Cutting Edge: Role of C-C Chemokine Receptor 5 in Organ-Specific and Innate Immunity to Cryptococcus neoformans

Gary B. Huffnagle, Lisa K. McNeil, Roderick A. McDonald, Juneann W. Murphy, Galen B. Toews, Nobuyo Maeda and William A. Kuziel

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The objective of our studies was to determine whether expression of CCR5 was required for host defense against the encapsulated yeast \textit{Cryptococcus neoformans}. Protective immunity against this AIDS-associated opportunistic pathogen requires the development of T cell-mediated immunity (reviewed in Ref. 19). \textit{C. neoformans} is acquired via the respiratory tract and disseminates to the CNS (20). Cryptococcal meningitis is common late in the pathogenesis of HIV infection (20). In murine models of pulmonary cryptococcosis, the T cell-mediated immune response in the lungs is a Th1-type response that requires CD4$^+$ T cells, CD8$^+$ T cells, monocyte chemotactic protein-1, and MIP-1a for leukocyte recruitment and cryptococcal clearance (reviewed in Ref. 19). After dissemination from the lungs, host defense in the CNS also requires CD4$^+$ T cells, IFN-$\gamma$, TNF-$\alpha$, and mononuclear cell recruitment (reviewed in Refs. 21 and 22). Our studies focused on the role of CCR5 in immunity against \textit{C. neoformans} at the sites of primary infection (lung) and disseminated infection (brain).

Materials and Methods

C. neoformans

\textit{C. neoformans} strain 145 was obtained from the American Type Culture Collection (62070; Rockville, MD). Strain 145 was originally isolated from the cerebrospinal fluid of a patient with cryptococcal meningitis. \textit{C. neoformans} organisms were grown and prepared as previously described (23). Pulmonary infection was initiated in mice via intratracheal inoculation of \textit{C. neoformans} using a 30-$\mu$l inoculum containing 10$^7$ CFU (23).

Mice

CCR5$^{+/+}$ mice (B6129F2/H, University of Michigan breeding colony and The Jackson Laboratory, Bar Harbor, ME) and CCR5$^{-/-}$ mice (B6129F2/
J-Cmkbr5tm1Kuz (University of Michigan breeding colony) were housed under specific pathogen-free conditions in enclosed filter top cages. The CCR5−/− and CCR5+/− lines of mice were B6/129F2 background and large enough in number values were used in the experiments to control for potential variance of responses within this strain of mice. CCR5+/− and CCR5−/− mice were generated that were homozygous for 129-derived sequences flanking the CCR5 locus (as described previously (17)) but differed in deletion or presence of the single CCR5 exon. These mice were bred as homozygotes since their derivation. CCR5+/− 129B6F2/J mice supplied by The Jackson Laboratory were also used in some experiments, and there was no significant difference between the two sources of CCR5+/− mice. Mice were 8–16 wk of age at the time of infection and there was no age-related differences in the responses of these mice to C. neoformans infection.

Leukocyte recruitment into C. neoformans Ag-instilled gelatin sponges

The Ag preparation for the assay (CnFe) was prepared by concentrating and dialyzing the supernatant from a C. neoformans broth culture (24). The predominant products in CnFe are polysaccharide capsule (glucuronoxylomannan and galactoxylomannan) and a Con A-binding mannanprotein (24). Two blocks of gelatin sponges (17 × 18 × 10 mm, Gelsoft sterile absorbent gelatin sponge; The Upjohn Co., Kalamazoo, MI) were implanted s.c. Four days after implantation, one sponge was injected with 0.1 ml CnFe and the other with 0.1 ml saline. The sponges were removed 24 h after sponge injection. Sponges were digested and dispersed with an enzyme mixture followed by lysis of erythrocytes. Viable cell counts were made using a hemocytometer and trypan blue exclusion. Cell differentials were determined with a modified Wright-Giemsa stain. The results represent specific leukocyte recruitment in response to CnFe challenge and are expressed as the difference in total leukocytes between the CnFe sponges and the saline only sponges.

Results and Discussion

The studies were performed with CCR5 knockout mice (CCR5−/−) generated by targeted deletion of the entire CCR5 coding exon without disruption of the CCR2 gene (4). As reported previously for this type of knockout, CCR5−/− mice develop normally; do not have observable histological abnormalities in the lungs, brain, spleen, or other organs compared with wild-type mice (CCR5+/+); and have no overt defects in T cell or macrophage numbers in the spleen or lungs (13). CCR5 knockout mice also do not display major defects in leukocyte recruitment as reported for CCR1 and CCR2 knockout mice (13–18, 25, 26). CCR5−/− mice do not differ from CCR5+/− mice in numbers of elicited macrophages after i.p. thioglycolate (13) or in size of liver granuloma formation after i.v. yzmosan or glucan (W. A. Kuziel et al., manuscript in preparation). CCR5−/− mice also do not differ significantly from CCR5+/− mice in survival after intratracheal inoculation of Klebsiella pneumoniae (data not shown). CCR5−/− mice display slightly reduced clearance of Listeria from the liver but not the spleen and have moderately reduced survival after intermediate but not high or low dose LPS treatment (13). Thus, the relatively normal immunological phenotype of the CCR5−/− mouse appears to be consistent with the normal immune status reported for humans possessing mutant copies of the CCR5 gene (9–12).

To test whether expression of CCR5 is required for protection against C. neoformans, CCR5−/− and CCR5+/− mice were inoculated intratracheally with 107 CFU of C. neoformans (strain 145). Intratracheal inoculation of this highly virulent strain of C. neoformans causes a pulmonary infection in immunocompetent mice that disseminates from the lungs by wk 2 and establishes an infection in the CNS (23). Greater than 90% of the CCR5+/− mice infected with C. neoformans survived through wk 12 (Fig. 1). In contrast, less than 25% of the CCR5−/− mice survived (median, 8.4 wk) through wk 12 (Fig. 1). These experiments demonstrated that expression of CCR5 is required for host defense and survival after pulmonary-acquired C. neoformans infection.

We next investigated whether the striking decrease in survival of CCR5-deficient mice was due to an inability to recruit leukocytes into the lungs and control the pulmonary infection. CCR5 expression in the lungs during a C. neoformans infection was readily detected in CCR5+/− but not CCR5−/− mice (data not shown). To quantitate the leukocytic infiltrate in the lungs in response to C. neoformans infection, total lung leukocytes were isolated by enzymatic dispersion of the lungs and then counted (23). Deletion of CCR5 did not inhibit leukocyte recruitment into the lungs; rather, there was a trend toward increased leukocyte recruitment in CCR5−/− mice (7.42 ± 2.66 × 107 (+/−) vs 8.78 ± 3.78 × 107 (−/−) lung leukocytes at wk 5, p > 0.05). This was predominantly due to increased mononuclear cell recruitment in CCR5−/− mice (51% (+/+) vs 64% (−/−)) (Fig. 2). The leukocytic infiltrate in the lungs of C. neoformans-infected CCR5+/+ and CCR5−/− mice is clearly evident in histological sections (Fig. 2). Interestingly, CCR5−/− mice had lower lung CFU of C. neoformans at this time point than CCR5+/− mice (7.54 ± 0.29 (+/+) vs 6.04 ± 0.31 (−/−) log10 CFU; p < 0.05). These results demonstrated that CCR5 is expressed in the lungs during a C. neoformans infection but that expression of CCR5 is not absolutely required for leukocyte recruitment into the lungs or to control the pulmonary C. neoformans infection. We then analyzed the role of CCR5 in protecting against dissemination and growth of C. neoformans in the brain. At wk 2, the infection had not disseminated from the lungs to the brain in either group of mice (<100 CFU, data not shown). At wk 5, low level dissemination to the brain was evident in both CCR5+/− and CCR5−/− mice (3.03 ± 0.82 (+/+) vs 2.52 ± 0.85 (−/−) log10 CFU). After wk 5, C. neoformans-infected CCR5−/− mice displayed clinical manifestations before death that were consistent with cryptococal “meningitis” (cranial swelling, ruffled fur, staggered gait, lethargy and unresponsiveness, and decreased limb function). In contrast, C. neoformans-infected CCR5+/− mice did not develop these symptoms. By wk 8, both groups of mice had equivalent colonization of the brain (7.52 ± 0.30 (+/+) vs 7.53 ± 0.55 (−/−) log10 CFU of wk 8 survivors), but 40% of the CCR5−/− mice had already died by wk 8 (Fig. 1), displaying signs of severe CNS infection before death. Further histological analysis revealed minimal mononuclear leukocyte recruitment into the brains of CCR5-deficient mice (Fig. 3). In sharp contrast, significant numbers of mononuclear leukocytes were recruited into the meninges of CCR5+/− mice after colonization by C. neoformans (Fig. 3). This was consistent with previous studies demonstrating that growth of C. neoformans in the brain of immunocompetent mice stimulates the recruitment of leukocytes into the CNS (reviewed in Refs. 21 and 22). Thus, our studies demonstrate that

4 W. A. Kuziel, T. C. Dawson, R. L. Reddick, and N. Maeda. Atherogenesis in apolipoprotein E-deficient mice that also lack the chemokine receptor CCR5. Submitted for publication.
expression of CCR5 is required for leukocyte recruitment into the CNS during *C. neoformans* infection.

The most striking histological feature was the excessive capsule deposition and swelling in the brains of CCR5−/− mice (Fig. 3C). Distension of the cranium and loss of neural tissue integrity was also clearly evident in gross necropsy of these mice, and altogether, the immunopathogenesis of the infection in the CNS correlated with the dramatic decrease in survival of CCR5-deficient mice. Large amounts of extracellular cryptococcal polysaccharide capsule accumulated in the brains of CCR5−/− mice as detected by mucicarmine stain (histology not shown). Cryptococci could be seen in sections from CCR5−/− mice as clusters of organisms (Fig. 3C), whereas only single yeast or small groups of organisms with minimal polysaccharide capsule were present in the brains of CCR5+/+ mice (Fig. 3B). One caveat is that the large amount of capsule in the brains of CCR5−/− mice could have interfered with the enumeration of brain CFU, leading to an underestimate of the cryptococcal burden in the brains of CCR5−/− mice. The clinical presentation and histological evidence in CCR5−/− mice are consistent with the cause of death being hydrocephalus due to excessive cryptococcal polysaccharide accumulation (and breakdown of the osmotic gradient in the brain). *C. neoformans* infections of the CNS are often referred to as “meningitis.” However, the CNS pathology of a *C. neoformans* infection in CCR5−/− mice was strikingly similar to that in AIDS patients (27), and cranial swelling in *C. neoformans*-infected CCR5−/− mice was not due to inflammation of the meninges. Thus, CCR5 expression is critical for elimination of extracellular polysaccharide from the brain during *C. neoformans* infection.

CCR5 expression on T cells appears to correlate with a Th1 phenotype (8, 28–30); thus, we examined the role of CCR5 in the
These studies have demonstrated that 1) a cell-mediated immune response to a single pathogen can be differentially regulated at different sites in the body (lungs vs brain), 2) CCR5 plays a role in tissue-specific recruitment of leukocytes, and 3) CCR5 is not required for leukocyte recruitment during a Th1 response but may play a role in innate recognition of shed microbial products. CCR5 deficiency prevents mononuclear cell recruitment only into the brain, not into the lungs (Figs. 2 and 3). In contrast, T cell deficiency inhibits leukocyte recruitment into both the lungs and brain during a *C. neoformans* infection (reviewed in Refs. 19 and 21). It is not clear at this point how CCR5 mediates tissue-specific trafficking during a cell-mediated immune response because CCR5 is also expressed on a number of parenchymal cells (5). However, these studies clearly demonstrate that CCR5 plays a critical role in trafficking of leukocytes to different tissue sites during infection.

One interesting feature of cryptococcal infections is that HIV-induced immunosuppression is by far the greatest predisposing factor for cryptococcal meningitis when compared with any other mechanism of immunosuppression (20). *C. neoformans* infections in the CNS occur late in the pathogenesis of HIV disease when the viral infection is well established (20). Our studies open the possibility that dysregulated macrophage CCR5 function (potentially caused by HIV or gp120 binding to CCR5) could be a significant predisposing factor for *C. neoformans* infection of the CNS. Also, in Africa and Asia where the rate of environmental *C. neoformans* exposure is high, CCR5 mutations are not found in the indigenous population (10, 12, 20). The studies presented here demonstrate that CCR5 plays a critical organ-specific role in host defense against disseminated *C. neoformans* infection and provide a testable hypothesis for why cryptococcal meningitis is far more prevalent in HIV-induced immunosuppression than any other type of immunosuppression.

References


FIGURE 4. Role of CCR5 in macrophage migration into CneF-instilled gelatin sponges in nonimmune mice. Sponges were instilled into mice of different genotypes (CCR5+/+ and CCR5−/−) and challenged with CneF (in the footpad or sponge) 1 wk later, as described previously (31–34). Immune CCR5+/+ mice were immunized by a single s.c. injection of CneF mixed in CFA and challenged with CneF in the footpad or sponge 1 wk later, as described previously (31–34). Immune CCR5+/+ and CCR5−/− mice both developed comparable, vigorous footpad DTH responses to *C. neoformans* antigen (CneF) Ag (~15 in−3, p > 0.05). Immune CCR5+/+ and CCR5−/− mice also had equivalent numbers of leukocytes infiltrating the CneF sponges (~33 × 106 leukocytes, p > 0.05). Leukocyte differentials in the Ag sponges of immune CCR5+/+ and CCR5−/− mice were identical: macrophages, 69–75%; neutrophils, 13–16%; eosinophils, 7–10%; and lymphocytes, 5–6%. Thus, CCR5 expression is not required for the development or manifestation of the Th1 cell-mediated DTH response to *C. neoformans*.

However, the sponge model experiments demonstrated a potentially novel role for CCR5 in leukocyte recruitment and recognition of *C. neoformans* by innate immunity. As previously described for other mouse strains (35), CneF instillation causes the influx of macrophages into the sponges of nonimmune CCR5+/+ mice (Fig. 4). Unexpectedly, few macrophages migrated into the CneF sponges in nonimmune CCR5−/− mice (an 80% decrease compared with CCR5+/+ mice (Fig. 4)). There is no overall defect in macrophage recruitment in CCR5−/− mice because macrophage recruitment is normal after thioglycolate instillation (13). DTH elicitation (described above), and pulmonary *C. neoformans* infection (Fig. 2). CneF is a concentrate of the products shed during growth of *C. neoformans* and is chemotactic for leukocytes (33, 35). The receptor for CneF-mediated chemotaxis is unknown; however, our studies demonstrate that CCR5 is required (directly or indirectly) for recruitment of macrophages in nonimmune mice in response to shed cryptococcal products.


