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Regulatory Effects of Macrophage Inflammatory Protein 1α/CCL3 on the Development of Immunity to *Cryptococcus neoformans* Depend on Expression of Early Inflammatory Cytokines

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Macrophage inflammatory protein 1α (MIP-1α)/CCL3 prevents the development of eosinophilic pneumonia (EP) driven by a nonprotective T2-type immunity during infection with a highly virulent strain of *Cryptococcus neoformans*. The present study evaluated the interaction of MIP-1α with other innate immune system cytokines by comparing the immune responses that followed pulmonary infections with high- (*C. neoformans* 145A) and low (*C. neoformans* 52D)-virulence strains. In contrast to what was found for *C. neoformans* 145A infection, lack of MIP-1α in *C. neoformans* 52D infection did not cause the development of EP. *C. neoformans* 52D induced tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and MCP-1 in the lungs of infected wild-type (WT) and MIP-1α knockout (KO) mice by day 7 postinfection. Both WT and MIP-1α KO mice subsequently cleared this infection. Thus, the robust expression of early inflammatory cytokines in *C. neoformans* 52D-infected mice promoted the development of protective immunity even in the absence of MIP-1α. Alternatively, *C. neoformans* 145A-infected WT and MIP-1α KO mice had diminished TNF-α, IFN-γ, and macrophage chemoattractant protein 1 (MCP-1) responses, indicating that virulent *C. neoformans* 145A evaded early innate host defenses. However *C. neoformans* 145A-infected WT mice had an early induction of MIP-1α and subsequently did not develop EP. In contrast, *C. neoformans* 145A-infected MIP-1α KO mice developed EP and had increased *C. neoformans* dissemination into the brain by day 35. We conclude that, in the absence of other innate immune response effector molecules, MIP-1α is crucial to prevent the development of EP and to control *C. neoformans* dissemination to the brain.

Cryptococcus neoformans is an encapsulated yeast that can cause persistent and often fatal opportunistic infections (3). *C. neoformans* infection occurs via the respiratory route; in the absence of appropriate clearance mechanisms the infection disseminates from the lung into internal organs, particularly the brain (3). The development of a protective T-cell-mediated immunity is required for clearance of *C. neoformans* (3).

Clinical isolates of *C. neoformans* in experimental murine infections can be classified into three categories: low virulent, moderately virulent, and highly virulent. Infection of immunocompetent mice with low or moderately virulent strains of *C. neoformans* such as 184A, YC-13, and 52D results in clearance (2, 6, 10, 14, 18, 32). This clearance is associated with a prominent inflammatory response in the lung and the development of strong T1-driven specific immunity. Infection of immunocompetent mice with highly virulent *C. neoformans* strains such as 145A, YC-11, and NU-2 results in a chronic infection (2, 6, 14, 23–25). In these cases the inflammatory response in the lung is reduced or delayed (2, 6, 14, 24). In addition, immune system deviation leading to the development of nonprotective eosinophilic pneumonia (EP) occurs when susceptible mice (C57BL/6, CC chemokine receptor 2 [CCR2] knockout [KO], or gamma interferon [IFN-γ]- and interleukin-12 [IL-12]-neutralized mice) are infected with *C. neoformans* (12, 13, 31) Thus, persistence of the *C. neoformans* infection is associated either with minimal immune responses or with the deviation of the immune response leading to EP.

In light of the latter observation, it is not clear why immunocompetent mice infected with highly virulent strains do not develop EP in response to infection (2, 6, 11, 14, 25). However, EP does develop in macrophage inflammatory protein 1α (MIP-1α) KO mice infected with highly virulent *C. neoformans* (27). MIP-1α KO mice developed a nonprotective T2 pulmonary immune response following infection with highly virulent *C. neoformans* strain 145A. This infection resulted in (i) EP, (ii) increased pulmonary IL-4 and IL-13 expression, (iii) highly elevated immunoglobulin E levels, (iv) severe lung pathology, and (v) dramatically increased mortality (27). These recent data demonstrate that, just like IFN-γ, CC chemokine MIP-1α plays a role in preventing the development of nonprotective EP.

There is accumulating evidence from other systems that MIP-1α and other chemokines and chemokine receptors can regulate the development and polarization of immune responses. MIP-1α is a classic CC chemokine, more recently designated CCL3 (33). MIP-1α has been shown to (i) promote chemotaxis of Th1, but not Th2, cell lines in vitro (30), (ii) promote differentiation of T-cell receptor transgenic Th0 cells

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to Th1 cells in vitro (19), and (iii) decrease IL-4 production from cultured Th2-type lymphocytes stimulated with schistosomal egg antigen (20, 26). Anti-MIP-1α antibodies inhibit the development of T1-mediated experimental autoimmune encephalitis (19, 21). Thus, MIP-1α can be classified as a factor that is associated with T1 immune responses and that prevents T2-type responses.

The interaction of MIP-1α with other inflammatory cytokines, such as IFN-γ, tumor necrosis factor alpha (TNF-α), and macrophage chemoattractant protein 1 (MCP-1), in the regulation of immunity to infection has not been studied. Infection by high- and low-virulence strains of *C. neoformans* provides a useful set of tools to study the interaction of MIP-1α with other inflammatory cytokines in driving T-cell-mediated responses to infection. Our objective was to assess the role of MIP-1α in the presence and absence of inflammatory signals in preventing the development of EP.

MATERIALS AND METHODS

Mice. Wild type (WT) mice (B6129F2/J; Jackson Laboratories, Bar Harbor, Maine) and MIP-1α KO mice (B6129-Scya3^{tm1Coo}) were used for these studies (5). These mice were housed under specific-pathogen-free conditions in enclosed filter top cages at the University of Michigan Laboratory Animal Facility. Clean food and water were given ad libitum. The mice were handled and maintained using microisolator techniques with daily veterinarian monitoring. The MIP-1α KO mice lack a promoter region, as well as exon 1 and part of exon 2, of the MIP-1α gene (5). Mice were 8 to 16 weeks of age at the time of infection. There were no age-related differences in the responses of these mice to *C. neoformans* infection.

Cultures of *C. neoformans*. Highly virulent *C. neoformans* strain 145A (ATCC 62070) and low-to-moderately virulent strain 52D (ATCC 24067) were obtained from the American Type Culture Collection (Manassas, Va.) (17). Both strains were isolated originally from cerebrospinal fluid of infected human patients, and both are encapsulated and laccase and urease positive; they have similar rates of in vitro growth. For infection, yeast was grown to stationary phase (at least 72 h) at 36°C in Sabouraud dextrose (SD) broth (1% neopeptone, 2% dextrose; Difco, Detroit, Mich.) on a shaker. The cultures were then washed in nonpyrogenic saline (Travenol, Deerfield, Ill.), counted on a hemocytometer, and diluted to 3.3×10^5 yeast cells/ml in sterile nonpyrogenic saline.

Intratracheal inoculation of *C. neoformans*. Mice were anesthetized by intraperitoneal injection of pentobarbital (0.074 mg/g of body weight; Butler, Columbus, Ohio) and restrained on a small board. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A bent 30-gauge needle (Becton Dickinson, Rutherford, N.J.) was attached to a tuberculin syringe (BD & Co., Franklin Lakes, N.J.) filled with the diluted *C. neoformans* culture. The needle was inserted into the trachea, and 30 μl of inoculum was dispensed into the lungs (10^4 yeast cells). The skin was closed with cyanoacrylate adhesive. The mice recovered with minimal visible trauma. Aliquots of the inoculum were collected periodically and plated on SD agar to monitor the number of CFU being delivered.

Preparation of lung leukocytes. The lungs from each mouse were excised, washed in phosphate-buffered saline, minced with scissors, and digested enzymatically at 37°C for 30 min in 15 ml of digestion buffer (RPMI 1640, 5% fetal calf serum, antibiotics, 1 mg of collagenase [Boehringer Mannheim Biochemical, Chicago, Ill.]/ml, 30 μg of DNase [Sigma]/ml)/lung. The cell suspension and tissue fragments were further dispersed by repeated aspiration through the bore of a 10-ml syringe and centrifuged. Erythrocytes in the pellets were lysed by addition of 3 ml of NH_4Cl buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, 0.0372% Na_2-EDTA, pH 7.4) for 3 min, followed by a 10-fold excess of RPMI 1640. Cells were resuspended again, and a second cycle of syringe dispersion and filtration through a sterile 100-μm-pore-size nylon screen (Nitex, Kansas City, Mo.) was performed. The filtrate was centrifuged for 25 min at $1,500 \times g$ in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Leukocyte pellets were resuspended in 10 ml of complete media and enumerated in a hemocytometer following dilution in trypan blue. Leukocyte recruitment was calculated using the following formula: recruited leukocytes in an infected mouse = total number of leukocytes in the infected mouse − mean number of leukocytes in uninfected mice. Values of leukocyte recovery from uninfected WT and MIP-1α KO mice were $(23.26 \pm 1.78) \times 10^6$ leukocytes ($n = 18$) and $(24.36 \pm 1.81) \times 10^6$ leukocytes ($n = 16$), respectively.

Assessment of leukocyte populations. For the differential count of lung cell suspensions, samples were cytospun (Shandon Cytospin, Pittsburgh, Pa.) onto glass slides and stained by a modification of the Diff-Quik whole-blood stain (VWR Scientific Products). Samples were fixed and prestained for 2 min in a one-step methanol-based Wright-Giemsa stain (Harleco, EM Diagnostics, Gibbstown, N.J.), rinsed in water, and stained using steps 2 and 3 of the Diff-Quik stain procedure. This modification of the Diff-Quik stain procedure improves the resolution of eosinophils from neutrophils in the mouse. A total of 200 to 400 cells were counted for each sample from randomly chosen, high-power microscope fields.

Lung and brain CFU assays. Aliquots of the lung digest solutions (prior to the first centrifugation) were collected for lung CFU assays. Dissected brains were homogenized in 2 ml of sterile water. Lung and brain suspensions were serially diluted in sterile water. Dilution samples (10 μl each) were plated on SD agar and incubated at room temperature for 48 h. Colony counts were performed and adjusted to reflect the total lung and brain CFU.

Histology. Lungs were fixed by inflation with 1 ml of 10% neutral buffered formalin, excised en bloc, and immersed in neutral buffered formalin. Brains were carefully excised and immersed in neutral buffered formalin. After paraffin embedding, 5-μm-thick sections of both organs were cut and stained with hematoxylin and eosin. Sections were analyzed with light microscopy.

Detection of cytokine mRNA by RT-PCR. Whole lungs were removed, homogenized in TRIzol reagent (Gibco BRL, Gaithersburg, Md.), extracted as outlined in the TRIzol protocol, and precipitated with isopropanol. The RNA was washed with 70% ethanol, dissolved in nuclease-free H_2O, and quantified by UV spectrophotometry using absorbance at 260 nm. One-step reverse transcriptase PCR (RT-PCR; Access RT-PCR kits; Promega) on equal aliquots of RNA was performed in accordance with the manufacturer's protocol. Three fivefold dilutions of the RNA product (1, 0.2, and 0.04 μg) were used for the RT-PCR to control for possible overamplification of the cDNA in the samples. The oligonucleotide primers for PCRs were as follows: for β-actin, 5′-GTG-GGC-CGC-TCT-AGG-CAC-CA-3′ (sense) and 5′-CTC-AGC-TGT-GGT-GGT-GAA-GC-3′ (antisense); for TNF-α, 5′-AGC-ACA-GAA-AGC-ATG-ATC-CGC-G-3′ (sense) and 5′-GAC-TTT-CTC-CTG-GTA-TGA-GAT-AGC-3′ (antisense); for IFN-γ, 5′-GGC-TGT-TTC-TGG-CTG-TTA-CTG-CCA-CG-3′ (sense) and 5′-GAC-AAT-CTC-TTC-CCC-ACC-CCG-AAT-CAG-3′ (antisense); for MIP-1α, 5′-AAG-GTC-TCC-ACC-ACT-GCC-CTT-G-3′ (sense) and 5′-CTC-AGG-CAT-TCA-GTT-CCA-GGT-C-3′ (antisense).

The number of PCR cycles for β-actin was 25, whereas the cycle number for MIP-1α, IFN-γ, and TNF-α was 35. Annealing temperatures were as follows: 55°C for β-actin, TNF-α, and MIP-1α and 63°C for IFN-γ. RT-PCR products were electrophoresed and visualized by ethidium bromide staining. The sizes of the RT-PCR products were confirmed by comparison with a 100-bp ladder run in parallel on the same gel. RT-PCR products were transferred from the gels onto a Zeta Probe blotting membrane (Bio-Rad, Hercules, Calif.) for approximately 2 h on a vacuum blotter (model 735; Bio-Rad). DNA was cross-linked to the membranes in a UV Stratalinker (model 1800; Stratagene). Specific DNA products were detected via hybridization with labeled internal probes for DNA products of interest: for β-actin, 5′-GGG-ACG-ACA-TGG-AGA-AGA-TCT-GG-3′; for TNF-α, 5′-CCT-GTA-GCC-CAC-GTC-GTA-GC-3′; for IFN-γ, 5′-CAG-CGA-CTC-CTT-TTC-CGC-TT-3′; for MIP-1α, 5′-GTC-AAC-GAT-GAA-TTG-GCG-TGG-AAT-C-3′. The presence of specific DNA products was determined via exposure of the blots on X-ray film.

MCP-1 and IFN-γ detection in BAL fluid. Bronchoalveolar lavage (BAL) fluid was obtained via a 3-mm-diameter Teflon catheter inserted directly into the exposed trachea of the mouse directly after euthanasia. The catheter was secured with surgical silk, and two 1-ml washes of ice-cold, sterile PBS were instilled into the lungs and withdrawn. BAL fluid was transferred into polyethylene microtubes and frozen until tested. Cytokines in BAL fluid were quantified using a murine enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen). Reactions were performed in 96-well ELISA plates (Costar) in accordance with the manufacturer's instructions. The optical densities (ODs) were read on a microplate reader (Ultra Micro EL 808; Biotek Instruments) at a wavelength of 450 nm. The cytokine content in each well was estimated by interpolation of sample OD values with an appropriate standard by a four-parameter curve-fitting program.

Calculations and statistics. Data (means ± standard errors [SE]) for each experimental group were derived from at least two independent infections (of compared groups of animals) and analyzed via *t* test or either one-way or two-way analysis of variance, depending on the number of groups. For individual comparisons of multiple groups, a post hoc test for simple main effects was used to calculate *P* values. Means with *P* values <0.05 were considered statistically significant.

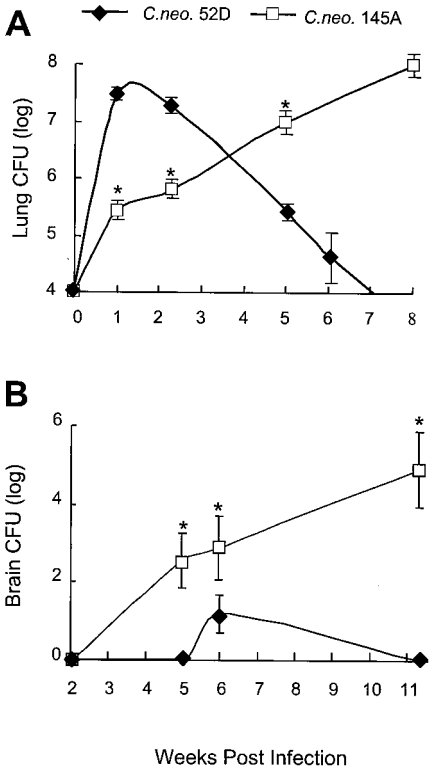

FIG. 1. Comparison of the growth of *C. neoformans* 52D and 145A in lungs (A) and brains (B). B6129F2/J mice were infected intratracheally with *C. neoformans* 52D or *C. neoformans* 145A (10^4 CFU) and analyzed ($n = 5$ to 22 per time point per group). Data pooled from separate matched experiments are expressed as the mean CFU per lung or brain ± SE. Asterisk, $P < 0.05$ in comparison with *C. neoformans* 52D-infected group.

RESULTS

Pulmonary growth and clearance of *C. neoformans* in infections with low- (52D) versus high (145A)-virulence strains. It was important to first determine the dynamics of pulmonary growth of *C. neoformans* 52D and *C. neoformans* 145A in B6129F2/J, i.e., WT mice, prior to our studies using MIP-1α KO mice. Rapid growth of *C. neoformans* 52D within the lung occurred over the first week of infection, resulting in a nearly 1,000-fold increase in lung CFU over the inoculum dose (Fig. 1A). This rapid phase of *C. neoformans* growth in the lung was followed by a gradual decrease over a period of several weeks and subsequent clearance of *C. neoformans* 52D from the lungs. In contrast, *C. neoformans* 145A proliferated in the lung less vigorously, approaching a 30-fold increase over the inoculum during the first week of infection (Fig. 1A). However, it continued to grow progressively during the 7 weeks of infection, approaching 10^8 CFU at day 70 (10,000-fold increase). Thus, pulmonary infection in B6129F2/J mice with *C. neoformans* 52D results in the development of a protective response and clearance, while pulmonary infection with *C. neoformans* 145A is chronic and characterized by progressive pulmonary growth of cryptococci.

Pathology of *C. neoformans* 52D versus *C. neoformans* 145A infections. We performed histological examination of lungs 11 to 12 weeks postinfection to compare the long-term pulmonary infections by *C. neoformans* 52D and *C. neoformans* 145A. Consistent with the CFU analysis, *C. neoformans* 52D was no longer present in the lungs by 12 weeks and almost all of the inflammatory response had resolved in the lungs. Lung histology of infected animals resembled lung histology of uninfected animals. Histological features of the repair process—groups of fibroblasts surrounded by modest mononuclear cell infiltration—were noted. At the same time point, lungs of mice infected with *C. neoformans* 145A contained large numbers of cryptococci, which formed large nodular clusters and masses. These *C. neoformans* clusters were surrounded by walls of mononuclear cell infiltrates forming diffuse granulomas containing large cryptococci. Thus, while *C. neoformans* 52D is entirely cleared from lungs by week 12, leaving only minimal pathological changes, *C. neoformans* 145A progressively grows in the lungs, resulting in chronic inflammation and focal destruction of the lung tissue.

Differential capacity for cerebral dissemination of *C. neoformans* strains. The capacity for cerebral dissemination of both strains of *C. neoformans* was evaluated. *C. neoformans* 52D was detected only at 6 weeks postinfection in two of eight animals. All remaining WT mice infected with *C. neoformans* 52D had no *C. neoformans* in the brain at any time point analyzed (Fig. 1B). Cerebral dissemination began between weeks 2 and 5 in animals infected with *C. neoformans* 145A. Cryptococci were cultured from the brains of 50% of the mice at 5 weeks. The mean brain CFU, as well as the percentage of mice with positive brain cultures, increased progressively after this time point (Fig. 1B). Thus, in contrast with *C. neoformans* 52D, *C. neoformans* 145A rapidly disseminates into the central nervous system (CNS) and establishes a permanent CNS infection.

Pulmonary recruitment of leukocytes. To determine if the differences in clearance were caused by differential host responses to these strains of *C. neoformans*, we analyzed pulmonary recruitment of leukocytes. Pulmonary leukocyte recruitment in mice infected with *C. neoformans* 145A was delayed compared with recruitment in mice infected with *C. neoformans* 52D (Fig. 2A). The numbers of leukocytes in the two groups were similar by day 35 postinfection. Significant clearance of *C. neoformans* 52D had occurred by this time point, and pulmonary leukocyte numbers were already declining in these mice. Thus, infection with strain 145A produced a much less vigorous and delayed cellular response.

Characterization of pulmonary leukocyte subsets. To determine if qualitative or quantitative differences in pulmonary leukocyte recruitment occurred, we performed analysis of cell subsets recruited into the lungs at 2 weeks postinfection. Leukocyte recruitment in the *C. neoformans* 145A-infected group was only about 28% of the recruitment in *C. neoformans* 52D-infected groups; thus recruitment of each individual cell type was significantly higher in the latter. Therefore, we compared the relative numbers of pulmonary leukocytes expressed as percentages. The percentages of eosinophils and lymphocytes were similar in both types of infection. We observed a higher proportion of neutrophils to macrophages in *C. neoformans* 52D-infected animals than in mice infected with *C. neoformans* 145A (Fig. 2B), indicating that *C. neoformans* 52D-infected mice developed a more pronounced acute inflammatory response.

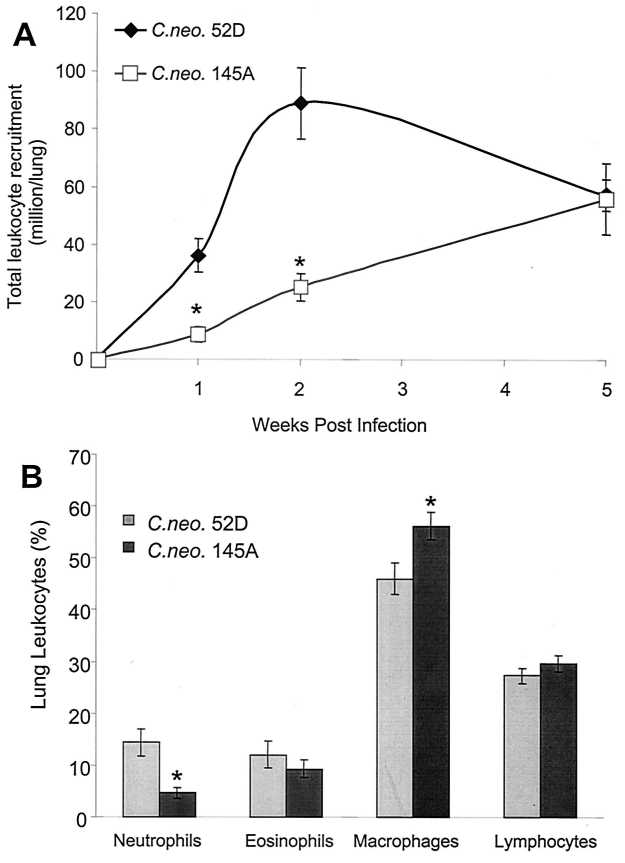

FIG. 2. Comparison of pulmonary leukocyte recruitment in mice infected with *C. neoformans* 52D or 145A. Leukocytes were isolated from infected lungs following enzymatic dispersion of whole lungs (see Materials and Methods). (A) Lung leukocyte recruitment at weeks 1, 2, and 5 postinfection was calculated by subtraction of the mean number of leukocytes in uninfected mice from the total number of leukocytes in infected animals. (B) Relative composition of leukocyte subsets in the lungs 2 weeks postinfection. Data (means ± SE) are percentages of subsets in the samples of total lung leukocyte isolates. Asterisk, $P < 0.05$ in comparison with *C. neoformans* 52D-infected group ($n = 18$ uninfected mice and $n = 5$ to 22 infected mice per time point per group).

Comparison of the proinflammatory cytokine profiles. We next analyzed early expression of the proinflammatory cytokines, because leukocyte recruitment was delayed in lungs infected with *C. neoformans* 145A compared with that in lungs infected with *C. neoformans* 52D. By day 7, *C. neoformans* 52D-infected mice demonstrated robust induction of TNF-α, IFN-γ, and MIP-1α in their lungs (Fig 3). In mice infected with *C. neoformans* 145A, expression of TNF-α was absent in the lungs; the expression of IFN-γ was dramatically reduced (Fig. 3). Interestingly, MIP-1α was induced following infection by both strains of *C. neoformans*. Thus, mice infected with *C. neoformans* 52D demonstrated strong induction of TNF-α, IFN-γ, and MIP-1α by day 7 of infection, while mice infected with *C. neoformans* 145A expressed only MIP-1α.

Comparison of MIP-1α effects on the development of EP in infections with *C. neoformans* 52D versus *C. neoformans* 145A. We reported previously that infection of MIP-1α KO mice with *C. neoformans* 145A caused a switch to a T2-type immune

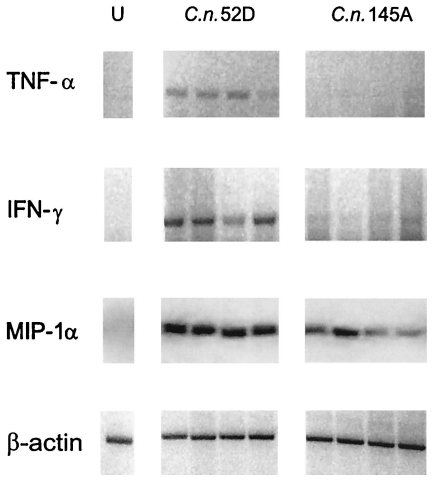

FIG. 3. Comparison of pulmonary TNF-α, IFN-γ, and MIP-1α gene expression in WT mice infected intratracheally (*C. neoformans* 52D or *C. neoformans* 145A; 10^4 CFU). Total lung RNA was isolated from the uninfected mice (U) on day 7 postinoculation. Expression of cytokine mRNA was analyzed via RT-PCR. The cytokine PCR products were confirmed by Southern blotting with specific DNA probes.

response, resulting in EP, immunoglobulin E production, and subsequent lung pathology. To determine if MIP-1α prevents the development of EP during development of immune responses to *C. neoformans* 52D, we analyzed eosinophil recruitment in *C. neoformans* 52D-infected WT and MIP-1α KO mice 2 weeks postinfection. Interestingly, the absence of MIP-1α did not result in increased eosinophil influx into the lungs of MIP-1α KO mice compared with WT mice (Fig 4). However, consistent with our previous report, we observed the develop-

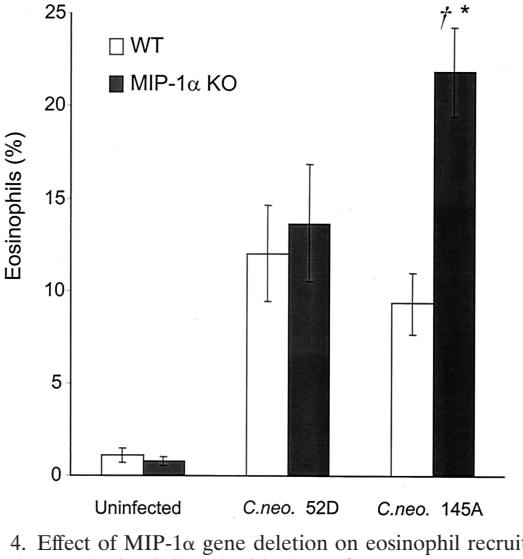

FIG. 4. Effect of MIP-1α gene deletion on eosinophil recruitment into the lungs of mice infected with *C. neoformans* 52D or 145A (2 weeks postinfection). Leukocytes were isolated from infected lungs of WT and MIP-1α KO mice following enzymatic dispersion of whole lungs. Data (means ± SE) are percentages of eosinophils in cytospun preparations of lung leukocytes ($n = 15$ to 17 mice per group). Asterisk, $P < 0.05$, in comparison with 52D-infected group; dagger, $P < 0.05$, in comparison with WT mice.

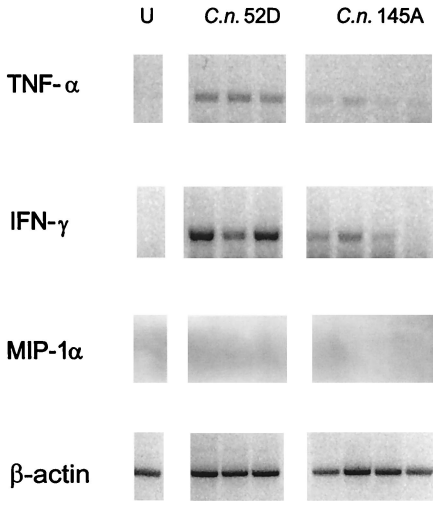

FIG. 5. Comparison of pulmonary TNF-α, IFN-γ, and MIP-1α gene expression in MIP-1α KO mice infected intratracheally (*C. neoformans* 52D or *C. neoformans* 145A; 10^4 CFU). Total lung RNA was isolated from the uninfected mice (U) on day 7 postinoculation. Expression of cytokine mRNA was analyzed via RT-PCR. The cytokine PCR products were confirmed by Southern blotting with specific DNA probes.

ment of EP in MIP-1α KO mice infected with *C. neoformans* 145A (Fig. 4). Thus, deletion of MIP-1α does not result in the development of EP in response to infection with *C. neoformans* 52D.

Comparison of the proinflammatory cytokine profiles in WT versus MIP-1α KO mice. To dissect the molecular mechanism of the differential effects of MIP-1α in the regulation of the immune response to *C. neoformans* 52D and *C. neoformans* 145A, we analyzed whether expression of MIP-1α was required for induction of TNF-α, IFN-γ, and MCP-1 at 7 days postinfection. Consistent with the MIP-1α KO genotype, MIP-1α mRNA was undetectable in both types of infection. Just like WT mice (Fig. 3) MIP-1α KO mice infected with *C. neoformans* 52D demonstrated robust induction of TNF-α and IFN-γ in their lungs (Fig. 5). The level of MCP-1 in BAL fluid was elevated (Fig. 6) in both groups infected with *C. neoformans* 52D. In contrast, both WT and MIP-1α KO mice infected with *C. neoformans* 145A demonstrated dramatically reduced expression of TNF-α and IFN-γ compared with *C. neoformans* 52D-infected mice (Fig. 3 and 5). MCP-1 induction in *C. neoformans* 145A-infected WT and MIP-1α KO mice was minimal compared to that in animals infected with *C. neoformans* 52D (Fig. 6). A small increase in MCP-1 level could be detected in *C. neoformans* 145A-infected WT mice but not in MIP-1α KO mice (Fig. 6). Thus, MIP-1α is not required for the robust early induction of TNF-α, IFN-γ, or MCP-1 in response to *C. neoformans* 52D but may be required for the modest production of MCP-1 seen in response to *C. neoformans* 145A.

Comparison of MIP-1α effects on pulmonary growth of *C. neoformans*. Our next objective was to determine the role of MIP-1α in the control of short- and long-term pulmonary infection with *C. neoformans* 52D and *C. neoformans* 145A. In contrast with *C. neoformans* 145A infection, there were no observed differences in *C. neoformans* 52D-infected lung CFU between WT and MIP-1α KO mice at 2 weeks postinfection

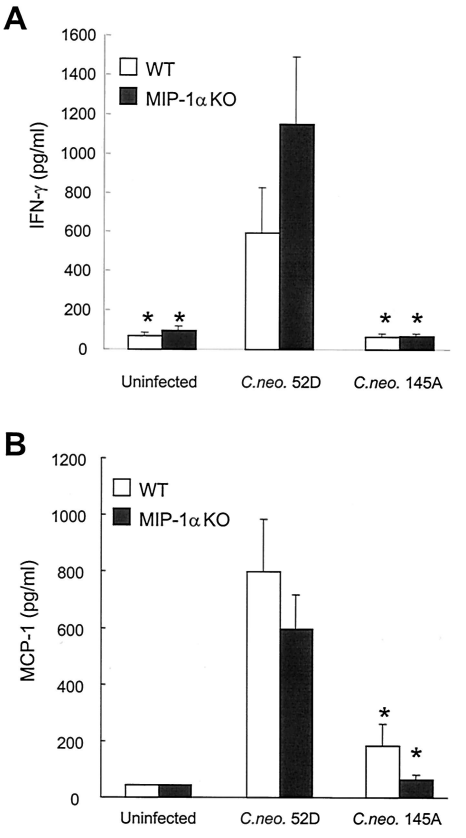

FIG. 6. Comparison of IFN-γ (A) and MCP-1 (B) levels in BAL fluid. The cytokines were detected by ELISA in BAL samples from infected (1 week postinfection) and uninfected WT and MIP-1α KO mice. Bars, means ± SE ($n = 9$ or 10 per group [infected] and 4 per group [uninfected]). Asterisk, $P < 0.05$ in comparison with *C. neoformans* 52D-infected group.

(Fig. 7). Both MIP-1α KO and WT mice cleared the *C. neoformans* 52D infection. In contrast, neither MIP-1α KO nor WT mice could control growth of *C. neoformans* 145A in the lungs (Fig. 7). Thus, MIP-1α was not required to control the pulmonary growth of *C. neoformans* 52D, and the beneficial effect of MIP-1α at week 2 in *C. neoformans* 145-infected mice was not apparent at 5 weeks postinfection.

Comparison of MIP-1α effects on control of *C. neoformans* in the CNS. To determine if MIP-1α deletion had an effect on the control of *C. neoformans* dissemination and growth in the CNS, we analyzed brain CFU at 5 weeks postinfection. One of eight animals in the MIP-1α KO group infected with *C. neoformans* 52D contained *C. neoformans* in the CNS; no *C. neoformans* was detected in the CNS of the remaining 7 MIP-1α KO animals (Fig 8). In contrast, brain dissemination occurred in both WT and MIP-1α KO mice infected with *C. neoformans* 145A. However, the number of cryptococci was nearly 2 log units greater in MIP-1α KO mice than in WT mice (Fig. 8). Thus, dissemination of the more-virulent *C. neoformans* 145A is much greater than that of *C. neoformans* 52D, and MIP-1α is required to control the growth of *C. neoformans* 145A in the CNS.

Comparison of CNS pathology between WT and MIP-1α KO mice infected with *C. neoformans* 145A. Our final objective

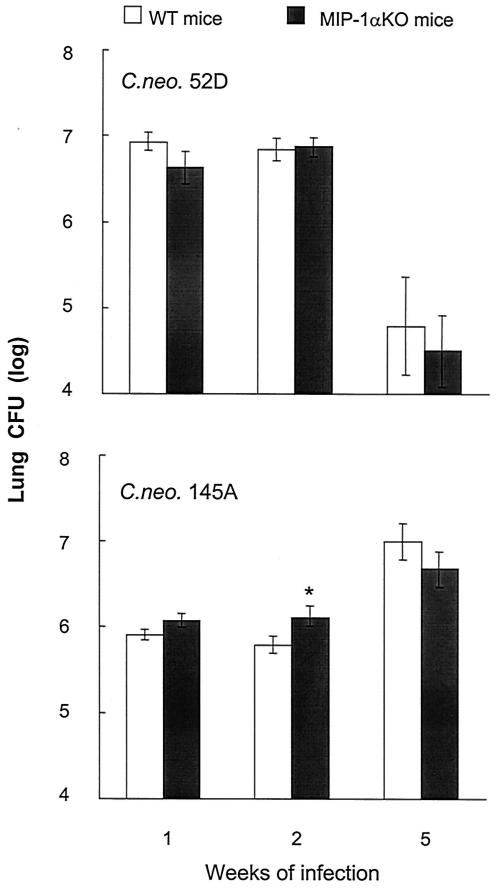

FIG. 7. Effect of MIP-1α gene deletion on pulmonary clearance of *C. neoformans* in mice infected with *C. neoformans* 52D (top) and *C. neoformans* 145A (bottom). Mice were infected intratracheally (*C. neoformans* 52D or *C. neoformans* 145A; 10^4 CFU) and analyzed (n = 5 to 22 per time point per group). Data, pooled from at least two separate matched experiments, are mean CFU per whole lung ± SE. Asterisk, $P < 0.05$, in comparison with 52D-infected group.

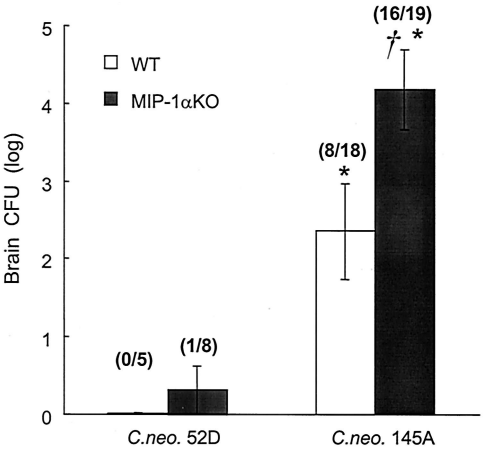

FIG. 8. Effect of MIP-1α gene deletion on cerebral dissemination of *C. neoformans* in mice infected with *C. neoformans* 52D and *C. neoformans* 145A. Mice were infected intratracheally (*C. neoformans* 52D or *C. neoformans* 145A; 10^4 CFU) and analyzed 5 weeks postinfection (n = 5 to 19 per group). Data, pooled from separate matched experiments, are mean CFU per brain ± SE. Asterisk, $P < 0.05$, in comparison with 52D-infected group; dagger, $P < 0.05$, in comparison with WT mice.

was to compare brain pathologies in WT and MIP-1α KO mice infected with *C. neoformans* 145A. Cryptococcal brain masses were increased in number and size in the brains of MIP-1α KO mice compared with WT mice (Fig. 9). The lesions in the brains of MIP-1α KO mice included the following: (i) profound meningitis (swelling and infiltration of the meninges and numerous cryptococci and mononuclear leukocytes throughout the entire perimeter of brain sections) and (ii) multiple intracerebral lesions (round foci expanding within the brain, containing large cryptococci with no or few inflammatory cells separating them from uninfected brain tissue) (Fig. 9A). In the WT mice, meningitis was far less pronounced; we observed 0 to 2 small lesions/section of the brain, demarcated by the inflammatory cell infiltrate (Fig. 9B). Thus, severe pathology and evidence of uncontrolled growth of *C. neoformans* were found in the CNS of *C. neoformans* 145A-infected MIP-1α KO mice.

DISCUSSION

In this paper, we report that deletion of MIP-1α did not cause development of nonprotective EP in mice infected with moderately virulent strain *C. neoformans* 52D while infection of MIP-1α KO mice with more-virulent strain *C. neoformans* 145A resulted in the development of EP. Early induction of inflammatory cytokines TNF-α, IFN-γ, and MCP-1 followed *C. neoformans* 52D infection but did not occur in *C. neoformans* 145A-infected animals. The observation that MIP-1α prevents the development of EP following *C. neoformans* 145A infection, even in the absence of TNF-α, IFN-γ, and MCP-1, documents that MIP-1α is a potent factor regulating the phenotype of the inflammatory response which develops in the lung upon infection with *C. neoformans*. On the other hand MIP-1α is not required for the development of protective immunity to *C. neoformans* 52D.

MIP-1α functions as a part of a complex regulatory network. A relationship between TNF-α and MIP-1α induction exists, since neutralization of TNF-α during the first 2 weeks of infection decreases MIP-1α levels in the BAL fluid of mice infected with *C. neoformans* 52D (14). Thus, we were surprised to observe MIP-1α induction in response to infection by *C. neoformans* 145A, during which TNF-α induction (RNA [Fig. 3] or protein level [16]) was minimal or absent. The difference between these two strains in the induction signals for MIP-1α likely relates to virulence factor or surface structural differences between these two isolates and is a point of future investigation. The lack of effect of MIP-1α deletion on the immune response to *C. neoformans* 52D, during which TNF-α, IFN-γ, and MCP-1 are abundant, suggests that cytokines and/or chemokines have overlapping functions with MIP-1α in regulation of the immunity to *C. neoformans*. Thus, the regulatory function of MIP-1α is particularly important in situations in which other strong proinflammatory molecules are absent.

Differential inflammatory responses were associated with subsequent differences in the clearance of *C. neoformans* 52D and *C. neoformans* 145A. The B6129F2/J mice generate a strong inflammatory response and are resistant to *C. neofor-*

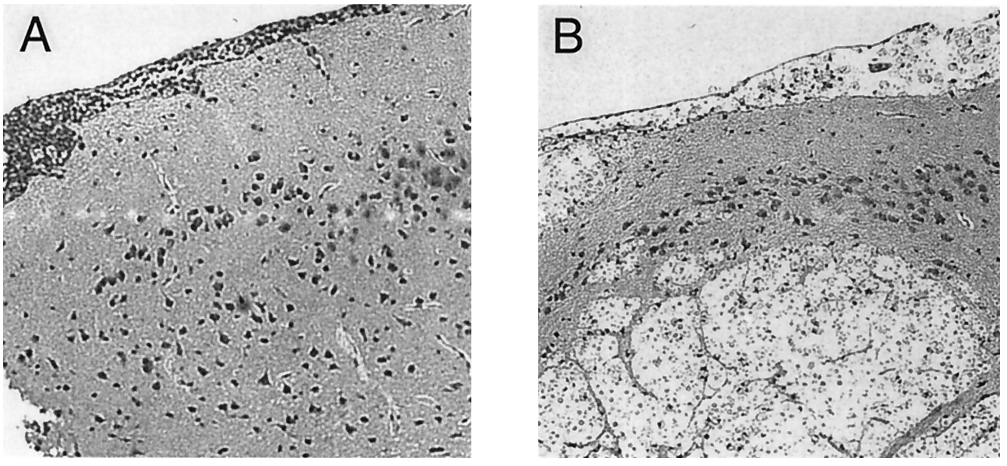

FIG. 9. Histology of brain sections 10 weeks post-intratracheal infection with *C. neoformans* 145A. Magnification, ×33. (A) WT mice. Note the well-organized, local inflammatory cell infiltrate colocalized with cryptococci and the good separation of infected areas of meninges from uninfected brain tissue. (B) MIP-1α KO mice. Note the swelling and infiltration of the meninges and multiple intracerebral lesions, the round foci expanding within the brain with numerous cryptococci with large capsules, no or few inflammatory cells.

mans 52D infection. Our studies demonstrate that this strain is useful for studying protective immunity, as are BALB/c, C.B-17, CDF1, and CBA/J mice. Information about the response in B6129F2/J mice is important since this strain is utilized often for KO studies. Similar to CBA/J mice, these mice do not generate significant early inflammation during *C. neoformans* 145A infection and the infection becomes chronic (6, 14). However, MIP-1α is expressed in response to the infection, which prevents the development of a T2 response even in the absence of TNF-α and IFN-γ. Our studies document that the generation of protective immunity in cryptococcal infection is crucially dependent on the early innate immune response. A vigorous early inflammatory response is associated with the development of strong protective immunity and clearance of the infection. On the other hand, a delayed inflammatory response is associated with a "deficient" immune response and chronic infection. Circulating cryptococcal polysaccharide is likely a factor in modulating leukocyte recruitment to the site of infection (8). However, the effect of circulating polysaccharide on leukocyte recruitment is secondary if the appropriate inflammatory signals and chemotactic factors are not generated at the site of infection (such as in a *C. neoformans* 145A infection). In this situation, leukocytes will not be recruited to the site whether or not there is circulating polysaccharide. Thus, down-regulation or absence of early "danger signals" such as TNF-α is an important factor determining *C. neoformans* virulence (14, 17, 22). The net result of decreased early danger signals is limited inflammation, altered cytokine production, limited or altered immune responses, and a chronic infection.

The early induction of MCP-1 in *C. neoformans* 52D infection suggests that MCP-1 may also play a role in early inflammatory cell recruitment. MCP-1 was previously shown to be important molecule during the efferent phase of immune response to *C. neoformans* (16). The dramatically lower induction of MCP-1 in *C. neoformans* 145A-infected lungs corresponds to decreased early inflammation in this infection and subsequent problems with clearance of the infection. Kawakami et al. also observed very little or no production of chemokines, including MCP-1, during infection with a highly virulent strain of *C. neoformans* (24). Moreover, mice deficient in CCR2, the receptor for MCP-1, also have decreased recruitment of mononuclear cells early in *C. neoformans* 52D infection (31). Thus, down-regulation of early MCP-1 production is consistent with a lack of danger signals induced by *C. neoformans* 145A.

Meningoencephalitis is the cause of excessive mortality previously reported in *C. neoformans* 145A-infected MIP-1α KO mice. In spite of the more-severe lung pathology in MIP-1α KO mice (27), no differences in lung CFU between *C. neoformans* 145A-infected WT and KO mice were noted on day 35. Thus, pulmonary cryptococcal burden itself was not the likely cause of the increased mortality that occurred between weeks 6 and 12 postinfection in MIP-1α KO mice. However, *C. neoformans* 145A disseminates earlier and in greater numbers in MIP-1α KO mice than in WT mice. Moreover, *C. neoformans* 145A multiplies in the CNS; CFU values $>10^7$ were noted in MIP-1α KO animals, as were multiple cryptococcal mass lesions with proliferating cryptococci (Fig. 9). Neurological abnormalities were present in premoribund animals (paralysis, abnormal gait). Thus, increased mortality in MIP-1α KO mice was predominantly associated with cryptococcal meningoencephalitis rather than pulmonary pathology.

The CNS pathology, survival times, and day 35 lung CFU burdens parallel those from our observations of CCR5 KO mice infected with *C. neoformans* 145A (15). MIP-1α is a high-affinity ligand of CCR5, and these findings provide further support for our notion that expression of CCR5 and its ligands is crucial for protection of the CNS from *C. neoformans* infection. The absence of MIP-1α results in pronounced brain dissemination and CNS pathology, showing that the MIP-1α/CCR5 axis is particularly crucial to organ-specific protection of the CNS (15). The importance of the MIP-1α/CCR5 axis may potentially be even greater in human immunodeficiency virus type 1 (HIV-1) patients infected with *C. neoformans*. Both MIP-1α and HIV gp120 are ligands for CCR5 (28, 29). Thus, CCR5, a receptor via which MIP-1α exerts its protective anti-

cryptococcal effect in the CNS, is also a coreceptor for HIV infection (1, 4, 7, 9). Our studies raise the possibility that dysregulated MIP1α/CCR5 function (potentially caused by HIV or gp120 binding to CCR5) is a significant predisposing factor for *C. neoformans* infection of the CNS (21).

Microbes persist in mammalian hosts, in part, because they do not elicit innate immune system danger signals. Well-characterized danger signals include TNF-α and IFN-γ, cytokines essential for the development of a protective immune response to *C. neoformans*. Our data document that MIP-1α also plays a crucial role as an early danger signal in certain microbe-host interactions. MIP-1α alone can prevent the development of a nonprotective EP immune response, particularly in circumstances when a pathogen does not elicit other danger signals.

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