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Melanization of *Cryptococcus neoformans* Affects Lung Inflammatory Responses during Cryptococcal Infection

Aron J. Mednick,[1] Joshua D. Nosanchuk,[1] and Arturo Casadevall[1,2]*

Division of Infectious Disease, Department of Medicine,[1] and Department of Microbiology and Immunology,[2] Albert Einstein College of Medicine, Bronx, New York

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The production of melanin pigments is associated with virulence for many microbes. Melanin is believed to contribute to microbial virulence by protecting microbial cells from oxidative attack during infection. However, there is also evidence from various systems that melanins have immunomodulatory properties, which conceivably could contribute to virulence by altering immune responses. To investigate the effect of melanin on the immune response, we compared the murine pulmonary responses to infection with melanized and nonmelanized *Cryptococcus neoformans* cells. Infection with melanized cells resulted in a greater fungal burden during the early stages of infection and was associated with higher levels of interleukin-4 and MCP-1 and greater numbers of infiltrating leukocytes. Infection with laccase-positive (melanotic) *C. neoformans* cells also elicited higher MCP-1 levels and more infiltrating leukocytes than did infection with laccase-negative cells. Melanization interfered with phagocytosis in vivo for encapsulated *C. neoformans* but not for nonencapsulated cells. The results provide strong evidence that cryptococcal melanization can influence the immune response to infection and suggest that immunomodulation is an additional mechanism by which the pigment contributes to virulence.

Melanin production is common in pathogenic fungi, and melanization during mammalian infection has been described for *Cryptococcus neoformans* (23, 29), *Histoplasma capsulatum* (20), *Paracoccidioides brasiliensis* (6), and *Sporothrix schenckii* (17). The current thinking in the field is that melanins contribute to microbial virulence by protecting fungal cells against host immune cells (reviewed in reference 19). However, there is also considerable evidence that melanins are immunomodulators with both pro- and anti-inflammatory properties, depending on the type of melanin and host response. In humans, melanin is implicated in the pathogenesis of acute anterior uveitis, an inflammatory process that can lead to blindness (33). Individuals with heavily pigmented eyes are at greater risk for uveitis following eye injury or surgery than those with lightly pigmented eyes, possibly as a result of the proinflammatory properties of melanin released into the eye chamber (12, 32). In the human gingiva, there is a significant correlation between the distribution of melanin pigmentation and the presence of gingival inflammation (26). However, there is also evidence that certain types of melanin can interfere with immune responses. For example, grape melanin inhibits inflammation in the rat by interfering with prostaglandin and cytokine production (2).

Fungal cell melanization could affect inflammatory responses directly and/or indirectly. The observation that synthetic melanin suppresses cytokine production in macrophages stimulated with lipopolysaccharide (16) suggests that melanins in fungal cells could influence the release of inflammatory mediators. However, melanins could also elicit foreign-body-like reactions since they are composed of insoluble polymers that are not easily degraded by macrophages. Consistent with this mechanism, injection of *C. neoformans* melanin ghosts into mice promotes granuloma formation (27). Given that melanins can reduce the susceptibility of fungal cells to oxidative damage (38), melanization may increase the survival of fungal cells in tissue, which could alter the inflammatory response by virtue of an increased microbial burden. Furthermore, since laccase can be released from the fungal cell (40), it could conceivably polymerize host substrates into melanin in tissue, which could affect local homeostasis and the immune response. For *C. neoformans* melanin, one study found that the immune response to *C. neoformans* strains differing in melanin production varied with respect to tumor necrosis factor alpha (TNF-α) levels and T-cell lymphoproliferation, suggesting that melanin contributed to virulence by interfering with the host inflammatory response (8).

In summary, there is a significant amount of evidence that melanins are powerful immunomodulators that possess both pro- and anti-inflammatory properties. Melanin effects on host immune function could represent a common mechanism of virulence for melanotic fungi that can be targeted for drug discovery. In this regard, inhibition of fungal melanization in mice with glyphosate was shown to be therapeutic in experimental cryptococcal infection (21). Melanin is immunogenic and elicits antibodies that can inhibit fungal growth (1, 22, 28). This study investigated how melanin may contribute to the virulence of *C. neoformans* by characterizing the early inflammatory changes in the lung associated with infection with melanized and nonmelanized cells. The results provide strong evidence that melanin may contribute to virulence through powerful effects on the host immune response.

* Corresponding author. Mailing address: Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2215. Fax: (718) 430-8968. E-mail: casadeva@aecom.yu.edu.

MATERIALS AND METHODS

***C. neoformans* and culture conditions.** Strain ATCC 34873 (B-3501) is serotype D. Strain Cap67 is a nonencapsulated mutant derived from B-3501 that was used

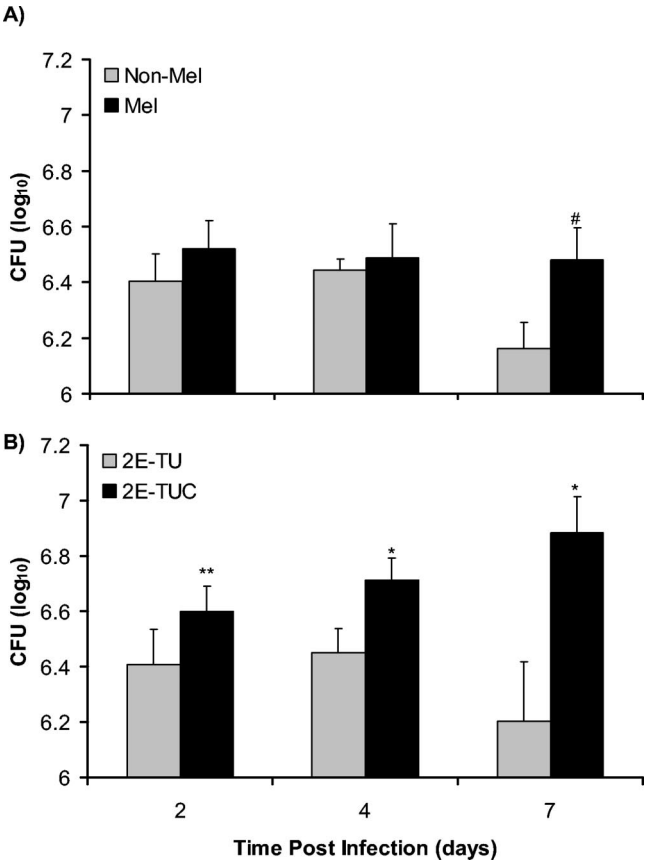

FIG. 1. Lung fungal burdens (CFU per lung) of mice infected intratracheally with 5×10^6 *C. neoformans* cells. Panels: A, in vitro melanized and nonmelanized 3501 (#, $P < 0.01$); B, 2E-TU-4 and 2E-TUC-4 (**, $P < 0.05$ on day 2; *, $P < 0.01$ on days 4 and 7). The experiment was done twice, and the results were consistent. For each group, the sample size was 10 mice. Error bars denote the standard deviation of the mean.

to compare phagocytosis of melanized encapsulated and nonencapsulated *C. neoformans*. 2E-TU-4 (*cnlac1* Lacc$^-$) is a laccase-deficient strain of *C. neoformans* (31). 2E-TUC is the conjugated strain of 2E-TU-4 (*CNLAC1* Lacc$^+$) and is laccase competent (31). The 2E-TU-4 strains are isogenic strains that have been backcrossed, trace their ancestry to B-3501 (31), have been used in several studies of laccase function (14, 15, 25), and were a generous gift from Peter Williamson (Chicago, Ill.). For experimental work, *C. neoformans* cultures were grown for 24 h at 30°C in Sabouraud dextrose (SAB) broth. An inoculum from the culture in SAB broth was then used to start cultures in defined minimal medium (15 mM glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, 3.0 μM vitamin B_1) with or without L-dopa supplementation for the generation of melanized and nonmelanized cells, respectively. The cultures were grown for 7 days, allowing for heavy melanization that manifested itself by a black cell suspension in cultures containing L-dopa. Melanin "ghosts" were generated as previously described (30) and washed with lipopolysaccharide-free phosphate-buffered saline (PBS; Gibco) before instillation into mouse lungs.

Intratracheal infection. Female 6- to 8-week-old BALB/c mice were obtained from the National Cancer Institute (Bethesda, Md.). Intratracheal infection was done as described previously (3). Mice were anesthetized with ketamine (10 mg/kg) and xylazine (125 mg/kg) in PBS administered intraperitoneally. Mice received 5×10^6 *C. neoformans* cells intratracheally or an equal number of melanin ghosts in 0.05 ml of PBS. In some experiments, mice naive to any manipulation were used. All animal work was done in accordance with the regulations of The Institute for Animal Studies at Albert Einstein College of Medicine.

CFU count determination and histological preparation. To determine the fungal burden in the lungs and spleen, mice were killed on days 2, 4, 7, and 14 and the lungs were removed, weighed, homogenized, and plated on SAB plates for CFU count determination. The right upper lung, which consists of approximately 10% of the lung mass, was placed in 10% formalin for histological examination. The other 90% of the lung was weighed and homogenized through a metal screen into 10 ml of PBS. Dilutions of the suspension were plated on SAB agar plates and incubated at 30°C for 48 h for CFU count determination (1 colony = 1 CFU).

Cytokine and chemokine analysis. Five groups of mice were used for cytokine and chemokine analysis consisting of infection with melanized yeast cells, infection with nonmelanized yeast cells, melanin ghost inoculation, PBS inoculation, and use of naive uninfected mice. Cytokine and chemokine analysis of the laccase mutant used five groups: mice infected with 3501, 2E-TU-4, or 2E-TUC-4; mice inoculated with PBS; and naive mice. Mice were killed on days 2, 4, and 7 postinfection or postinoculation. The left lung was removed and homogenized for 60 s in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim, Indianapolis, Ind.). The homogenates were centrifuged at $6{,}000 \times g$ for 10 min, and the supernatant was collected and frozen at −80°C until tested for cytokine production. The supernatants were tested for interleukin-2 (IL-2), IL-4, IL-10, IL-12p70, TNF-α, gamma interferon (IFN-γ), and MCP-1 with enzyme-linked immunosorbent assay kits (Pharmingen, San Diego, Calif.) with detection limits of 3.1, 7.8, 31.3, 62.5, 15.6, 31.3, and 15.6 pg/ml, respectively.

In vivo phagocytosis assay. BALB/c mice were infected intratracheally with melanized 3501, nonmelanized 3501, melanized Cap67, or nonmelanized Cap67 as described above. Mice were sacrificed 3 h later, and their lungs were subjected to lavage 10 times with 1 ml of PBS for a total volume of approximately 9 ml of bronchoalveolar lavage fluid. The cells were collected by centrifugation at 3,000 $\times g$ for 10 min. The supernatant was then decanted, and cells were suspended in 500 ml of Dulbecco's modified Eagle's medium. Cells were transferred to 4 wells of a 96-well plate and allowed to adhere at 37°C for 1 h. Supernatant was

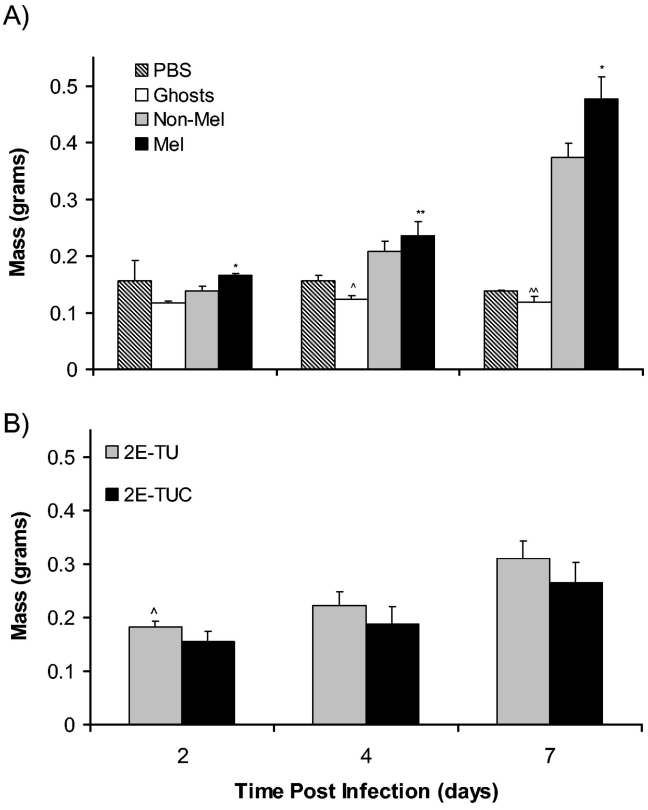

FIG. 2. Masses of lungs infected intratracheally with 5×10^6 *C. neoformans* cells. Panels: A, in vitro melanized (Mel) and nonmelanized (Non-Mel) 3501 (*, $P < 0.01$; **, $P < 0.05$ [between melanized and non-melanized cells]; ^, $P < 0.01$; ^^, $P < 0.05$ [between ghosts and PBS]); B, 2E-TU-4 and 2E-TUC-4 (^, $P < 0.05$ on day 2). Error bars denote the standard deviation of the mean.

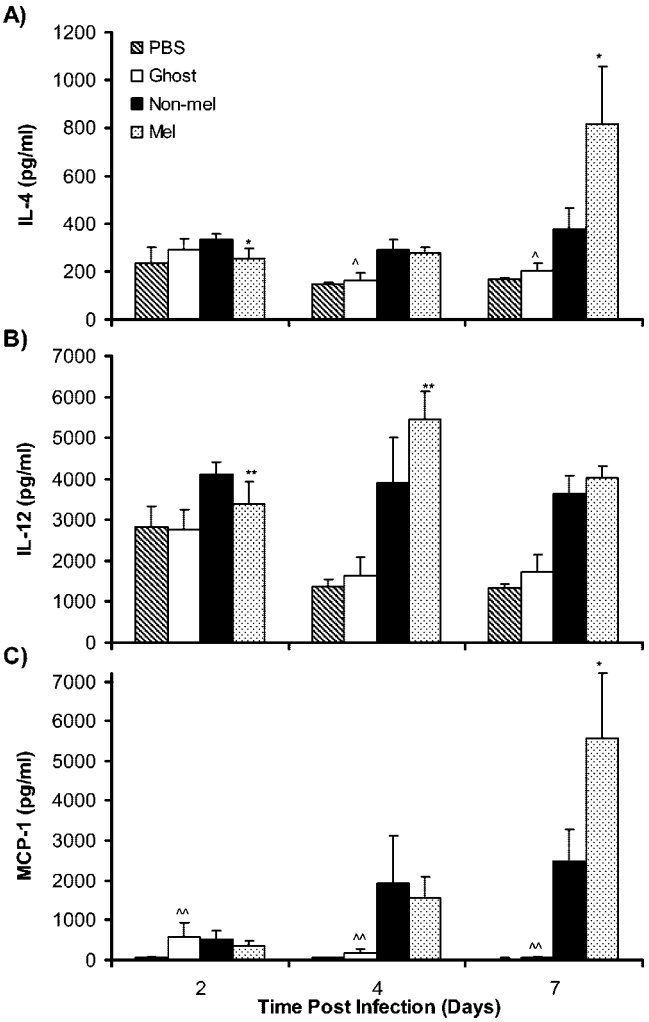

FIG. 3. Cytokine concentrations per milliliter in lungs of mice infected intratracheally with 5×10^6 melanized (Mel) or nonmelanized (Non-Mel) *C. neoformans* cells. Panels: A, IL-4 (*, $P < 0.01$ between melanized and nonmelanized cells on days 2 and 7; ^, $P < 0.01$ between ghosts and PBS on days 4 and 7); B, IL-12 (**, $P < 0.05$ between melanized and nonmelanized cells on days 2 and 4); C, MCP-1 (*, $P < 0.01$ between melanized and nonmelanized cells on day 7; ^^, $P < 0.05$ between ghosts and PBS on days 2, 4, and 7). This experiment was done twice, and the results were consistent. For each group, the sample size was six mice. Error bars denote the standard deviation of the mean.

aspirated, and cells were fixed with 100 μl of ice-cold methanol and incubated at room temperature for 30 min. Methanol was aspirated and discarded. Cells were washed three times with 200 μl of PBS. Fifty microliters of a 1:20 dilution of Giemsa stain was added, and the mixture was kept at room temperature for 24 h. Cells were washed with PBS, and the percentage of macrophages with internalized *C. neoformans* was measured.

Preparation of lung leukocytes and FACS analysis. At days 2, 4, and 7 postinfection, lungs were excised, minced, homogenized with a sterile 70-μm nylon mesh (Becton Dickinson), and digested for 60 min at 10 ml/lung with digestion buffer containing RPMI medium, 10% fetal calf serum, 1 mg of collagenase (Boehringer Mannheim, Chicago, Ill.) per ml, and 30 μg of DNase I (Sigma, St. Louis, Mo.) per ml. The cell suspension was collected by centrifugation, and erythrocytes were lysed by suspension in ice-cold 0.17 M NH_4Cl for 10 min. A 10-fold excess of RPMI solution was then added to make the solution isotonic, the cells were collected by centrifugation and suspended in staining buffer (PBS, 1% fetal bovine serum), and the number of cells was determined by counting in a hemocytometer chamber. For fluorescence-activated cell sorter (FACS) analysis, lung leukocytes (10^6) were stained for 30 min on ice with 100 μl of one of the following antibodies diluted in staining buffer: 2 μg of R-phycoerythrin-labeled anti-CD45 per ml, 5 μg of fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 per ml, 2 μg of Cy-Chrome-labeled anti-mouse CD8 per ml, 5 μg of FITC-labeled anti-mouse CD19 per ml, and 5 μg of FITC-labeled anti-mouse Mac-3 per ml (all monoclonal antibodies were from Pharmingen, San Diego, Calif.). The samples were washed twice in staining buffer and fixed in 1% paraformaldehyde. Stained samples were stored in the dark at 4°C until analyzed on a FASCalibur flow cytometer (Becton Dickinson, Mountain View, Calif.) with CellQuest software (Becton Dickinson). Live cells were gated as judged from forward and side laser scatter and $CD45^+$ staining.

Statistics. All data were analyzed by Student's *t* test, the Kruskal-Wallis test (Primer; McGraw-Hill, New York, N.Y.), or log rank analysis (Sigmastat, Chicago, Ill.). For multiple comparisons, the *P* value was adjusted by the Bonferroni correction.

RESULTS

Infection of BALB/c mice with melanized and nonmelanized *C. neoformans*. Given that *C. neoformans* in the environment is melanized, there is a significant likelihood that initial infection occurs with melanized organisms (24). Hence, we compared the fungal burdens in and masses of lungs infected with melanized and nonmelanized 3501. Mice were sacrificed at days 2, 4, and 7 after infection with 5×10^6 *C. neoformans* cells in one experiment and on days 7 and 14 postinfection in another. Lung CFU counts were similar in the melanized and nonmelanized groups on days 2, 4, and 14, but on day 7 the fungal burden in the lungs of mice receiving melanized 3501 cells was significantly greater than that in the lungs of mice receiving nonmelanized cells ($P < 0.01$) (Fig. 1A). The higher CFU count was accompanied by a statistically significant increase in the mass of lungs infected with melanized fungi compared to that of lungs infected with nonmelanized *C. neoformans* on days 2, 4, and 7 (Fig. 2A). No comparable increase in mass was observed in lungs of mice instilled with ghost particles (Fig. 2A). Lungs of mice infected with laccase-complemented strain 2E-TUC-4 had higher CFU counts on days 2, 4, and 7 relative to those of mice infected with laccase-deficient strain 2E-TU-4 (Fig. 1B). However, in mice infected with the 2E-TU pair, the lung masses were comparable (Fig. 2B). Histological analysis of the lung for each strain revealed granulomatous inflammation with cellular infiltrate including neutrophils, eosinophils, lymphocytes, and macrophages (data now shown). On visual examination, there were no major histological differences between the various groups. No colonies were recovered from the spleen.

Lung cytokine contents. The lung homogenate concentrations of several cytokines and chemokines measured in mice inoculated intratracheally with melanized 3501 cells, nonmelanized 3501 cells, melanin ghosts, or PBS resulted in different cytokine profiles. No differences were detected in IL-2, IL-10, TNF-α, and IFN-γ levels between melanized and nonmelanized infection groups or between mice inoculated with melanin ghosts and those that received a PBS inoculation (data not shown). However, larger changes were measured in IL-4, IL-12, and MCP-1 concentrations. The level of IL-4 in mice infected with melanized cells was approximately twice that measured in the lungs of mice infected with nonmelanized cells (Fig. 3). Lung IL-12 concentrations remained relatively constant in mice infected with the nonmelanized strain but fluctuated in mice infected with melanized cells such that it was

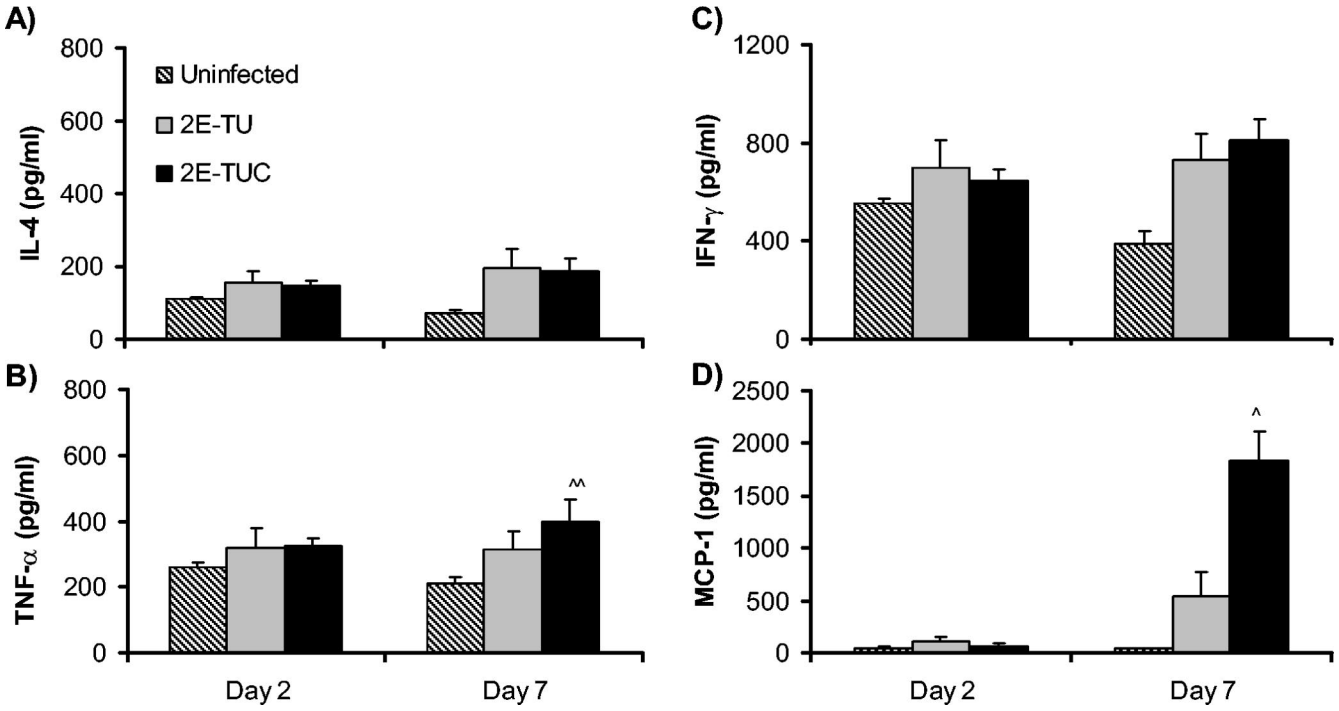

FIG. 4. Cytokine concentrations per milliliter in lungs of mice infected intratracheally with 5×10^6 2E-TU-4 or 2E-TUC-4 cells. Panels: A, IL-4; B, TNF-α (^^, $P < 0.05$ between 2E-TUC-4 and 2E-TU-4 on day 7); C, IFN-γ; D, MCP-1 (^, $P < 0.01$ between 2E-TU-4 and 2E-TUC-4 on day 7). Error bars denote the standard deviation of the mean.

higher at day 2, lower at day 4, and not different at day 7. Lung MCP-1 concentrations increased with the time of infection in both melanized and nonmelanized groups and was significantly higher in the melanized group only at day 7. In contrast, inoculation of melanin ghosts had relatively small effects on IL-4 or IL-12 levels but was associated with higher MCP-1 levels compared to those of the PBS control on days 2, 4, and 7 (Fig. 3C).

Studies of lung cytokine content after infection with the laccase mutant 2E-TU-4 and laccase-complemented 2E-TUC-4 revealed few differences, except for TNF-α and MCP-1 levels (Fig. 4). On day 7, the lungs of mice infected with 2E-TUC-4 had a modest yet significant increase in TNF-α levels relative to those of mice infected with 2E-TU-4. However, the level of MCP-1 in the lungs of 2E-TUC-4 mice was threefold higher than that in the lungs of 2E-TU-4 mice. A small increase in the IL-10 level was measured on day 7 of infection in lungs infected with 2E-TUC-4 relative to those infected with 2E-TU-4 (data not shown). No differences in IL-2 expression were detected between lungs infected with 2E-TU-4 and 2E-TUC-4.

FACS analysis of lung infiltrate. The cellular infiltrate resulting from lung inoculation with 5×10^6 melanized or nonmelanized *C. neoformans* cells or melanin ghosts or PBS was analyzed by FACS. On day 4 after infection, there was a significant increase in the total number of leukocytes recruited to the lungs in mice infected with melanized 3501 relative to that in mice infected with nonmelanized 3501 ($P < 0.05$) (Fig. 5). Also on day 4 there was a trend toward a higher leukocyte count in mice inoculated with melanin ghost treatment compared to those receiving PBS, although it did not achieve statistical significance ($P = 0.11$). On days 2 and 7 of infection, there were no significant differences in lung leukocyte counts between mice infected with melanized and nonmelanized 3501 or between the melanin ghost-treated mice and PBS controls. FACS analysis was used to identify the cell types recruited to the lungs during infection for days 4 and 7 (Fig. 6). The number of CD4- and CD8-positive leukocytes was significantly increased on day 4 in mice infected with melanized cells relative to those infected with nonmelanized cells. Melanin ghosts increased the number of Mac-3-positive cells recruited to the lungs on day 4 ($P < 0.01$). On day 7, CD19-positive cell counts were increased in mice with a melanized infection compared to those with a nonmelanized infection ($P < 0.05$) and decreased in mice that received a melanin ghost inoculation compared to those in PBS controls ($P < 0.001$).

Analysis of infection with the laccase mutant strains revealed a significant increase in leukocyte recruitment in mice receiving 2E-TUC-4 on days 2 and 4 compared with mice receiving 2E-TU-4 (Fig. 7). FACS analysis revealed an increase in CD4-, CD19-, and Mac-3-positive cells in lungs infected with strain 2E-TUC-4 relative to those infected with 2E-TU-4 on day 2, but not in CD8-positive cells. Lungs infected with 2E-TUC-4 had significantly more Mac-3-positive cells than did those infected with 2E-TU-4 (data not shown)

In vivo phagocytosis. The phagocytosis of melanized and nonmelanized *C. neoformans* cells was studied in vivo by harvesting bronchoalveolar lavage fluid 3 h after infection and assaying it for the percentage of ingested versus extracellular yeast. Furthermore, we compared an encapsulated strain (3501) with an acapsular mutant (Cap67). In the encapsulated strain, melanization was associated with a reduced efficiency of phagocytosis ($P < 0.01$). In contrast, melanization of the acap-

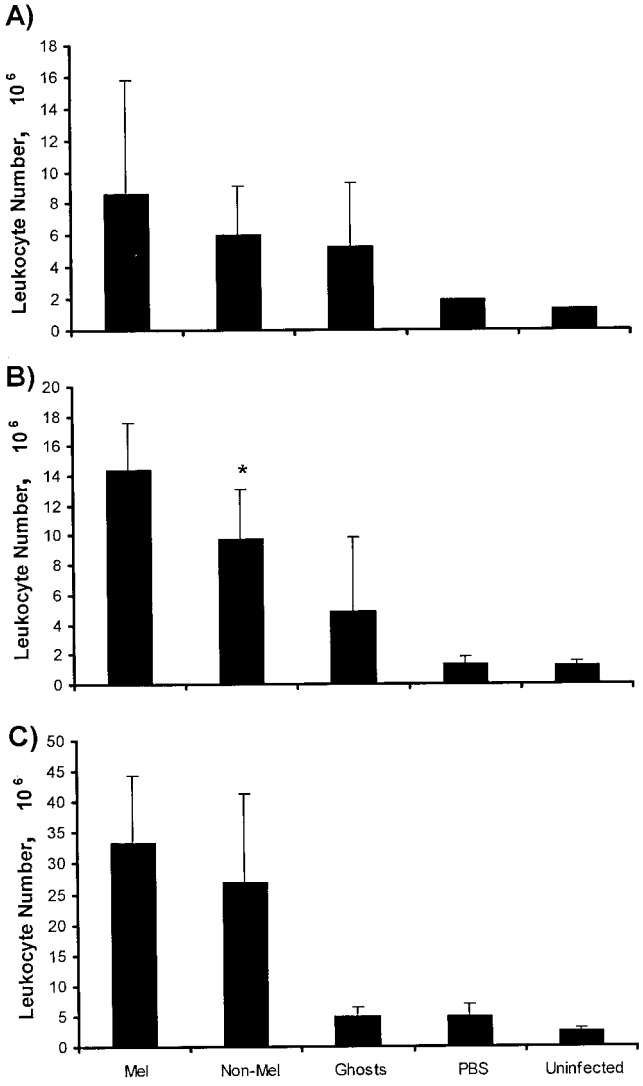

FIG. 5. Total leukocytes in the lungs of mice infected intratracheally with 5×10^6 melanized (Mel) or nonmelanized (Non-Mel) *C. neoformans* cells. Panels: A, day 2; B, day 4 (*, $P < 0.05$); C, day 7. Error bars denote the standard deviation of the mean.

sular strain was associated with an increased efficiency of phagocytosis ($P < 0.01$) (Fig. 8).

DISCUSSION

The capacity for melanization is common among pathogenic microbes, and there is considerable evidence linking melanin synthesis to virulence (19). Current views favor the hypothesis that melanization contributes to virulence by enhancing the survivability of melanotic cells confronted with host defense mechanisms (19). This concept has emerged from in vitro studies showing that melanized cells are less susceptible to microbicidal oxidants (10, 11, 38, 39). Recently, studies comparing the outcomes of pulmonary infections in mice with the 2E-TU strain pair revealed that laccase was important for extrapulmonary dissemination (25). However, evidence that melanin could modulate host immune responses prompted us to evaluate the effect of melanization on murine cryptococcal infection. The *C. neoformans* system is attractive for evaluating the contribution of melanin because the fungus does not produce melanin unless provided with an exogenous substrate, the fungal burden is easily quantifiable by CFU, and the mouse provides an excellent model for studying pathogenesis. Three types of experiments were done. One compared the inflammatory responses to melanized and nonmelanized *C. neoformans* cells, another compared the inflammatory responses to melanin ghosts, and another compared the inflammatory responses to *C. neoformans* cells with and without laccase activity. Each experiment provided complementary evidence that fungal melanin has potent immunomodulatory properties in this system.

Comparison of organ fungal burdens in mice infected with melanized and nonmelanized *C. neoformans* cells revealed a difference in CFU counts only on day 7. The absence of significant differences in CFU counts at days 2, 4, and 14 strongly argues against a role for melanin in protecting *C. neoformans* cells against the type of effector cells found in the lungs at the early stages of infection. Given that in vivo melanization takes at least 3 days (29), one would have expected lower lung CFU counts if melanin were protecting *C. neoformans* cells against

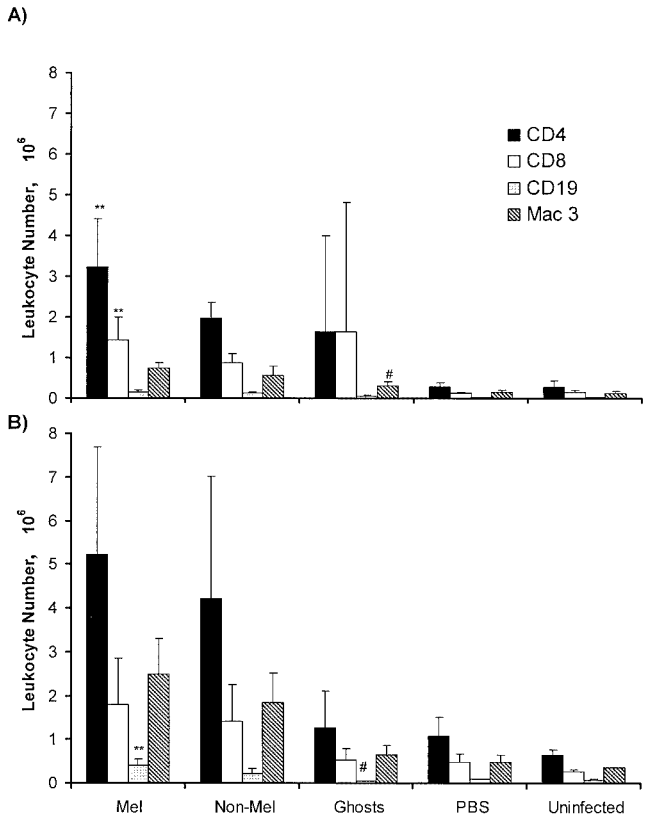

FIG. 6. Absolute differential of leukocytes determined by FACS analysis in lungs after intratracheal infection with 5×10^6 melanized (Mel) or nonmelanized (Non-Mel) *C. neoformans* cells. Panels: A, day 4 (**, $P \leq 0.05$ between melanized and nonmelanized cells for CD4 and CD8 cells; #, $P < 0.01$ between ghosts and PBS for Mac-3-positive cells); B, day 7 (**, $P < 0.05$ between melanized and nonmelanized cells for CD19-positive cells; #, $P < 0.01$ between ghosts and PBS for CD19-positive cells). Error bars denote the standard deviation of the mean.

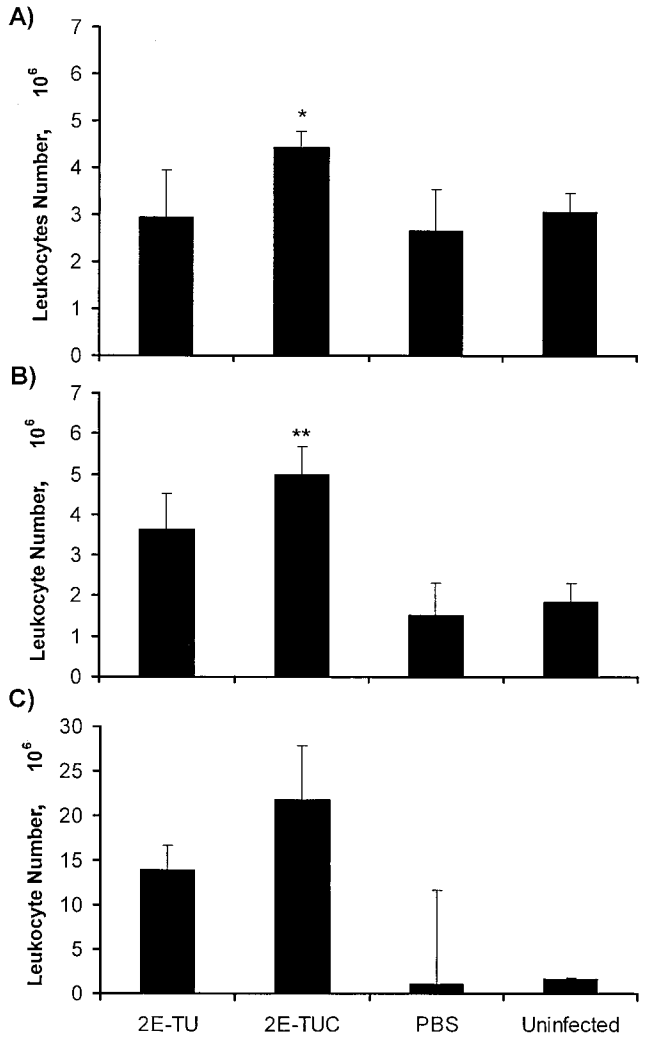

FIG. 7. Total numbers of leukocytes in the lungs of mice infected with 5×10^6 2E-TU-4 or 2E-TUC-4 cells. Panels: A, day 2 (*, $P < 0.01$ between 2E-TUC-4 and 2E-TU-4); B, day 4 (**, $P < 0.05$ between 2E-TUC-4 and 2E-TU-4); C, day 7. Error bars denote the standard deviation of the mean.

the fungicidal mechanisms of lung macrophages and early responder effector cells in the lung. The difference in CFU counts observed at day 7 results from a reduction in the CFU counts in lungs infected with nonmelanized cells, relative to those infected with melanized cells, and indicates killing of yeast cells in lungs infected with nonmelanized cells. The fact that day 7 is coincident with the development of a strong cellular inflammatory response suggests that this effect reflects either resistance to killing of *C. neoformans* cells in mice infected with melanized cells or a less effective immune response in the presence of melanized cells. However, the effect appears to be transient since at day 14 lung CFU counts in mice infected with melanized cells and those infected with nonmelanized cells were comparable, a phenomenon that could reflect the disappearance of differences as melanization occurs under in vivo conditions. In this regard, we note that melanin ghosts can be recovered from mice infected with nonmelanized cells by day 3 of infection (29).

Comparison of the inflammatory responses to melanized and nonmelanized *C. neoformans* cells revealed significant differences in cellular recruitment and lung cytokine content. There was a slight but significant increase in the number of leukocytes in the lungs of mice infected with melanized *C. neoformans* cells at day 4 but not at day 2 or 7. However, the concentrations of IL-4 and MCP-1 were significantly higher in the lungs of mice infected with melanized cells whereas there was no significant difference in IL-2, TNF-α, or IFN-γ. The finding of higher IL-4 levels in the lungs of mice infected with melanized cells suggests that the presence of melanin tilts the polarity of the response toward Th2. Since control of *C. neoformans* infection in the lung is associated with strong Th1 responses (13), the altered cytokine milieu suggests a potential explanation for the transiently higher CFU counts observed in mice infected with melanized cells. However, we note that instillation of melanin alone into the lungs elicited only minimal inflammatory and cytokine changes relative to those observed with an active *C. neoformans* infection. Hence, the effects observed on cellular recruitment and cytokine production reflect a requirement for both melanin and live *C. neoformans* cells.

In vitro studies have shown that melanized encapsulated *C. neoformans* cells are more resistant to antibody-mediated phagocytosis than nonmelanized cells are (38). This phenomenon was not understood, but subsequent studies suggested that melanization imparts a significant negative charge on both encapsulated and nonencapsulated cells (18). In this study, we revisited that question by measuring the efficiency of ingestion of melanized and nonmelanized encapsulated and nonencapsulated cells in the lung. Prior studies have shown that encapsulated *C. neoformans* cells are rapidly ingested in alveolar spaces after intratracheal infection in nonimmune mice (5). Melanized encapsulated cells were ingested less efficiently by alveolar macrophages than were nonmelanized cells, whereas the opposite was observed for melanized and nonmelanized nonencapsulated cells. This phenomenon could be relevant

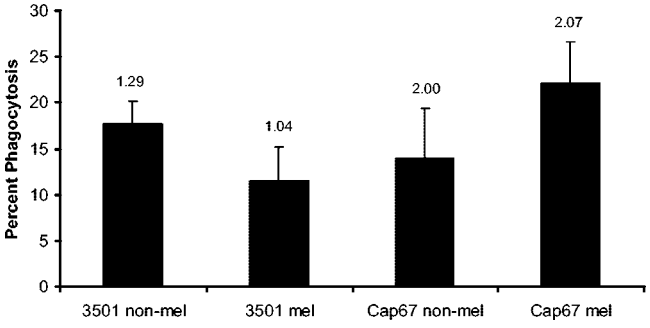

FIG. 8. Percentages of macrophages with ingested cryptococci from alveolar lavage 3 h postinfection intratracheally with 5×10^6 melanized or nonmelanized *C. neoformans* cells. The differences between the melanized (mel) and nonmelanized (non-mel) encapsulated cryptococci and the melanized and nonmelanized nonencapsulated strain Cap67 cryptococci were statistically significant ($P < 0.01$ for melanized 3501 versus non-melanized 3501 and melanized Cap67 versus nonmelanized Cap67). The value above each bar represents the total number of internalized *C. neoformans* cells divided by the total number of macrophages with internalized *C. neoformans*. Error bars denote the standard deviation of the mean.

since there is evidence that *C. neoformans* cells are melanized in the environment, and consequently, initial infection may occur with melanized cells (24). For encapsulated cells, this phenomenon parallels the prior in vitro observations (38) and may reflect an increased negative charge on cryptococcal cells. Paradoxically, melanization enhanced phagocytosis of nonencapsulated cells. Although melanization increases the charge of nonencapsulated cells, any electrostatic effects on the efficiency of phagocytosis may be overwhelmed by the fact that melanin is also a potent activator of the alternative complement pathway (27). Hence, increased melanin activation of the complement system may have been responsible for the significantly enhanced opsonization of melanized nonencapsulated cells relative to that of nonmelanized nonencapsulated cells. Differences in the efficiency of phagocytosis of melanized and nonmelanized cells could translate into differences in the immune response since fungal cell ingestion can affect antigen presentation, cytokine production, dendritic cell activation, intracellular fungal growth, and macrophage cell viability (4, 34–37).

In another experiment, we compared the responses to *C. neoformans* strains sufficient and deficient in laccase, which catalyzes the formation of melanin from L-dopa and other precursors. The comparison of 2E-TU-4 to 2E-TUC-4 revealed that infection with the complemented strain resulted in a greater fungal burden, as would be expected from the fact that laccase is an important virulence factor. However, the comparison of cytokine levels in mice infected with strains 2E-TU and 2E-TUC revealed few differences, with the notable exception of the MCP-1 level, which was threefold higher in lungs infected with 2E-TUC. The higher MCP-1 level correlated with increased numbers of leukocytes and Mac-3-positive cells in the lungs, a finding consistent with the proinflammatory properties of this chemokine. Our results obtained with the 2E-TU-4 pair showing a greater burden in lungs infected with 2E-TUC-4 differ from the finding that these strains produced similar lung fungal burdens in another strain of mice (25). Since mouse strains can differ greatly in their responses to *C. neoformans* infection (7, 9), we attribute the differences between our results and those of the other study to differences in host genetic backgrounds. However, the finding of relatively few changes in the inflammatory response is similar to and consistent with the findings of that study (25).

Comparison of the immune response parameters measured after instillation of ghosts into the lungs and pulmonary infection with melanized and nonmelanized cells and the 2E-TU-4 strains revealed changes that were not always consistent from one group to the other. For example, the changes in IL-12, TNF-α, and MCP-1 levels were different for these groups, suggesting that factors other than melanin were influencing the immune response. The differences between the experiments with the 2E-TU-4 strains and the experiments comparing infection with melanized and nonmelanized cells may reflect the initial effects of melanin on the immune response, an event that would not be expected in the 2E-TU-4 experiment, where the initial infection used nonmelanized cells. Furthermore, laccase not only catalyzes the synthesis of melanin but also has direct effects on macrophages (14). Since pulmonary macrophages produce critical early inflammatory signals, laccase effects on macrophage function could translate into additional effects on the inflammatory response. The differences between mice infected with live cells and those instilled with melanin ghost particles are understandable if one considers that one involves active infection with a replicating microbe and the other involves a response to melanin particles in the lungs. Despite the intergroup variation in the inflammatory response, the presence of fungal melanin and/or laccase consistently affected the inflammatory response relative to conditions under which these components where not present.

In summary, melanized cells and cells capable of melanization elicited inflammatory changes different from those elicited by nonmelanized cells and laccase-deficient cells. Our results provide strong evidence that melanin pigment can have immunomodulatory effects during the course of a cryptococcal infection that could contribute to virulence. Therapeutic strategies that inhibit the formation of melanin (21) or specifically target melanin (1, 28) may prove useful as adjunctive therapy for diseases caused by melanotic fungi.

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