Urokinase Is Required for the Pulmonary Inflammatory Response to *Cryptococcus neoformans*

A Murine Transgenic Model

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Abstract

Urokinase (uPA) is hypothesized to provide proteolytic activity enabling inflammatory cells to traverse tissues during recruitment, and it is implicated as a cytokine modulator. Definitive evaluation of these hypotheses in vivo has previously been impossible because uPA could not completely and irreversibly be eliminated. This limitation has been overcome through the development of uPA-deficient transgenic mice ($uPA^{-/-}$). Using these mice, we evaluated the importance of uPA in the pulmonary inflammatory response to *Cryptococcus neoformans* (strain 52D). *C. neoformans* was inoculated into $uPA^{-/-}$ and control mice ($uPA^{+/+}$), and cell recruitment to the lungs was quantitated. The number of CFU in lung, spleen and brain was determined to assess clearance, and survival curves were generated. By day 21 after inoculation, $uPA^{-/-}$ mice had markedly fewer pulmonary inflammatory ($CD45^+$), $CD4^+$, and $CD11b/CD18^+$ cells compared with $uPA^{+/+}$ controls ($P < 0.007$); pulmonary CFUs in the $uPA^{-/-}$ mice continued to increase, whereas CFUs diminished in $uPA^{+/+}$ mice ($P < 0.005$). In survival studies, only 3/19 $uPA^{+/+}$ mice died, whereas 15/19 $uPA^{-/-}$ mice died ($P < 0.001$). We have demonstrated that uPA is required for a pulmonary inflammatory response to *C. neoformans*. Lack of uPA results in inadequate cellular recruitment, uncontrolled infection, and death. (*J. Clin. Invest.* 1996. 97:1818–1826.) Key words: transgenic mice • cellular recruitment • fungal pathogens • lung inflammation • plasminogen activators

Introduction

The expression of proteases by leukocytes is thought to be critically important for the ability of cells to degrade matrix proteins and traverse tissue planes during recruitment to inflammatory sites (1–3). Substantial evidence implicates the urokinase-type plasminogen activator (uPA)[1]/plasmin system as a central mediator in this process (4, 5). Leukocytes express uPA that converts the inactive proenzyme plasminogen to plasmin, a protease of broad substrate specificity (6).

The postulate that uPA mediates proteolysis associated with migrating cells is further strengthened by the presence of specific receptors for uPA (urokinase receptor, CD87) on the cell surface of monocytes and neutrophils (5, 7, 8). We have shown that CD87 clusters to the leading front of cellular migration in monocytes in response to an FMLP gradient, thus focusing the proteolytic activity of uPA in the direction of migration. But whereas CD87 is required for monocyte and neutrophil chemotaxis, uPA activity is not (9, 10). The independence of chemotaxis from uPA proteolytic activity is not surprising, since the assays were done under matrix-free conditions where there was no requirement for cells to penetrate matrix protein to effect chemotaxis. However, even under these conditions, chemotaxis was diminished in monocytes treated with antisense-uPA oligonucleotides, suggesting that uPA binding to its receptor may be involved in cell movement (9). In vitro support of the role of uPA in cell migration has been demonstrated not only in hematopoietic cells, but also in tumor cells, in which migration through basement membrane and defined matrices can be impeded by inhibitors to uPA or plasmin (4, 11–13). The roles that uPA and uPA proteolytic activity play in the increased complexities of inflammatory cell recruitment in vivo, where inflammatory cells must become activated, migrate, and proteolytically degrade multiple, varied tissue planes over a defined time period, are largely unexplored.

Although work done in vitro has suggested that uPA may play a role in inflammatory responses, in vivo testing of this hypothesis has previously been limited by the inability to eliminate uPA from any system completely and irreversibly, especially over the time required to assess complex host defenses. The development of transgenic mice lacking the uPA gene has allowed us to develop an animal model system to test the importance of uPA in cell recruitment and in the generation of protective pulmonary inflammatory responses.

Initial characterization of these uPA-deficient ($uPA^{-/-}$) mice raised doubts as to whether uPA is important in cellular recruitment. The $uPA^{-/-}$ mice were shown to recruit peritoneal macrophages in response to thioglycolate in a way identical to uPA-replete ($uPA^{+/+}$) controls, suggesting that uPA is

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1. *Abbreviations used in this paper*: CD87, urokinase-type plasminogen activator receptor; IT, intratracheal; Mac1, complement receptor 3; MØ, mononuclear phagocyte; uPA, urokinase-type plasminogen activator; $uPA^{-/-}$, uPA-deficient (knock-out) transgenic mice; $uPA^{+/+}$, uPA-replete control mice.

not required (14). Further, uPA$^{-/-}$ mice have normal fertility rates and produce litters indistinguishable from those of uPA$^{+/+}$ controls in terms of number and size of pups (14). Since trophoblast implantation and embryo development are thought to be critically dependent on uPA, further doubts were raised about the importance of uPA in invasive cell systems (15, 16).

Despite the initial findings suggesting that uPA's role in cell migration had previously been overstated, uPA$^{-/-}$ mice permit powerful animal modeling for the evaluation of the role of uPA in host defenses. In this study, by comparing uPA$^{-/-}$ mice with background-matched control uPA$^{+/+}$ mice, we sought to determine whether uPA is required for pulmonary defenses against a clinically relevant pathogen, *Cryptococcus neoformans. C. neoformans* rarely causes disease in the immunocompetent, but it is an increasingly important lethal fungal pathogen in AIDS and other immunocompromised patients (17). Using a murine model of intratracheal (IT) inoculation of *C. neoformans,* we mimic the natural route of acquisition of the infection. We have previously shown that *C. neoformans* strain 52D is nonlethal in normal mice but disseminates and causes death by meningitis in immunocompromised mice (SCID or CD4 depleted) (18, 19). Thus, the pattern of host susceptibility in mice parallels that seen clinically and is critically dependent on the adequacy of cellular inflammatory responses.

In this study, we investigate the importance of uPA in the pulmonary inflammatory response to *C. neoformans.* Comparing uPA$^{-/-}$ mice with uPA$^{+/+}$ controls, we evaluate the role uPA plays in recruitment of pulmonary inflammatory cells, the containment and irradication of the fungal infection, and in the overall effectiveness of defenses as reflected in survival of the host.

Methods

Animals. Mice were housed in specific pathogen-free isolation rooms in the University of Michigan Department of Laboratory Animal Medicine, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. This study was approved by the University of Michigan Committee on Use and Care of Animals (UCUCA). Mice were periodically checked for murine hepatitis virus and were found to be negative, and they were fed standard animal chow (Rodent Lab chow No. 5008; Purina, St. Louis, MO) and chlorinated tap water ad libitum. Mice were used at 8–12 wk of age.

Transgenic uPA deplete mice (uPA$^{-/-}$) and background matched control mice (uPA$^{+/+}$) were generous gifts from Dr. Peter Carmeliet (14). The uPA gene was "knocked out" by homologous recombination with the uPA/neomycin construct in ES cells derived from strain 129 mice. The ES cells were injected into C57/B6 blastocysts, and the resulting chimeric males were bred with C57/B6 females to produce transgenic mice heterozygous for the uPA gene. Breeding of these progeny produced the homozygous mice used to establish the uPA$^{-/-}$ and uPA$^{+/+}$ lines we used to generate our colonies.

Parental strains C57/B6 and 129 were obtained from The Jackson Laboratory (Bar Harbor, ME).

Confirmation of genotype of uPA$^{-/-}$ and uPA$^{+/+}$ mice. We confirmed the genotype of the uPA$^{-/-}$ and uPA$^{+/+}$ mice by PCR analysis. Genomic DNA was extracted from tail samples by proteinase K digestion followed by chloroform extraction and ethanol precipitation (20). Samples were amplified with primers specific for the wild-type uPA gene (sense 5′-ctgtctgtcatccaaccagtcc, anti-sense 5′-gacacgcatacacctccgttct; (21) or the knock-out uPA/neomycin construct (sense 5′-ctgtctgtcatccaaccagtcc, anti-sense 5′-ctcctgtcatctcaccttgctc; sequence pMC1Neo from Stratagene Inc., La Jolla, CA), designed with the aid of the program Primer 2 (Scientific and Educational Software, State-line, PA). Reaction mixtures (0.02 mg/ml DNA; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 2.5 mM $MgCl_2$; 0.25 mM each nucleotide [dCTP, dATP, dTTP, dGTP]; 0.2 mM sense and antisense primers; 0.025 U/ml Taq polymerase [Life Technologies, Gaithersburg, MD)], were denatured (94°C, 3 min) and amplified through 45 cycles (93°C 30 s, 62°C 45 s, 72°C 120 s) with a one cycle extension of 72°C for 5 min. Products were electrophoresed in agarose, stained with ethidium bromide, Southern blotted, and hybridized with labeled gene-specific internal oligonucleotide probes (22) to check product length and sequence specificity. Amplification products of the wild-type and knock out genes are 1,153 bp and 1,292 bp, respectively.

C. neoformans. C. neoformans strain 52 D was obtained from the American Type Culture Collection (ATCC; Rockville, MD) (ATCC No. 24067; serotype D) (23). Stock cultures of *C. neoformans* were passed monthly on Sabouraud's dextrose agar slants (Difco Laboratories, Inc., Detroit, MI) and stored at 4°C. For infection, yeast was grown from stock in Sabouraud's dextrose broth medium (1% neopeptone, 2% dextrose; Difco Laboratories, Inc.) for 48 h at 35°C on a shaker, washed twice in nonpyrogenic saline (NPS; Travenol Laboratories, Deerfield, IL), counted on a hemocytometer, and diluted to 3.3×10^5 organisms $\cdot$ ml^{-1} in NPS.

IT inoculation. Mice were lightly anesthetized with pentobarbital (64 mg $\cdot$ kg^{-1} intraperitoneally, Butler, Columbus, OH) and restrained on a small board. Each mouse received an IT inoculum of 1×10^4 *C. neoformans* in 30 μl NPS as previously described (19). This technique results in a highly reproducible pulmonary infection (24). Aliquots of the inoculum were serially diluted and plated out to confirm the number of CFU of *C. neoformans* being delivered. Control mice that received an IT inoculation with normal saline were examined histologically at various times after inoculation and showed no evidence of pulmonary inflammation.

Euthanasia of mice and collection of tissues. At various times after inoculation, mice were killed with an overdose of pentobarbital (250 mg $\cdot$ kg^{-1} intraperitoneally). Lungs were excised, minced, and digested enzymatically with 15 ml/lung of digestion solution (RPMI 1640, 5% FCS, penicillin and streptomycin, 1 mg/ml collagenase [Boehringer Mannheim Biochemicals, Indianapolis, IN], and 30 μg/ml DNase [Sigma Chemical Co., St. Louis, MO]) for 30 min at 37°C. The undigested fragments in the cell suspension were dispersed further by repeated aspiration through a 10-ml syringe. This digestion procedure results in minimal contamination with epithelial cells (25). A 100-μl digest was removed to assay for lung CFU. The remaining cell suspension was then pelleted, and erythrocytes were lysed by resuspending in lysis buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, 0.0372% Na_2EDTA, pH 7.4). The cells were then washed twice with HBSS, resuspended in complete medium (RPMI 1640, 5% FCS, nonessential amino acids, sodium pyruvate, glutamine, and antibiotics) and filtered through 100-μm nylon mesh. An aliquot was removed and stained with trypan blue, and the cells were counted on a hemocytometer. Absolute numbers of each leukocyte subset were obtained by multiplying the total number of cells in that lung sample by the percentage of that cell type, as determined by immunofluorescent flow cytometry.

For assaying CFUs in brains and spleens, individual organs were placed in sterile plastic bags with 2 ml sterile water and homogenized by crushing with a pipette side.

For histologic sections, the lungs were perfused, the trachea cannulated, and the lungs inflated in situ with 10% formalin in PBS. Next the entire thoracic contents were dissected and fixed by immersion in 10% formalin in PBS for 18–24 h. The fixed tissues were transferred to 70% ethanol until use.

Preparation and grading of histologic specimens. Parasagittal sections through the fixed lungs were cut, embedded in paraffin, and sectioned at 5-μm thickness. The slides, each representative of both lungs from a single mouse, were stained with hematoxylin and eosin. Each slide was scanned at low power, and representative sections were identified.

Immunofluorescent cell staining and flow cytometry. To quantify

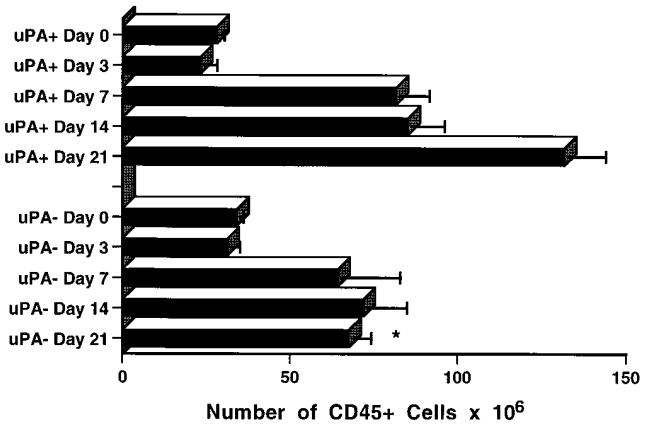

Figure 1. Comparison of inflammatory cell ($CD45^+$) recruitment to the lungs of $uPA^{+/+}$ vs $uPA^{-/-}$ mice during pulmonary cryptococcal infection. Lungs were minced and enzyme digested to a single-cell suspension. $CD45^+$ cells were identified by immunofluorescent flow cytometry. The data are expressed as millions of pulmonary $CD45^+$ cells per mouse on the day of inoculation (day 0) and on postinoculation days 3, 7, 14, and 21; $*P = 0.0001$, $n = 8$.

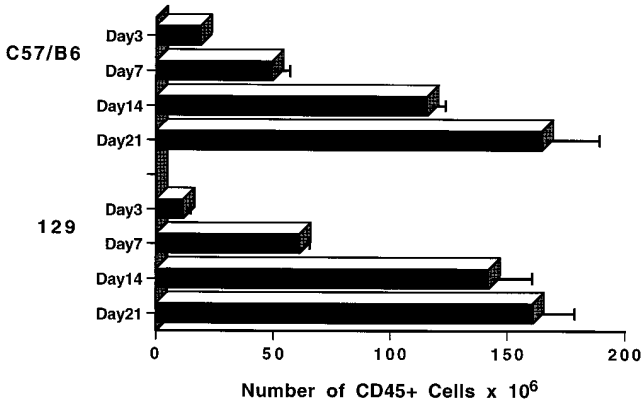

Figure 2. Comparison of background strains C57/B6 and 129. Recruitment of inflammatory cells ($CD45^+$) to the lungs during pulmonary cryptococcal infection. Lungs were minced and enzyme digested to a single-cell suspension. $CD45^+$ cells were identified by immunofluorescent flow cytometry. The data are expressed as millions of pulmonary $CD45^+$ cells per mouse on postinoculation days 3, 7, 14, and 21; comparison of C57/B6 to 129 at day 21; P = NS, $n = 5$.

leukocyte subsets, individual lungs were processed as described above and analyzed separately. Aliquots of lung cells (5×10^5 cells in 0.1 ml) were stained for 30 min on ice with 20 μl of staining buffer (PBS, 0.1% NaN_3, 1% FCS) containing 1 μg of phycoerythrin-labeled anti–common leukocyte antigen (CLA), an epitope of CD45 (clone 30F11.1, PharMingen, San Diego, CA), and 1 μg of one of the following FITC-labeled mAbs (PharMingen): RM-4-5 (anti-CD4), 53-6.7 (anti-CD8), RA3-6B2 (anti-B220), M1/70 (anti-CD11b, Mac-1), or isotype-matched rat IgG. The samples were washed in staining buffer, fixed in buffered 1% paraformaldehyde (Sigma Chemical Co.), and stored in the dark at 4°C until analyzed.

Samples were analyzed on a flow cytometer with accompanying software (EPICS Elite; Coulter Corp., Hialeah, FL). At least 20,000 cells/sample were analyzed, gated for $CD45^+$ (red fluorescence) cells (forward and side laser scatter) and analyzed for staining by the specific FITC-labeled antileukocyte markers. To confirm that cryptococci did not fall within the laser scatter gates of the leukocytes, separate samples were treated before analysis with Triton X-100 (Sigma Chemical Co.) to lyse mouse leukocytes.

CFU assay. The CFU assay was performed as described (26). Briefly, serial 10-fold dilutions of each organ homogenate were plated on Sabouraud's dextrose agar (Difco Laboratories, Inc.) in duplicate and incubated at 37°C. Cryptococcal colonies were counted 2 d later, and the number of CFU was calculated on a per-organ basis.

Statistical analysis. Comparisons between group means were performed using an unpaired, two-tailed Student's t test; survival study comparisons were performed using Chi-square analysis (27). Statistical calculations were done using StatView 4.01 software (Abacus Concepts, Berkeley, CA). n = number of mice in each experimental group. Data are expressed ±SEM. Statistical difference was accepted at $P \leq 0.05$.

Results

Inflammatory cell recruitment in $uPA^{+/+}$ compared with $uPA^{-/-}$ mice in response to pulmonary C. neoformans infection. $uPA^{+/+}$ and $uPA^{-/-}$ mice were inoculated IT with *C. neoformans* (10^4 CFU), and the total number of inflammatory cells ($CD45^+$) present in lung minces was determined over time (Fig. 1). Although at baseline (time 0) there was no difference between the $uPA^{-/-}$ and $uPA^{+/+}$ mice, the recruitment of inflammatory cells over the ensuing 21 d was diminished in $uPA^{-/-}$ mice. The $uPA^{+/+}$ mice vigorously recruited inflammatory cells (day 7 compared with day 21; $P = 0.007$). In contrast, the $uPA^{-/-}$ mice had a distinct flattening of their recruitment curve after day 7 (day 7 compared with day 21, P = NS). The number of inflammatory cells present in $uPA^{+/+}$ mice was markedly greater than in the $uPA^{-/-}$ mice on day 21 (131.8 ± 12.3 vs $67.7 \pm 6.6 \times 10^6$ $CD45^+$ cells/lung, respectively; $P = 0.0001$). Thus, cellular recruitment in response to a pulmonary *C. neoformans* infection was critically dependent on the presence of uPA.

Since the $uPA^{-/-}$ and $uPA^{+/+}$ mice are a mixture of strains 129 and C57/B6, we sought to eliminate the possibility that strain differences contributed to the diminished recruitment of $CD45^+$ cells seen in the $uPA^{-/-}$ mice. Purebred 129 mice and purebred C57/B6 mice were inoculated in parallel with *C. neoformans* IT, and recruitment of inflammatory cells was assessed (Fig. 2). The recruitment of inflammatory cells in response to pulmonary *C. neoformans* infection is the same in strain 129 and strain C57/B6 mice ($CD45^+$ cells day 21; 129 compared with C57/B6; P = NS) Further, there is no difference in recruitment of $CD45^+$ cells on day 21 comparing either 129 or C57/B6 mice to the $uPA^{+/+}$ control mice (P = NS). Thus, the two parental background strains recruit inflammatory cells identically in response to pulmonary *C. neoformans* infection and therefore do not contribute to the diminished recruitment observed in the $uPA^{-/-}$ mice compared with the $uPA^{+/+}$ background-matched control mice.

Recruitment of leukocyte subsets to the pulmonary parenchyma in response to C. neoformans infection. The importance of the phagocyte in host defense against *C. neoformans* is well established (28, 29). Mononuclear phagocytes (MØs) are critically involved in the phagocytosis and intracellular killing of *C. neoformans,* and in the amplification of inflammatory and immune responses (29, 30). Phagocytosis of *C. neoformans* is largely dependent on complement receptor 3 (CR3, Mac1, CD11b/CD18; expressed on MØs and neutrophils) (31). We therefore assessed recruitment of $Mac1^+$ cells to the pulmonary parenchyma in response to IT inoculation with strain 52D

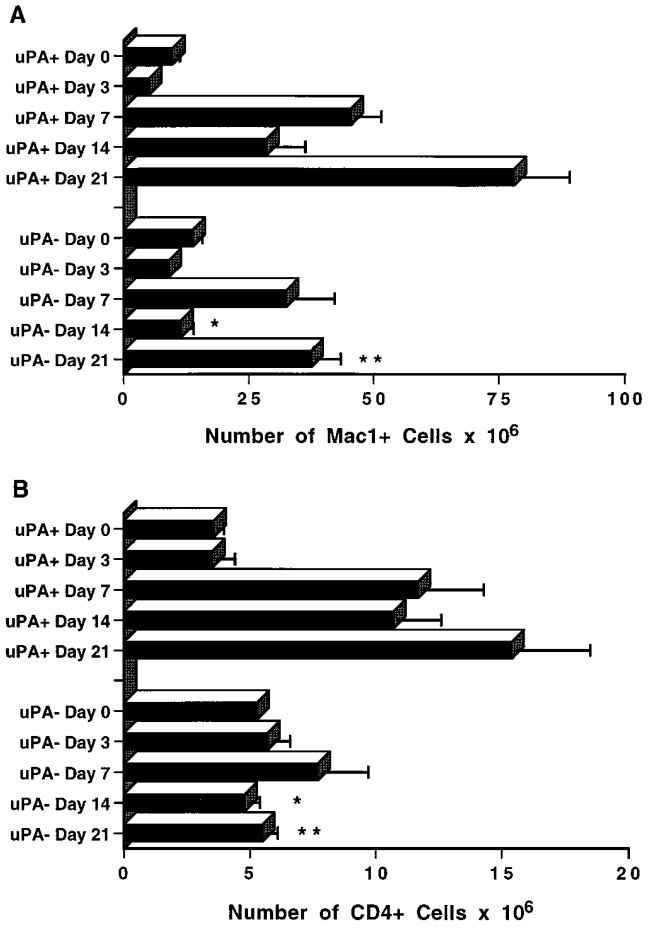

Figure 3. Recruitment of leukocyte subsets to the lungs of $uPA^{+/+}$ vs $uPA^{-/-}$ mice in response to pulmonary *C. neoformans* infection. (*A*) Recruitment of $Mac1^+$ cells to the lungs during pulmonary cryptococcal infection. Lungs were minced and enzyme digested to a single-cell suspension. Cells that were both $CD45^+$ and $Mac1^+$ were identified by double immunofluorescent flow cytometry. The data are expressed as millions of pulmonary $CD45^+/Mac1^+$ cells per mouse on the day of inoculation (day 0) and on postinoculation days 3, 7, 14, and 21; $*P = 0.05$; $**P = 0.006$, $n = 8$. (*B*) Recruitment of $CD4^+$ cells to the lungs during pulmonary cryptococcal infection. Lungs were minced and enzyme digested to a single-cell suspension. Cells that were both $CD45^+$ and $CD4^+$ were identified by double immunofluorescent flow cytometry. The data are expressed as millions of pulmonary $CD45^+/CD4^+$ cells per mouse on the day of inoculation (day 0) and on postinoculation days 3, 7, 14, and 21; $*P = 0.01$; $**P = 0.007$, $n = 8$.

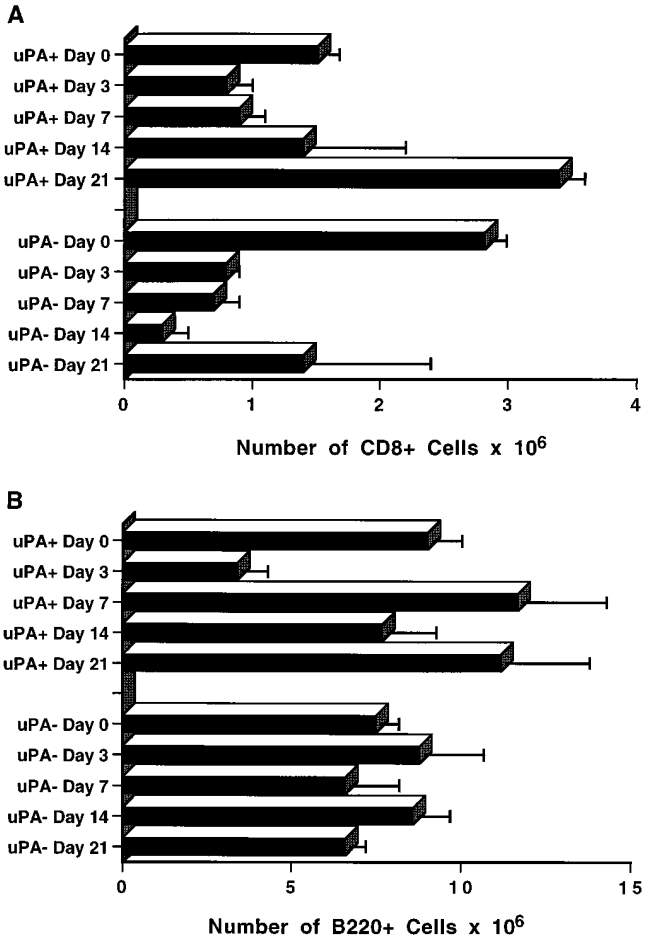

Figure 4. Recruitment of leukocyte subsets to the lungs of $uPA^{+/+}$ vs $uPA^{-/-}$ mice in response to pulmonary *C. neoformans* infection. (*A*) Recruitment of $CD8^+$ cells to the lungs during pulmonary cryptococcal infection. Lungs were minced and enzyme digested to a single-cell suspension. Cells that were both $CD45^+$ and $CD8^+$ were identified by double immunofluorescent flow cytometry. The data are expressed as millions of pulmonary $CD45^+/CD8^+$ cells per mouse on the day of inoculation (day 0) and on postinoculation days 3, 7, 14, and 21; comparison of $uPA^{+/+}$ to $uPA^{-/-}$ mice at day 21; P = NS, $n = 8$. (*B*) Recruitment of B cells ($B220^+$) to the lung during pulmonary cryptococcal infection. Lungs were minced and enzyme digested to a single-cell suspension. Cells that were both $CD45^+$ and $B220^+$ were identified by double immunofluorescent flow cytometry. The data are expressed as millions of pulmonary $CD45^+/B220^+$ cells per mouse on the day of inoculation (day 0) and on postinoculation days 3, 7, 14, and 21; comparison of $uPA^{+/+}$ to $uPA^{-/-}$ mice at day 21; P = NS, $n = 8$.

C. neoformans. As shown in Fig. 3 *A,* while $Mac1^+$ cell number was the same in $uPA^{-/-}$ and $uPA^{+/+}$ mice at baseline and on day 7 after IT inoculation, by day 14 $uPA^{-/-}$ animals had substantially fewer $Mac1^+$ cells than $uPA^{+/+}$ mice did ($11.5 \pm 2.5 \times 10^6$ vs 28.6 ± 7.7 10^6 cells, respectively; $P = 0.05$). This was even more evident by day 21 ($uPA^{-/-}$, $37.5 \pm 5.9 \times 10^6$ vs $uPA^{+/+}$, $78.0 \pm 11.1 \times 10^6$ cells; $P = 0.006$). The bimodal pattern of $Mac1^+$ cell recruitment seen in both $uPA^{-/-}$ and $uPA^{+/+}$ mice is consistent with sequential recruitment of neutrophils and MØs in response to pulmonary *C. neoformans* infection (24). Thus, recruitment of cells that constitute the first line of defense against pulmonary *C. neoformans* infection is markedly diminished in $uPA^{-/-}$ mice compared with $uPA^{+/+}$ controls.

Mice depleted of lymphocytes are unable to defend adequately against pulmonary *C. neoformans* infection (19, 32). We therefore sought to determine whether recruitment of specific leukocyte subsets were selectively inhibited in $uPA^{-/-}$ mice. Lymphocytes were identified by immunofluorescent staining with anti-CD4 or anti-CD8 mAbs to identify T cell subsets, or with B220 mAbs to identify B cells.

As shown in Fig. 3 *B*, recruitment of $CD4^+$ lymphocytes in response to *C. neoformans* infections was markedly reduced in the $uPA^{-/-}$ mice compared with $uPA^{+/+}$ mice. Whereas at baseline the $uPA^{-/-}$ mice had more $CD4^+$ cells than $uPA^{+/+}$ mice ($P = 0.004$), at days 3 and 7 after IT inoculation there was

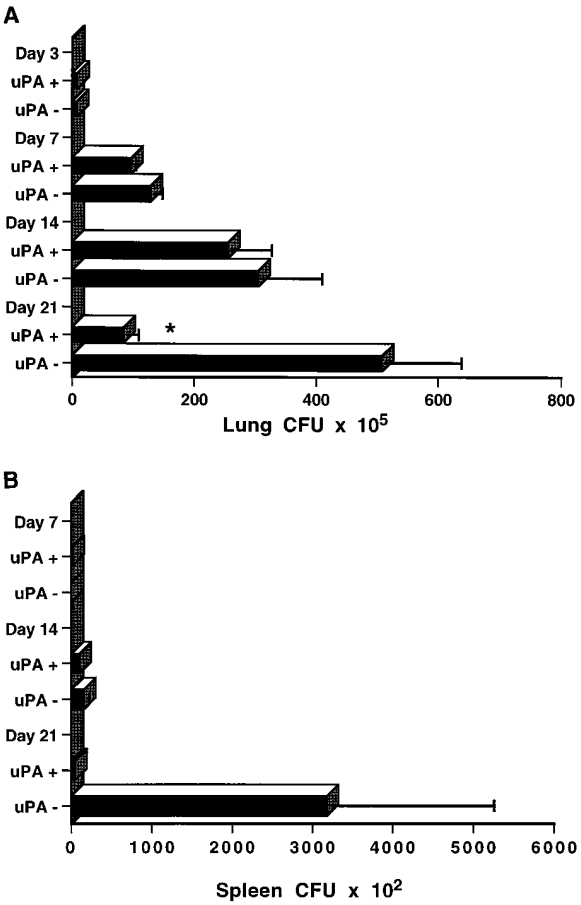

Figure 5. Containment vs dissemination of pulmonary *C. neoformans* infection in uPA$^{+/+}$ vs uPA$^{-/-}$mice. (*A*) Comparison of the number of lung CFUs in uPA$^{+/+}$ mice vs uPA$^{-/-}$ mice on postinoculation days 3, 7, 14, and 21 with strain 52D *C. neoformans*; *$P = 0.005$, $n = 11$. (*B*) Comparison of the number of spleen CFUs in uPA$^{+/+}$ mice vs uPA$^{-/-}$ mice on postinoculation days 7, 14, and 21 with strain 52D *C. neoformans*; comparison of uPA$^{+/+}$ to uPA$^{-/-}$ mice at day 21; P = NS, $n = 11$.

no difference between them. However, by day 14, uPA$^{-/-}$ mice had fewer CD4$^+$ cells than uPA$^{+/+}$ controls (4.8±0.6 vs 10.7±1.9 × 10^6, respectively; $P = 0.01$). This was even more evident by day 21, when the uPA$^{-/-}$ mice had profoundly diminished CD4$^+$ cells compared with uPA$^{+/+}$ controls (5.5±0.6 and 15.4±3.1 × 10^6, respectively; $P = 0.007$). Thus, not only is CD4$^+$ lymphocyte recruitment diminished, but it is diminished earlier in the inflammatory response to *C. neoformans* infection than seen in CD45$^+$ cellular recruitment. Since CD4$^+$ lymphocytes play a required role in *C. neoformans* defense, this suggests that the uPA$^{-/-}$ mice may have impaired abilities to combat *C. neoformans* infection effectively.

Although CD8$^+$ lymphocytes were increased in the uPA$^{-/-}$ mice at baseline ($P < 0.001$), CD8$^+$ lymphocyte recruitment tended to be less in uPA$^{-/-}$ mice compared with uPA$^{+/+}$ mice by day 21, but was variable and did not reach statistical significance in the time period studied (Fig. 4 *A*).

The pattern of diminished cellular recruitment was clearly not a generalized phenomenon. The pattern and magnitude of B cell recruitment (B220$^+$) was no different in the uPA$^{-/-}$ and uPA$^{+/+}$ mice, suggesting that the importance of uPA-mediated proteolysis in cellular recruitment may vary depending on the cell type (Fig. 4 *B*). Since uPA appears to mediate directional proteolysis by binding to cell surface-CD87 during cellular recruitment, and B cells lack CD87 expression, it is not surprising that the presence or absence of uPA was irrelevant to the recruitment of these cells (33).

Containment vs dissemination of C. neoformans infection in uPA$^{+/+}$ compared with uPA$^{-/-}$ mice. CFUs were quantitated in lung minces from the uPA$^{-/-}$ and uPA$^{+/+}$ mice (Fig. 5 *A*). Whereas there was no difference in lung CFUs comparing the uPA$^{-/-}$ and uPA$^{+/+}$ mice on days 7 and 14, the uPA$^{-/-}$ mice had markedly higher lung CFUs than uPA$^{+/+}$ mice did by day 21 (508.0±130.1 × 10^5 compared with 84.6±24.7 × 10^5; $P = 0.005$). Also, by day 21, it appeared that the uPA$^{-/-}$ mice were disseminating the *C. neoformans* infection to spleen (Fig. 5 *B*). Thus, whereas the number of CFUs in the lungs of uPA$^{+/+}$ mice was diminishing between days 14 and 21, the number of CFUs in the uPA $^{-/-}$ mice continued to increase. Further, the increased number of CFUs in the spleen on day 21 of the uPA$^{-/-}$ mice suggests the beginnings of dissemination of the organism.

Survival curves of C. neoformans–infected uPA$^{-/-}$ and uPA$^{+/+}$ mice. Based on the preceding data, we sought to determine whether the uPA$^{-/-}$ mice would disseminate *C. neoformans* to brain and die. We inoculated uPA$^{-/-}$ and uPA$^{+/+}$ mice in parallel with strain 52D *C. neoformans* and performed survival studies until day 85 after IT inoculation. 19 mice from each group were inoculated (Fig. 6). Three uPA$^{+/+}$ mice died before day 60, and the rest survived to day 85. In distinct contrast, there was a rapid die-off of the uPA$^{-/-}$ mice beginning at day 50. By day 85, all but four of the uPA$^{-/-}$ mice had died. The difference in survival between the uPA$^{+/+}$ and uPA$^{-/-}$ mice was highly significant at day 85 ($P < 0.0001$). At day 85, CFUs were determined in lung, spleen, and brain of all the surviving mice (Table I). In the uPA$^{+/+}$ mice, although there were a few CFUs in lung and spleen, no CFUs were found in the brain tissue of any of the 15 survivors. In distinct contrast, the four surviving uPA$^{-/-}$ mice had large numbers of CFUs in lung and spleen, and all had significant numbers of CFUs in brain tissue. Therefore, the uPA$^{-/-}$ mice are unable to combat a pulmonary *C. neoformans* infection adequately. These knockout mice disseminate the fungal pathogen and ultimately go on to

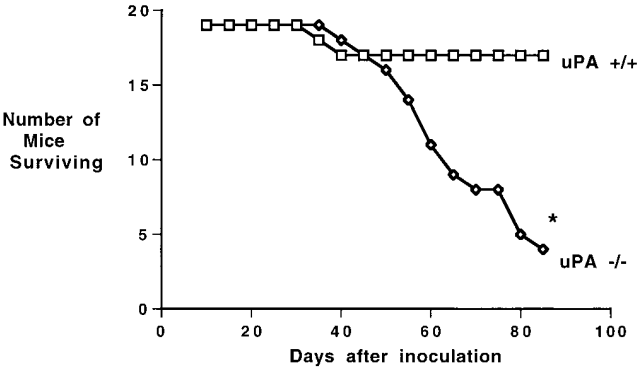

Figure 6. Survival curves of *C. neoformans*–infected uPA$^{-/-}$ and uPA$^{+/+}$ mice. 19 uPA$^{+/+}$ and 19 uPA$^{-/-}$ mice were IT inoculated with strain 52D *C. neoformans*. Survival curves were carried out to day 85. The difference in survival at day 85 between the uPA$^{+/+}$ and uPA$^{-/-}$ mice was significant; *$P < 0.0001$.

Table I. Organ CFU of uPA$^{+/+}$ and uPA$^{-/-}$ Mice Inoculated with C. neoforms Strain 52D

Mice	Organ CFU (mean±SEM)		
	Lung CFU	Spleen CFU	Brain CFU
	$\times 10^4$	$\times 10^4$	$\times 10^4$
uPA$^{+/+}$ ($n = 15$)	0.4±0.2	0.3±0.20	0.0±0.0
uPA$^{-/-}$ ($n = 4$)	47,575.0±18,811.7	58.0±36.4	125.0±58.7

die from fungal meningitis. Previously, this pattern has only occurred when classically immunodeficient mice (such as SCID) are inoculated with strain 52D.

Comparative pulmonary histology of uPA$^{+/+}$ and uPA$^{-/-}$ mice. To assess further the pulmonary inflammatory response to *C. neoformans*, hematoxylin and eosin–stained lung histology slides of uPA$^{+/+}$ and uPA$^{-/-}$ mice were compared at critical time points (at a magnification of 50). Mice were killed on days 7, 14, 24, and 52 to compare cellular recruitment and proliferation of *C. neoformans* within the pulmonary parenchyma. In Fig. 7, *A* and *B* represent postinoculation day-7 mice (*A,* uPA$^{+/+}$; and *B,* uPA$^{-/-}$). On day 7, the uPA$^{+/+}$ and uPA$^{-/-}$ mice both have scattered pockets of inflammatory cellular response throughout the lung fields. By day 14, (*C,* day 14 uPA$^{+/+}$; *D,* day 14 uPA$^{-/-}$), whereas the pattern is similar to that seen on day 7, the inflammatory cell recruitment appears to be greater in the uPA$^{+/+}$ mice than in the uPA$^{-/-}$ mice, especially in the peribronchial and perivascular areas. By day 24 (*E,* day 24 uPA$^{+/+}$; *F,* day 24 uPA$^{-/-}$), there is ongoing inflammation in the uPA$^{+/+}$ mice, with large numbers of inflammatory cells, whereas in the uPA$^{-/-}$ mice there is progressive accumulation of acellular inflammatory debris and evidence of ongoing proliferation of *C. neoformans* (pink staining, acellular, filling of alveolar spaces). At this stage of infection, the uPA$^{+/+}$ mice are beginning to decrease their pulmonary CFUs, whereas the uPA$^{-/-}$ mice are not (Fig. 5 *A*). By day 52 (*G,* day 52 uPA$^{+/+}$; *H,* day 52, uPA$^{-/-}$), we see that in the uPA$^{+/+}$ mice there is essentially complete restoration of normal pulmonary architecture. There is no evidence of inflammation, and the lung is free of *C. neoformans* organisms. In stark contrast, in the uPA$^{-/-}$ mice there are very few areas of normal lung left, and the lung tissue is nearly completely filled and obliterated with inflammatory debris and *C. neoformans* organisms. Fig. 8, *A* and *B*, shows day 52 uPA$^{+/+}$ and uPA$^{-/-}$ lungs, respectively, at a magnification of 200. In the uPA$^{+/+}$ mice, a normal alveolar capillary bed has been restored, and in the uPA$^{-/-}$ mice the alveo-

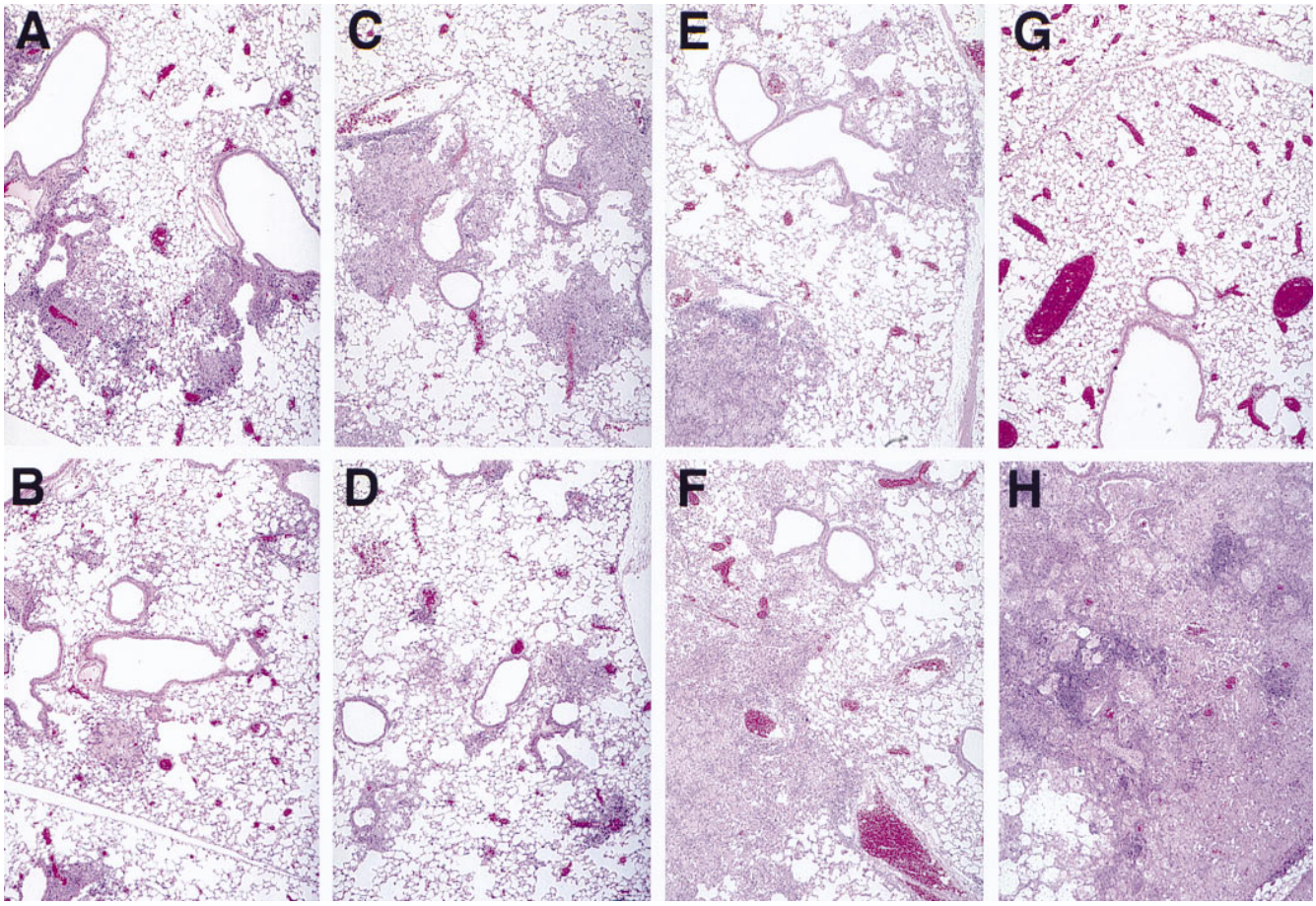

Figure 7. Comparative histology of uPA$^{+/+}$ and uPA$^{-/-}$ mice. Hematoxylin and eosin–stained lung histology slides of uPA$^{+/+}$ and uPA$^{-/-}$ mice were compared at low power (×50). (*A*) Postinoculation day 7, uPA$^{+/+}$; (*B*) postinoculation day 7, uPA$^{-/-}$; (*C*) postinoculation day 14, uPA$^{+/+}$; (*D*) postinoculation day 14, uPA$^{-/-}$; (*E*) postinoculation day 24, uPA$^{+/+}$; (*F*) postinoculation day 24, uPA$^{-/-}$; (*G*) postinoculation day 52, uPA$^{+/+}$; (*H*) postinoculation day 52, uPA$^{-/-}$.

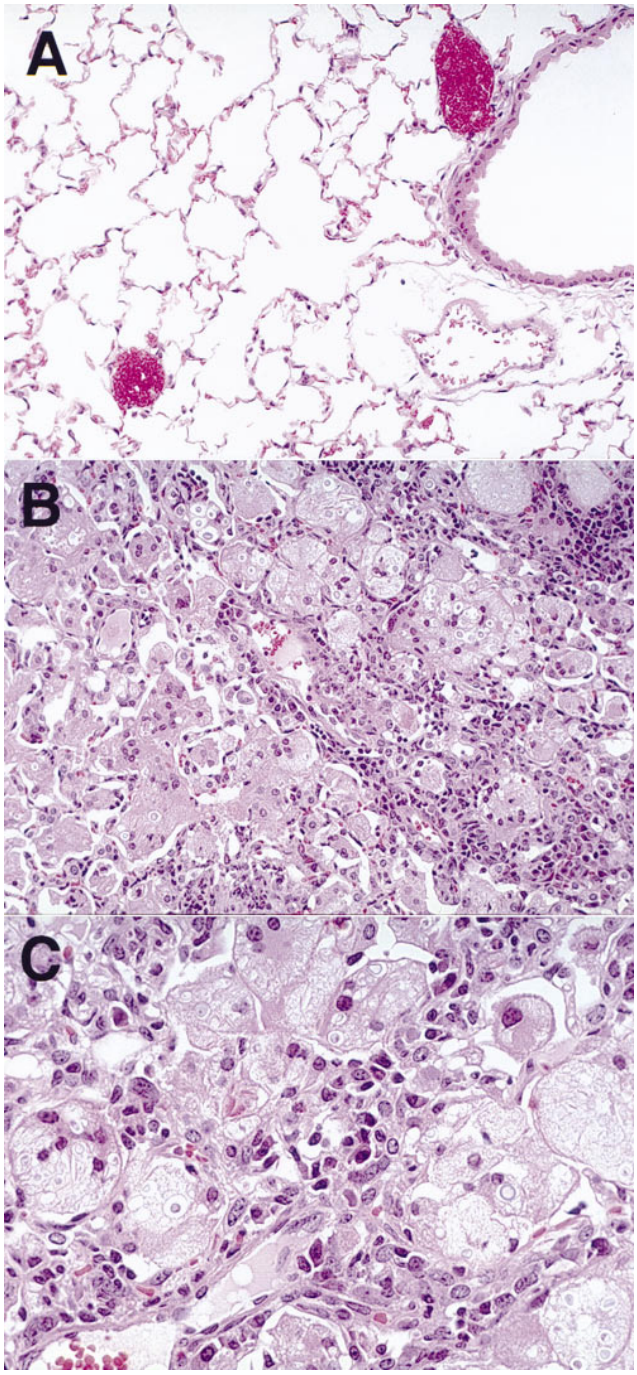

Figure 8. Comparative pulmonary histology of uPA$^{+/+}$ and uPA$^{-/-}$ mice. Hematoxylin and eosin–stained lung histology slides from uPA$^{+/+}$ and uPA$^{-/-}$ mice were compared. (*A*) Postinoculation day 52, uPA$^{+/+}$; (*B*) postinoculation day 52, uPA$^{-/-}$ (*A* and *B* at ×200); (*C*) postinoculation day 52, uPA$^{-/-}$ at ×400.

lar spaces are almost completely filled with *C. neoformans* and inflammatory debris. This is even more evident in *C*, at a magnification of 400. Inflammatory cells are relatively scant given the degree of infection, and they are largely comprised of MØs, lymphocytes, and plasma cells. There are multinucleated giant cells and alveolar macrophages packed with *C. neoformans* made obvious by their capsule. Further, within single alveolar macrophages there are multiple *C. neoformans* organisms, indicating inadequate killing and perhaps even intracellular replication.

Discussion

We show that uPA is required for protective cellular recruitment and host defenses against a clinically relevant pulmonary pathogen. In this study we demonstrate the following points: (*a*) In the absence of uPA, recruitment of inflammatory cells in response to pulmonary *C. neoformans* infection is markedly diminished. Recruitment of Mac1^{+} cells (neutrophils and MØs) and CD4^{+} cells is particularly blunted; (*b*) In the absence of uPA, pulmonary *C. neoformans* infection is not adequately combated; pulmonary CFUs continue to increase through day 21 of infection, and the infection disseminates to spleen and brain. (*c*) The lack of uPA has profound physiological consequences. The uPA$^{-/-}$ animals do not survive infection with strain 52D *C. neoformans* and die of meningitis by day 85 after IT inoculation. The uPA$^{+/+}$ survive. (*d*) Whereas, in uPA$^{+/+}$ mice, pulmonary *C. neoformans* infection is eliminated by day 52 and normal lung architecture is restored, the lung tissue of uPA$^{-/-}$ mice is virtually completely filled with debris, proliferating *C. neoformans* organisms, and ongoing destruction.

Several aspects of the animal model used in this study are worthy of mention. First, the uPA$^{-/-}$ are phenotypically normal and, when housed under specific pathogen free conditions, live normal life spans. Because unchallenged uPA$^{-/-}$ animals are phenotypically indistinguishable from background controls, evaluation of host defenses over time can be evaluated from a common, uncompromised initiation point, allowing us to test the requirement for uPA during infectious challenge with greater precision. Second, the lack of uPA is specific, absolute, and irreversible. The uPA$^{-/-}$ mice lack uPA on the genomic, mRNA, protein, and enzyme activity levels (14). The expression of other genes, including tissue type plasminogen activator, is unaffected by the elimination of the native uPA gene (14). Thus, the role of uPA can be evaluated specifically without interfering with the activity of other proteases by using potentially nonspecific inhibitors. Third, we use a pathogen where adequate host defense is dependent upon MØs and lymphocytes, and where IT inoculation results in a massive recruitment of MØs, neutrophils, and lymphocytes to the lung over a predictable time period (24). And, finally, the model provides several clearly defined, measurable end points to evaluate the adequacy of host defense: (*a*) the number of cells of specific leukocyte subsets recruited to the lung; (*b*) the limitation or progression (and dissemination) of *C. neoformans* infection in the host, as measured by CFU in lung, spleen, and brain; and (*c*) the survival of the infected animals.

By using mice that genetically lack uPA expression, we have shown that uPA is required for inflammatory cell recruitment in response to pulmonary infection but is not involved in innate, resident defenses. There was a marked decrease in the number of inflammatory cells recruited in the uPA$^{-/-}$ mice compared with the uPA$^{+/+}$ mice in response to pathogen challenge by day 21 after inoculation. This could not be explained by a differential mix of the two background strains used in the development of these mice. Thus, although resident inflammatory cells are present equally in the uPA$^{-/-}$ and uPA$^{+/+}$ mice, recruitment of inflammatory cells in response to *C. neoformans* infection cannot occur in the absence of uPA.

Although some malignant cells use uPA/plasmin to invade (12, 34–36), no studies have previously addressed the invasive potential of inflammatory cells in vivo, particularly in the context of an active, physiologically relevant infection. Because inflammatory cells express other matrix-degrading enzymes in addition to uPA (37–39), it was previously unclear whether uPA was critically involved.

The $uPA^{-/-}$ mice had diminished recruitment of MØs, neutrophils, and T lymphocytes. Although the recruitment of these cells requires the elaboration of multiple different chemotactic signals (40), and these cells have disparate functions in host defenses, they share a common characteristic. MØs, neutrophils, and activated T lymphocytes express CD87, a specific high-affinity receptor for uPA (7, 8, 33). In response to a chemotactic gradient, monocytes tightly cluster CD87 at the leading edge of migration (9), and neutrophils rapidly mobilize pools of CD87 on their cell surface (8). Thus, both MØ and neutrophils use CD87 as a way to focus uPA, which this study demonstrates is required for their recruitment to infected lung.

uPA may also be involved in MØ activation. The numerous intracellular organisms seen in the day 52 $uPA^{-/-}$ mice suggest that, while phagocytosis occurs despite the lack of uPA, intracellular killing of *C. neoformans* is inadequate (Fig. 8 *C*). We hypothesize that a lack of uPA prevents alveolar macrophages from becoming appropriately activated. Our previous work, demonstrating that MØ-TNF transcription requires uPA, supports the hypothesis that uPA modulates activating cytokines critical for fungal killing (41).

Whereas it is known that activated (but not resting) $CD3^+$ cells express CD87, the role of uPA in lymphocyte-mediated proteolysis and recruitment has remained obscure (33).

It is possible that the role uPA plays in lymphocyte recruitment is not limited to enhancing matrix proteolysis during cellular migration. uPA may be important in modulating the cytokine networks that trigger lymphocyte recruitment and activation during the inflammatory response. uPA has been implicated in the release of active IL-1 from MØs (42). IL-2 expression is inhibited in the presence of serine protease inhibitors, a finding that suggests that serine proteases such as uPA or plasmin are involved (43). Diminished production of IL-2 results in inadequate lymphocyte proliferation and therefore may account for inadequate host defenses in the $uPA^{-/-}$ mice. Further, IL-2 stimulation upregulates CD87 expression by T cells (33). Therefore, IL-2 not only activates T cells but also endows them with the ability to focus uPA proteolytic activity during entry into inflammatory foci. Thus, at least two uPA-mediated processes, cell activation and proteolytic capacity, may be impaired in the $CD4^+$ cell population of the $uPA^{-/-}$ mice.

In contrast, B cell recruitment was unaffected by the absence of uPA. This is fully consistent with the lack of CD87 expression by B cells and thus serves as a confirming contrast for our findings in leukocytes that express CD87 either constitutively or when activated by inflammatory mediators (33).

The altered host defense seen in the $uPA^{-/-}$ mice has substantial physiologic consequences. By day 21, there was a marked reduction in the pulmonary cryptococcal burden in the $uPA^{+/+}$ mice, demonstrating containment and control of the infection. In contrast, the $uPA^{-/-}$ mice continued to have substantial increases in pulmonary CFUs on day 21. *C. neoformans* infection disseminates in the $uPA^{-/-}$ mice, resulting in markedly dissimilar survival curves compared with $uPA^{+/+}$ mice. Whereas the $uPA^{+/+}$ mice survived infection with *C. neoformans* and at autopsy (day 85) had no *C. neoformans* in their brains, the $uPA^{-/-}$ mice showed a substantial death rate by day 52 (15/19), and the four survivors had extraordinarily high burdens of *C. neoformans* present in their brain tissue (Table I).

C. neoformans has several characteristics that are classic for infection with an intracellular organism, (*a*) The organism is of low toxicity and results in a prolonged course of disease; (*b*) host defense requires macrophage activation and (*c*) is T cell dependent, but (*d*) is antibody independent (44). As the population of immunocompromised patients afflicted by intracellular infections has increased, investigation has been directed to the elucidation of the cellular requirements these patients lack for adequate host defenses. However, although these organisms are considered opportunistic, a substantial number of apparently normal hosts are vulnerable and are unable to combat infection (45). Identification of the immunologic defect(s) in these patients remains an ongoing dilemma. Our data demonstrate that a previously unconsidered effector molecule, uPA, is involved in normal host defense against an intracellular pathogen. Since potency of the fibrinolytic system is known to vary substantially among patients (46, 47) and even a complete lack of uPA does not appear to exert negative selection pressure (14), it is possible that inadequate or dysregulated uPA expression may play a role in the vulnerability of these so-called normal hosts.

This is the first study showing a requirement for uPA for the generation of an adequate in vivo inflammatory response. We have demonstrated that uPA is required for normal pulmonary host defenses against *C. neoformans*. The absence of uPA results in the inadequate recruitment of neutrophils, MØs, and $CD4^+$ lymphocytes to the pulmonary parenchyma. The diminished recruitment of the cells most critically involved in host defenses against *C. neoformans* results in the proliferation of the organism within the lung, and wide dissemination. Whereas uPA-replete mice recover completely from pulmonary *C. neoformans* infection and restore normal lung architecture, the uPA-deficient mice die from *C. neoformans* infection, exhibit ongoing destruction of lung parenchyma, and intrapulmonary proliferation of the organism. We conclude that uPA plays a critical role in protective pulmonary inflammatory responses.

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