**CNLAC1 Is Required for Extrapulmonary Dissemination of Cryptococcus neoformans but Not Pulmonary Persistence**

Mairi C. Noverr,1,2 Peter R. Williamson,3 Ryan S. Fajardo,1 and Gary B. Huffnagel1,2*

**Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine,**1,2 **and Department of Microbiology and Immunology,**3 **University of Michigan Medical School, Ann Arbor, Michigan 48109, and Division of Infectious Diseases, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60612**

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The pathogenic yeast *Cryptococcus neoformans* produces a laccase enzyme (CNLAC1), which catalyzes the synthesis of melanin in the presence of phenolic compounds. A number of genes have been implicated in the regulation of laccase and melanization, including IPC1, GPA1, MET3, and STE12. Albino mutants derived from random mutagenesis techniques may contain mutations in genes that regulate multiple virulence factors, including CNLAC1. The goal of our study is to investigate the role of CNLAC1 in virulence and evasion of pulmonary host defenses after infection via the respiratory tract. Using a set of congenic laccase-positive (2E-TUC-4) and laccase-deficient (2E-TU-4) strains, we found that both strains are avirulent at a lower dose (10^4 CFU/mouse) in mice. After the infectious dose was increased to 10^6 CFU/mouse, 70% mortality was observed in mice infected with 2E-TUC-4 compared to no mortality in mice infected with 2E-TU-4 at 30 postinfection. This observation confirms the requirement for CNLAC1 in virulence. Interestingly, we observed no differences between the two strains in pulmonary growth or in elicitation of cellular immune responses in the lung. The only measurable defect of 2E-TU-4 was in dissemination to extrapulmonary sites. To examine the role of CNLAC1 in dissemination, mice were infected intravenously. By week 3 postinfection, equal numbers of strains 2E-TUC-4 and 2E-TU-4 were recovered from the brain and spleen. This observation indicates that CNLAC1 facilitates escape from the lung, but not growth in the lungs or brain, and suggests a novel role for CNLAC1 in virulence during an infection aquired via the respiratory tract.

*Cryptococcus neoformans* is an opportunistic pathogenic yeast acquired via the respiratory tract. Clearance of a pulmonary infection requires the development of adaptive immunity. In immunocompromised hosts, *C. neoformans* can disseminate to extra-pulmonary sites, particularly the central nervous system (CNS), where infection can lead to fatal meningitis. *C. neoformans* produces a number of factors that are required for virulence, including growth at 37°C (13), the presence of polysaccharide capsule (5), urease (9), phospholipase B (7), and virulence, including growth at 37°C (13), the presence of polysaccharide capsule (5), urease (9), phospholipase B (7), and CNLAC1. The only measurable defect of 2E-TU-4 was in dissemination to extrapulmonary sites. To examine the role of CNLAC1 in dissemination, mice were infected intravenously. By week 3 postinfection, equal numbers of strains 2E-TUC-4 and 2E-TU-4 were recovered from the brain and spleen. This observation indicates that CNLAC1 facilitates escape from the lung, but not growth in the lungs or brain, and suggests a novel role for CNLAC1 in virulence during an infection acquired via the respiratory tract.

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In vitro studies have identified a number of possible molecular mechanisms for the role of laccase during pathogenesis. Melanin provides increased resistance to antifungal drugs (37), antibody-mediated phagocytosis (36), and defensins (10), and it is an antioxidant both in vitro (12) and in macrophages (18). The goal of our study is to define the role of CNLAC1 in evasion of host defenses in the lung. In vivo, melanin has been implicated in inhibition of early recognition events by the immune system and interfering with T-cell responses (11). These studies used laccase high- and low-producing strains, limiting definitive conclusions on the role of CNLAC1 in vivo. In addition, other studies investigating the role of melanin and/or laccase in pathogenesis have used albino mutants that may contain mutations in regulatory genes that control melanization (2, 15, 31). To specifically investigate the role of CNLAC1, a set of congenic mutants of *C. neoformans* differing only in CNLAC1 production (2E-TU-4/2E-TUC-4) was used for the present study. Strain 2E-TU-4 is a laccase-deficient strain (contains mutations and/or deletions in the CNLAC1 gene), and 2E-TUC-4 is a laccase-positive reconstituted transformant of 2E-TU-4 containing an integrated functional copy of the CNLAC1 gene (33). Using these congenic *C. neoformans* strains, we tested the requirement for CNLAC1 in virulence and in evasion of murine pulmonary immune responses after infection via the respiratory tract.

**MATERIALS AND METHODS**

*C. neoformans*. Strains 2E-TU-4 and 2E-TUC-4 are laccase-deficient and laccase-positive congenic strains of *C. neoformans*, respectively, and were generated as previously described (33). Briefly, parent strain B-3501 (ATCC 34873) was mutagenized with ethyl methanesulfonate treatment, and melanin-negative mutants were selected on dopamine plates. Albino strain mel2 was then backcrossed once with strain B-4476. This strain, termed 2E, was transformed with a plasmid containing *URA4* to generate strain 2E-TU or with a plasmid containing *URA4* and *CNLAC1* to generate strain 2E-TUC. Strains 2E-TU and 2E-TUC were then
backcrossed with strain B-4476 four times to generate strains 2E-TU-4 and 2E-TUC-4. Strain 2E-TU-4 was negative for phenoloxidase activity, recessive sterility, and suppression of mutant phenotype by CuSO4. The CNLAC1 gene from 2E-TU-4 was sequenced and contains six differences at the amino acid level, including a His-Tyr substitution in the highly conserved histidine copper-binding site. Southern blot analysis confirmed that transformant 2E-TUC-4 contains an integrated copy of the CNLAC1-containing plasmid. For infection, yeast were grown to stationary phase (72 h) at 37°C in Sabouraud dextrose broth (1% neopeptone, 2% dextrose; Difco, Detroit, Mich.) with shaking. The cultures were then washed in nonpyrogenic saline (Abbott Laboratories, Chicago, Ill.), counted with a hemocytometer, and diluted to 3.3 × 10⁵ CFU/ml in sterile nonpyrogenic saline.

Mice. Female CBAJ mice (18 ± 2 g) were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed in specific-pathogen-free conditions in enclosed filter-top cages. Food and sterile water were given ad libitum. The mice were maintained by the Unit for Laboratory Animal Medicine at the University of Michigan (Ann Arbor, Mich.), and protocols were approved by an animal institutional review board.

Intratracheal inoculation. Infection was established via intratracheal inoculation with 10⁵ or 10⁶ CFU of C. neoformans. Four animals per group per time point were infected in two independent experiments for a total of eight mice per group per time point. Mice were anesthetized with ketamine-xylazine solution (2.5 mg of ketamine/mouse [Fort Dodge Animal Health, Fort Dodge, Iowa] plus 0.1 g of xylazine/mouse [Lloyd Laboratories, Shenandoah, Iowa]) and restrained on a small board. A small incision was made in the skin over the trachea, and the underlying tissue was separated. A tuberculin syringe (Monoject, St. Louis, Mo.) was filled with a dilute C. neoformans culture and a 30-gauge needle (Becton Dickinson, Rutherford, N.J.) was attached and bent. The needle was inserted into the trachea and a 30-μl inoculum was delivered. The skin was sutured with a cyanoacrylate adhesive, and the mice recovered with no visible trauma. Aliquots of the inoculum were analyzed for CFU to monitor the amounts delivered.

Intravenous inoculation. Infection was established via intravenous inoculation with 10⁵ CFU of C. neoformans with 8 to 10 mice per group per time point. Mice were warmed under a heating lamp for 15 min prior to intravenous inoculation. A tuberculin syringe (Monoject) was filled with a dilute C. neoformans culture and a 30-gauge needle (Becton Dickinson) was attached. The needle was inserted into the lateral tail vein, and a 250-μl inoculum was delivered. Aliquots of the inoculum were analyzed for CFU to monitor the amount delivered.

Harvesting of tissues. Extrapulmonary organs were harvested subsequent to removal of the lungs. Lung-associated lymph nodes (LALN) were collected by excising the nodes from the junction of the azygos vein and the superior vena cava. Brains were collected by removing the top of the cranium and excising the brain from the brain stem. Organs were placed in tubes containing 2 ml of sterile water and homogenized mechanically using a Tissue-Tearor (Biospec Products, Bartlesville, Okla.).

CFU assay. Aliquots of the lungs digests (intratracheal infection), lung homogenates (intravenous infection), and brain, spleen, and LALN homogenates were plated out on Sabouraud dextrose agar (Difco) in 10-fold dilutions and incubated at room temperature. Colonies were counted 2 to 3 days later, and the numbers of CFU/organ were calculated.

Lung leukocyte isolation. Mice were euthanized by CO2. Lungs were excised, minced, and enzymatically digested for 30 min at 37°C by using 15 ml/lung digestion buffer (RPMI, 10% fetal calf serum), antibiotics, 1 mg of collagenase (Boehringer Mannheim Biochemical, Chicago, Ill.)/ml, and 30 μg of DNase (Sigma Chemical Co., St. Louis, Mo.)/ml. Cells were further dispersed by drawing the suspension up and down through the bore of a 10-ml syringe. A 100-μl aliquot was removed for the CFU assay. The cell suspension was pelleted, and erythrocytes were lysed by incubation in ice-cold NH4Cl buffer (0.829% NH4Cl, 0.1% KHCO3, 0.037% Na2EDTA [pH 7.4]; Sigma). Excess RPMI was added to make the solution isotonic, and the cells were pelleted and resuspended in complete medium (RPMI 1640, 10% fetal calf serum; Life Technologies), 5 × 10⁸ M 2-mercaptoethanol, sodium pyruvate, nonessential amino acids, glucose, and antibiotics (Sigma). Cell concentrations were determined by counting cells diluted in trypan blue by using a hemocytometer.

Lung leukocyte culture and cytokine ELISA. Isolated leukocytes (from enzymatic digestion) from individual mice were standardized to 1.5 × 10⁷ cells/ml and cultured in complete medium without additional stimulation at 37°C and 5% CO2. Supernatants were harvested at 24 h and assayed for cytokine production by enzyme-linked immunosorbent assay (ELISA; OptEIA; Pharmingen, San Diego, Calif.).

Cell staining. Leukocyte differentials (neutrophils, eosinophils, macrophages, and monocytes or lymphocytes) were visually counted after Wright-Giemsa staining of lung leukocyte samples cytospun onto glass slides (Shandon Cytocentrifuge, Pittsburgh, Pa.). The percentage of a leukocyte subset was calculated by multiplying the total number of leukocytes to yield the absolute number of that leukocyte subset.

RESULTS

CNLAC1 and survival in mice. To determine the role of CNLAC1 in virulence during infection acquired via the respiratory tract, we inoculated CBAJ mice intratracheally with 10⁶ CFU of C. neoformans strains 2E-TU-4 or 2E-TUC-4. Interestingly, no mortality was observed in mice infected with either strain 2E-TU-4 or 2E-TUC-4 by day 42 postinfection (data not shown). After the infectious dose was increased to 10⁶ CFU/mouse, mice infected with these strains exhibited significant differences in mortality (Fig. 1). By day 30 postinfection, 70% of mice infected with the laccase-positive strain 2E-TUC-4 died. In contrast, no mortality was observed in mice infected with strain 2E-TU-4 by day 63 postinfection. The mean survival time of 2E-TU-4-infected mice was >63 days, which was significantly longer than 2E-TUC-4-infected mice (27.7 ± 0.44 days) (P < 0.005). This demonstrates that CNLAC1 is required for virulence after infection via the respiratory tract.

CNLAC1 and pulmonary clearance of C. neoformans. Pulmonary cryptococcal burden was examined in mice infected at the 10⁶-CFU/mouse dose (Fig. 2a). Pulmonary burden remained between 10⁵ and 10⁶ CFU/lung at weeks 1, 2, and 4 postinfe-
Leukocyte recruitment. Mice were infected with $10^6$ CFU of strain 2E-TU-4 or 2E-TUC-4. No surviving animals remained for mice infected with 2E-TUC-4 after week 4 postinfection. The results are expressed as the mean CFU per organ $\pm$ the standard error of the mean (SEM). $n = 12$ to 17 mice per time point pooled from two separate experiments. For examination of recruited lung leukocytes, lungs were excised at weeks 1, 2, 4, and 5 postinfection. Leukocytes were isolated from whole lungs by enzymatic digestion and mechanical dispersion. The number of recruited leukocytes in infected mice was calculated as the total number of leukocytes in infected mice minus the mean number of leukocytes in uninfected mice. The results are expressed as the mean number of leukocytes per mouse $\pm$ the SEM. $n = 7$ to 8 mice per time point pooled from two separate experiments.

**FIG. 2.** Effect of CNLAC1 on pulmonary cryptococcal burden and leukocyte recruitment. Mice were infected with $10^6$ CFU *C. neoformans* strain 2E-TU-4 or 2E-TUC-4. (a) Pulmonary burden was determined at various time points postinfection. No surviving animals remained for mice infected with 2E-TUC-4 beyond week 4 postinfection due to mortality. How-ever, in mice infected with strain 2E-TU-4, pulmonary burden could not be obtained from mice infected with either strain were statistically equivalent ($P > 0.05$), indicating that both strains elicit Th1 responses. Cytokine production was also measured in lung leukocyte cultures harvested from infected mice (Fig. 5b and data not shown). Overall, these data provide evidence for the development of T-cell responses in mice infected with either strain of *C. neoformans* but do not identify differences in host response that correlate with mortality in mice infected with laccase-positive strain 2E-TUC-4.

**CNLAC1 and dissemination of *C. neoformans*.** Although no measurable differences were observed in lungs of mice infected with either 2E-TU-4 or 2E-TUC-4, we also examined cryptococcal burden in extrapulmonary organs in these mice. Significantly greater numbers of 2E-TUC-4 were recovered from extrapulmonary sites, including the brain and spleen, at week 3 postinfection (Fig. 6a). In addition, detectable cryptococci were recovered from greater numbers of animals infected with laccase-positive strain 2E-TUC-4 compared to laccase-deficient strain 2E-TU-4 (11 of 12 mice versus 4 of 13 mice for the brain and 13 of 16 mice versus 6 of 17 mice for the spleen). Significantly greater numbers of 2E-TUC-4 were also recovered from the brain (2E-TUC-4, log 5.5 CFU/organ; 2E-TU-4, log 5.1 CFU/organ) and spleen (2E-TUC-4, 5.1 CFU/organ; 2E-TU-4, log 5.4 CFU/organ) (data not shown). Thus, CNLAC1 plays a role in the ability of *C. neoformans* to disseminate.

Dissemination is a multistep process, involving escape from the lung, emptying into the draining lymph nodes en route to the lymphatics and bloodstream, and survival and growth in extrapulmonary sites. To determine at which step of dissemination CNLAC1 is involved, mice were infected intravenously. Equal numbers of strain 2E-TUC-4 and 2E-TU-4 were recovered from the brain and spleen at week 3 postinfection (Fig. 6b). At earlier time points (week 1), similar numbers of laccase-positive and laccase-negative *C. neoformans* were recovered from the brain (2E-TUC-4, log 5.5 CFU/organ; 2E-TU-4, 5.1 CFU/organ) and spleen (2E-TUC-4, 5.1 CFU/organ; 2E-TU-4, log 5.4 CFU/organ) (data not shown). At this time point both strains had exited the microcapillaries and were growing in the brain tissue, resulting in tissue damage (Fig. 7). This indicates that CNLAC1 does not play a role in (i) survival and/or growth in extrapulmonary sites, (ii) survival in the bloodstream en route to other destinations, or (iii) survival within the brain. Instead, these results support a role for CNLAC1 in escape from the lungs, which may explain the role of CNLAC1 in virulence.
DISCUSSION

The goal of our study was to define the role of CNLAC1 in evasion of host defenses in the lung. Increased mortality was observed in mice infected intratracheally with laccase-positive strain 2E-TUC-4, confirming that CNLAC1 is required for virulence. Surprisingly, we did not observe any differences in the ability of these strains to grow in the lungs of infected mice through week 4 postinfection. After week 4, mice infected with 2E-TUC-4 began to die, whereas mice infected with 2E-TU-4 began to clear the infection in the lungs. No differences were observed in leukocyte recruitment or IFN-γ production during the course of the infection. However, we observed significantly greater numbers of laccase-positive organisms in the brain, spleen, and LALN at week 4 postinfection. This indicates that CNLAC1 plays a role in dissemination of C. neoformans.

Dissemination is a multistep process, involving escape from the lung, passage into the draining lymph nodes en route to the lymphatics and bloodstream, and survival and growth in extrapulmonary sites. Significantly more organisms were recovered from the LALN in mice infected intratracheally. If dissemination from the lungs occurs through the lymphatics, the LALN would be the first extrapulmonary site infected. These results suggest that strain 2E-TUC-4 escapes from the lung with increased frequency compared to strain 2E-TU-4. To more specifically address this issue, mice were infected intravenously. By week 3 postinfection, equal numbers of each strain were recovered from the brains and spleens of infected mice, indicating that CNLAC1 does not play a role in growth or survival in extrapulmonary sites or in survival in the bloodstream.

The present study establishes a novel role for CNLAC1 in the virulence of a C. neoformans infection acquired via the respiratory tract. Our results indicate that CNLAC1 is involved in promoting escape of C. neoformans from the lung. Escape may be facilitated by intracellular survival within a cellular disseminatory vehicle. C. neoformans can grow both intracellularly and extracellularly in the lung. However, intracellular survival plays a role in escape from the lung (20). CNLAC1 has been shown to play a role in intracellular survival in alveolar macrophages, which may explain the increased ability of 2E-TUC-4 to disseminate (18). Our results also suggest that intracellular survival in alveolar macrophages may not be important in the growth of C. neoformans in the lung. This idea is supported by the fact that a mutant strain of C. neoformans in Apl1, which mediates antiphagocytic activity, exhibits decreased virulence in mice (20). Therefore, extracellular growth may be the preferred environment for C. neoformans in the lung for evasion of host defense mechanisms. Overall, we believe the escape of strain 2E-TUC-4 from the lung environment is mediated by a CNLAC1-dependent mechanism and that subsequent growth within the CNS is the likely cause of death in these studies.

C. neoformans has a unique predilection for the CNS, where it can cause fatal meningitis. C. neoformans is well known for its propensity to establish a CNS infection (25). Our results conflict with the notion that CNLAC1 plays a crucial role in the protection of cryptococci at the level of the CNS, where substrates for the enzyme are more abundant (26). However, it is still possible that CNLAC1 may facilitate a cryptococcal CNS infection.
infection after infection acquired via the respiratory tract. In this scenario, an immune response against \textit{C. neoformans} is initiated in the lungs and LALN. A secondary specific immune response elicited by \textit{C. neoformans} in the CNS could lead to recruitment and activation of effector cells specific to the CNS that are not present in the lung. These effector cells elicited as part of a secondary response also would be absent in an intravenous model of infection. \textit{CNLAC1} could provide another level of defense against activated cells in the CNS. In fact, melanin-negative mutants of \textit{C. neoformans} are more susceptible to the antifungal

FIG. 4. Photomicrographs of the lungs from week 2 (a and c) and week 4 (b and d) postinfection with \textit{C. neoformans} strain 2E-TUC-4 (a and b) or 2E-TU-4 (c and d). Mice were treated as outlined in Fig. 6. The absence of leukocytic recruitment at week 2 for each group (a and c) and increased leukocyte recruitment at week 4 for each group (b and c) is consistent with the total leukocyte recruitment reported in Fig. 2. A similar cryptococcal burden is evident between each group. Mucicarmine stained; magnification, x200.

FIG. 5. Effect of \textit{CNLAC1} on anticryptococcal DTH responses (a) and lung leukocyte IFN-\gamma production (b). CBA/J mice were infected intratracheally with 10^6 CFU of \textit{C. neoformans} strain 2E-TU-4 or 2E-TUC-4. (a) For measurement of DTH, right hind footpads of infected mice were injected with CheF antigen, and left hind footpads were injected with bovine serum albumin at day 28 postinfection. At 2 days after antigen injection, footpad swelling was measured by using a micrometer (swelling = right minus left) (b) For measurement of IFN-\gamma production, lung leukocytes were isolated from whole lungs and were cultured for 24 h without additional stimulation. Supernatants were collected and assayed for IFN-\gamma by ELISA. The results are expressed as the mean \pm the SEM. n = 6 mice per time point assayed in duplicate. The dashed lines represent DTH and IFN-\gamma levels in uninfected animals.
actions of activated microglial cells, which are effector cells involved in controlling an infection in the CNS (3). In addition, the humoral arm of cell-mediated immune responses (specific antibody) is also required to mediate antifungal activities of microglial cells (16). Further studies are needed to elucidate the finer details of the role of CNLAC1 in defense against innate and acquired defenses in the CNS.

These studies specifically redefine the role of laccase in virulence. Previous studies have compared cryptococcal strains with differences in the ability to melanize (2, 15, 31). The presumption that the mutations in these strains were specific to laccase may need to be reexamined. A number of genes have been implicated in the regulation of laccase and melanization, including IPC1 (21), GPA1 (1), MET3 (40), and STE12 (6). Albino mutants derived from random mutagenesis techniques may contain mutations in genes that regulate multiple virulence factors, including CNLAC1. This may provide an alternative explanation for the discrepancies between the present study and studies that examined the role of melanization. The present study is the first to use congenic strains differing specifically in laccase to define the role of laccase in cryptococcal pulmonary pathogenesis and dissemination.

We propose that the pathogenesis and tropism of C. neoformans is mediated in a stepwise fashion by specific virulence factors. C. neoformans is an environmental microbe (14, 27, 32) that is initially inhaled (28, 29) and yet exhibits a tropism for the CNS (17, 30, 34). We believe the dissemination from the lungs to the bloodstream occurs initially through the lymphatics draining the lungs. We can detect cryptococci in the LALN prior to the appearance of detectable numbers of organisms in the spleen or other organ sites (during the first week of infection; data not shown). From the blood, dissemination proceeds to extrapulmonary organs such as the CNS. Polysaccharide capsule protects C. neoformans from destruction by alveolar macrophages (22, 35, 38, 41). Phospholipase B and CNLAC1 allow the organism to survive inside the alveolar macrophage (18, 24). The mechanism of lung-lymph node dissemination is not known but is clearly defective in laccase-deficient and phospholipase B-deficient C. neoformans strains (8, 24, 42; the present study) and in mice depleted of alveolar macrophages (A. C. Herring et al., unpublished), implicating transport of intracellular C. neoformans by macrophages into the lymph nodes and subsequently into the bloodstream. Once in the bloodstream, urease appears to facilitate entry into the CNS (24a). The fact that CNLAC1-deficient cells can grow in the CNS but albino mutants cannot (2) suggests that unknown virulence factors (that are regulated along with CNLAC1) play a role in the neurotropism of C. neoformans. Thus, C. neoform-

![FIG. 6. Effect of CNLAC1 on extrapulmonary organ burden.](image)

(a) Mice were infected intratracheally with 10^6 CFU of C. neoformans strain 2E-TU-4 or 2E-TUC-4, and the brains and spleens were harvested at week 4 postinfection. n = 12 to 17 mice per time point pooled from two separate experiments. (b) Mice were infected intravenously with 10^6 CFU of C. neoformans strain 2E-TU-4 or 2E-TUC-4, and the brains and spleens were harvested at week 3 postinfection. n = 9 to 10 mice per time point. The results are expressed as the mean CFU per organ ± the SEM. *, P < 0.01 (as determined by the Student t test).

![FIG. 7. Photomicrographs of brains at week 1 postinfection in intravenously infected mice.](image)

Mice were infected with 10^6 CFU of C. neoformans strain 2E-TU-4 or 2E-TUC-4, and brains were harvested at week 1 postinfection. Similar cryptococcal burden and tissue destruction is evident between the two groups. Mucicarmine stained; magnification, ×100.
mans produces a number of factors that function in a stepwise fashion as site-specific virulence factors to promote the dissemination and virulence of this microbe after inhalation.

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