vesicles induced a significantly lower amount of this cytokine, although the level was significantly greater than in untreated macrophages. The increased production of IL-10 was recently associated with a protective immune response to cryptococcosis, which was stimulated by a peptide mimotope of GXM (13). This higher efficiency was inferred from (i) the lower concentration of CAP67 vesicles required for induction of high levels of NO, (ii) the enhanced ability to induce yeast internalization, and (iii) the requirement of a shorter incubation period to display potent microbicidal activity.

Transmission electron microscopy demonstrated that *C. neoformans* vesicle fractions contain subpopulations that differ in morphological characteristics, suggesting the existence of different biosynthetic steps for vesicle biogenesis (62). More importantly, the detection of different morphological types suggests that vesicles of diverse molecular compositions occur. In a recent report, we used gradient fractionation of vesicles to demonstrate that GXM concentrations differ considerably in fractions with different densities (57). The methods currently used for vesicle purification do not discriminate between vesicles of different characteristics, resulting in heterogeneous preparations. Here we showed that differences in molecular composition of vesicles between two strains were translated into functional differences in the modulation of host cell functions. Although this vesicle heterogeneity complicates the functional analysis of specific vesicle populations, it approximates the reality that *C. neoformans* simultaneously secretes different vesicle types (57). Assuming that *C. neoformans* secretes vesicles during interaction with host cells (65), the overall effects of the exposure of phagocytes to extracellular fractions could be better reflected in experiments using "crude" vesicle samples, although macrophage function could be differentially affected by distinct vesicle fractions.

Although our results provide strong evidence that cryptococcal vesicle preparations stimulate macrophage-like cells to alter their cytokine production and enhance their antifungal function, there are some experimental limitations that preclude more generalized conclusions at this time. Currently we do not have a reliable method to accurately enumerate vesicles in our preparations and consequently cannot estimate the number of vesicles required for the activation effect. It is conceivable, and probably likely, that the effect of the presence of vesicles on macrophages depends on the number of vesicles that interact with each phagocytic cell such that, at low vesicle/macrophage ratios, the effect results in activation whereas, at higher ratios, the effect is detrimental to the macrophage. The *in vivo* milieu may also impact the interactions; for instance, secreted host lipases could disrupt the vesicles, leading to earlier release of vesicular contents. Furthermore, the impact of vesicles produced by intracellular fungi may be different from that of extracellular vesicle preparations. Notwithstanding these uncertainties, the results further support our conclusion that fungal vesicles are biologically active; this information opens new venues of investigation in fungal-host cell interactions.

In this report, we demonstrate that the recently described extracellular fungal vesicles can modify the functions of mammalian cells, altering the fate of *C. neoformans* after phagocytosis by macrophages. These results strongly suggest the potential of these vesicular structures to modify the course of cryptococcal infections through their effects on host phagocytic cells. Fungal vesicles are apparently related to mammalian exosomes (58, 64). In this regard, it is noteworthy that exosome preparations from dendritic cells are being investigated as potential anticancer vaccines (26). The current results and the fact that vesicle-related proteins are immunogenic (1, 62, 64) suggest that vesicle preparations and/or their components could have utility as fungal vaccines, which will be a focus of future studies in our laboratory.

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