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Dissemination of *C. neoformans* to the central nervous system: role of chemokines, Th1 immunity and leukocyte recruitment

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Cryptococcus neoformans **is a fungus that possesses two properties unique for yeast: (1) production of a polysaccharide capsule and (2) neurotropism. The natural route of infection by *C. neoformans* is the respiratory tract; thus, factors that regulate the development and recruitment of memory Th1 cells and monocytes into the brain are critical for an effective response against disseminated *C. neoformans* infection. Production of TNFα prior to day 7 is required to prevent colonization of the central nervous system (CNS). Th1 type immunity is required to clear established foci. In contrast, Th2 type immunity is ineffective at eliminating the infection in the brain and results in decreased survival. *C. neoformans* infection of MIP-1α and CCR5 knockout mice has highlighted the complex role that some chemokines may play in different organs. MIP-1α knockout mice have decreased leukocyte recruitment and cryptococcal clearance from the brain compared to wild-type mice. Thus, the host defence mechanisms that clear *C. neoformans* from the CNS appear to be similar to those in the lungs: via a Th1 cell-mediated inflammatory response that requires chemokines for the recruitment of effector cells.**

Keywords: cryptococcus; lymphocytes; macrophage inflammatory protein-1; CCR5

Introduction

Cryptococcus neoformans is a fungus that possesses two properties unique for yeast: (1) production of a polysaccharide capsule and (2) neurotropism (Diamond, 1995). It is not entirely clear why *C. neoformans* is neurotropic but the ability to synthesize the anti-oxidant melanin from neurotransmitters (via the enzyme laccase) is a major factor (Kwon-Chung *et al*, 1982; Salas *et al*, 1996). *C. neoformans* enters the body by inhalation but the primary pulmonary infection is often undiagnosed or self-limiting (Diamond, 1995). Other unidentified virulence factors play a role in allowing the yeast to escape the lungs and colonize the brain through capillary embolization and destruction. The development of protective Th1 type cell-mediated immunity is required to eradicate the infection (Huffnagle and Lipscomb, 1998), control cryptococcal dissemination from the lungs, and eliminate subsequent colonization in the brain (Aguirre *et al*, 1995; Hill and Aguirre, 1994; Huffnagle *et al*, 1991b).

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Central nervous system (CNS) – the site of disseminated infection and secondary immune response

The lungs are the primary site of *C. neoformans* colonization and also the site at which the primary immune response develops. Protective immunity to *C. neoformans* requires both $CD4^+$ and $CD8^+$ T cells and clearance is mediated by the generation of an inflammatory response at the site of infection (Hill, 1992; Hill and Harmsen, 1991; Huffnagle *et al*, 1991a, 1994). Chemokines such as MIP-1α and MCP-1 are required for leukocyte recruitment into the lungs of *C. neoformans*-infected mice (Huffnagle *et al*, 1995b). Production of macrophage-activating cytokines such as TNFα, IFN-γ, IL-12, and GM-CSF are important for clearance of the infection (Huffnagle and Lipscomb, 1998). Clearance is mediated by a variety of cell types through both intracellular and

extracellular mechanisms (Diamond, 1995; Huffnagle and Lipscomb, 1998). If *C. neoformans* is inoculated intracranially, the host can develop a primary immune response and clear the infection (Blasi *et al*, 1992). However, when *C. neoformans* is acquired via the natural route of infection (the respiratory tract), the immune response in the brain is a secondary immune response (Diamond, 1995). Thus, the factors that regulate recruitment of memory Th1 cells and monocytes into the brain are critical for an effective response against disseminated *C. neoformans* infection.

Two of the factors that control clearance of *C. neoformans* from the brain include (1) the production of TNFα early in the response and (2) the production of Th1 type cytokines. Intratracheal inoculation of mice with a low virulence strain of *C. neoformans* (52) produces an infection that remains relatively contained in the lungs because the small numbers of organisms that escape from the lungs are cleared by cells of the reticuloendothelial system or circulating phagocytes (Huffnagle *et al*, 1991b). Opsonins such as complement and antibody also prevent establishment of extrapulmonary foci (Casadevall, 1995; Kozel, 1993). One role of $CD4^+$ T cells and specific immunity is to clear established cryptococcal foci from the CNS (Hill and Aguirre, 1994). Interestingly, treatment of mice with anti-TNFα antibodies to neutralize TNFα at the onset of infection blocks clearance of the yeast from the lungs and promotes colonization of the brain by a low virulence *C. neoformans* strain (strain 52) (Huffnagle *et al*, 1996). To further determine the 'window' when early production of TNFα is required to protect against subsequent colonization of the CNS, mice were given a single injection of anti-TNFα antibody at day 0, 3, 7, or 14 post-infection (Figure 1). Significant colonization of the CNS was observed only if the mice were treated at day 0 or day 3. This effect was not observed if the antibody treatment was delayed until day 7 or day 14 post-infection (Figure 1), time points by which protective cell-mediated immunity has developed (Huffnagle *et al*, 1991b). These observations demonstrate that production of TNFα prior to day 7 is required to prevent the establishment of cryptococcal foci in the CNS.

Intratracheal inoculation of a high virulence strain of *C. neoformans* (strain 145) produces an infection that disseminates from the lungs and establishes an infection in the brain in immunocompetent mice (Huffnagle *et al*, 1995a). The virulence factors have not been completely identified that account for the increased ability of *C. neoformans* strains such as 145 to colonize the CNS. However, one of the mechanisms that likely plays a role in the ability of strain 145 to effectively colonize the CNS in immunocompetent hosts is that it produces virulence factors, such as melanin and capsule, that can block TNFα production by

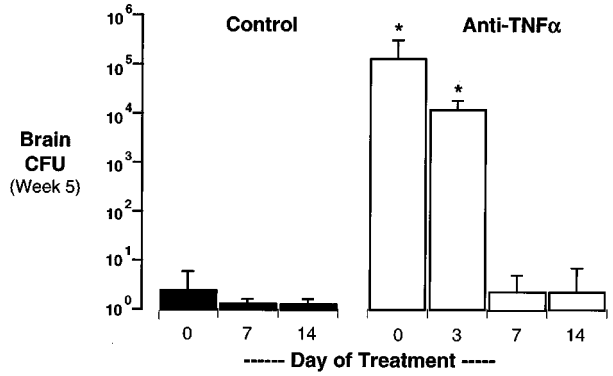

Figure 1 Effect of TNFα neutralization on *C. neoformans* colonization of the CNS following dissemination from the lungs. CBA/J mice were inoculated with 10^4 c.f.u. of a low virulence *C. neoformans* strain (52) on day 0 and treated with 1 mg of anti-TNFα Ab or control Ig at the time indicated. Brain c.f.u. were assayed at day 35 post-infection. $^*P<0.01$ compared to control Ig treated mice at the same time point (day 3 anti-TNFα treated mice are compared to day 0 or day 7 control mice). $n=5-10$ mice per group.

macrophages (Huffnagle *et al*, 1995a; Vecchiarelli *et al*, 1995).

The experiments in Figure 2 demonstrated that growth of *C. neoformans* strain 145 in the CNS is slowed by the development of Th1 type immunity while Th2 type immunity does not control the infection in the CNS. CBA/J mice normally develop Th1 type responses to *C. neoformans* 52 and 145 (Huffnagle *et al*, 1995a and Huffnagle *et al*, submitted) while C57BL/6 mice develop Th2 type responses to these two strains (Hoag *et al*, 1995 and Huffnagle *et al*, in preparation). Accordingly, C57BL/6 mice were not able to control the growth of strain 145 in the brain and the mice died by week 7 post-infection (Figure 2). In summary, (1) early production of TNFα is required to prevent establishment of cryptococcal foci in the CNS and (2) Th1 type immunity is required to clear established cryptococcal foci.

Role of leukocyte recruitment into the CNS

The effector mechanisms that clear *C. neoformans* from the CNS appear to be similar to those in the lungs, namely, via a T cell-mediated inflammatory response (Aguirre *et al*, 1995; Blasi *et al*, 1994; Hill and Aguirre, 1994; Salkowski and Balish, 1990). The experiments in Figure 3 suggest that growth of *C. neoformans* in the CNS is one of the signals that initiates inflammatory cell recruitment into the CNS. Leukocyte recruitment into the brain of CBA/J mice was initiated once the burden of *C. neoformans* in the brain became significant (Figure 3). The low virulence *C. neoformans* strain 52

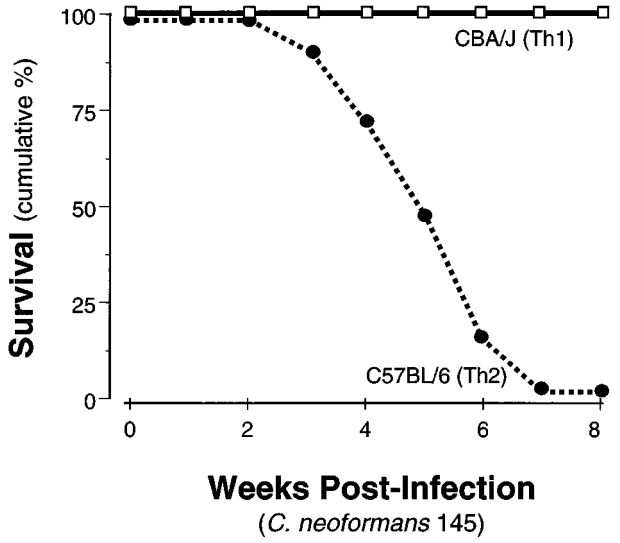

Figure 2 Role of Th1 *vs* Th2 immunity in survival following *C. neoformans* infection. Genetically resistant (Th1 response, CBA/J) and susceptible (Th2 response, C57BL/6) mice were inoculated intratracheally with 10^4 c.f.u. of a high virulence *C. neoformans* strain (145) on day 0. Strain 145 disseminates from the lungs to the CNS in all strains of mice. Death was due to CNS infection (by c.f.u. analysis and clinical observations) Brain c.f.u. at 4 weeks post-infection were 4.57 ± 0.39 ($\log_{10}$ c.f.u.) for CBA/J mice and 6.43 ± 0.56 for C57BL/6 mice ($\log_{10}$ c.f.u.). n=8–12 mice per group.

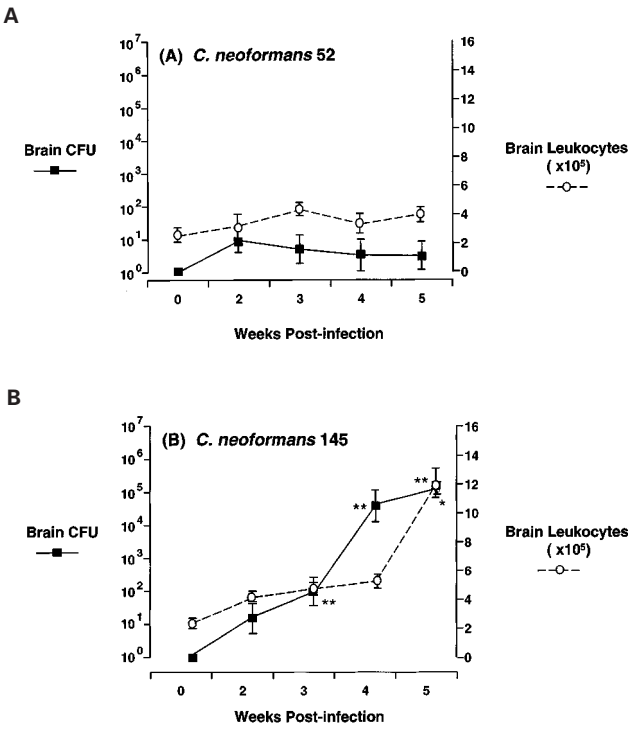

Figure 3 Leukocyte recruitment into the CNS in response to growth of *C. neoformans* in the brain. CBA/J mice were inoculated intratracheally with 10^4 c.f.u. of either (**A**) low virulence *C. neoformans* strain (52) or (**B**) high virulence strain (145) on day 0. Total leukocyte numbers and brain c.f.u. were quantitated from perfused, collagenase digested brains of mice at the times indicated. The low numbers of leukocytes in the brains of mice prior to infection (week 0) is most likely due to low level blood capillary leukocyte contamination of the preparation. **Time point at which dissemination is detected in >50% of the mice. *$P<0.05$ compared to uninfected mice. n=8–14 mice per time point.

disseminated to the CNS but did not establish a significant infection; leukocyte recruitment was low (Figure 3A). In contrast, the high virulence strain 145 established an infection in the brain ($>10^5$ c.f.u.) that evoked an influx of leukocytes into the CNS (Figure 3B). Other laboratories have reported that leukocyte recruitment into the brain is deficient in nude mice, SCID mice, and mice treated with anti-IFN-γ antibodies (Aguirre *et al*, 1995; Hill and Aguirre, 1994; Salkowski and Balish, 1990). Thus, the previous and current studies demonstrate that CNS inflammation during *C. neoformans* infection is an effector phase mechanism of Th1 cell-mediated immunity.

Role of chemokines in protecting the CNS from disseminated cryptococcosis

Chemokines are major mediators of leukocyte recruitment into sites of *C. neoformans* infection. In the brain, microglial cells are potent sources of IL-8, IP-10, MIP-1α, MIP-1β, RANTES, KC, and MCP-1 (Kunkel *et al*, 1995). The specific signals for chemokine production by microglial cells are not known; however, three possible signals are (1) *C. neoformans* products, (2) pro-inflammatory cytokines, or (3) disruption of the osmotic balance in the brain. *C. neoformans* products such as glucoronyloxylmannan (GXM) can directly induce IL-8 production by isolated microglial cells in culture (Lipovsky *et al*, 1998). Heat-killed *C. neoformans* injected intracranially induces the expression of a number of pro-inflammatory cytokines including TNFα resulting in protective immunity (Blasi *et al*, 1992, 1994). This protective effect can be mimicked by intracranial injection of other inflammatory stimuli such as *Toxoplasma gondii* and *Candida albicans* (Aguirre *et al*, 1995; Barluzzi *et al*, 1997). Isolated *C. neoformans* products such as GXM or mannoprotein also directly induce TNFα production by macrophages and peripheral blood monocytes (Levitz and North, 1997; Retini *et al*, 1996). TNFα, IFN-γ, and other pro-inflammatory cytokines are potent inducers of chemokine production by resident cells of the CNS. Neutralization of TNFα or IFN-γ after immunity has developed blocks leukocyte recruitment into the brains of *C. neoformans*-infected mice directly demonstrating a role for these cytokines in leuko-

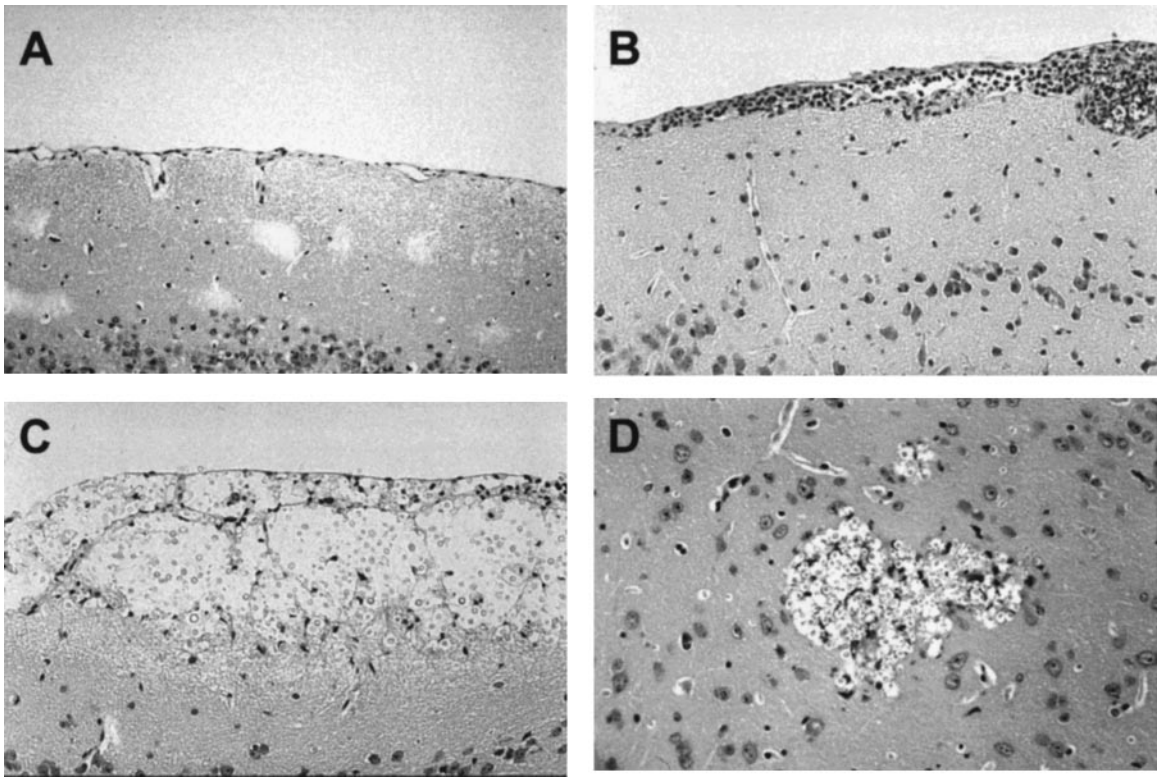

Figure 4 Leukocyte recruitment into the brains of MIP-1α knockout and wild-type mice. Photomicrograph of the brains of mice at week 10 post-infection. Mice were inoculated intratracheally with 10^4 c.f.u. of a high virulence *C. neoformans* strain (145) on day 0. (**A**) Uninfected brain, (**B**) 10 week infected brain from wild-type mouse, (**C**) 10 week infected brain from MIP-1α knockout mice illustrating meningeal infection, (**D**) 10 week infected brain from MIP-1α knockout mice illustratiing a deep brain cyst.

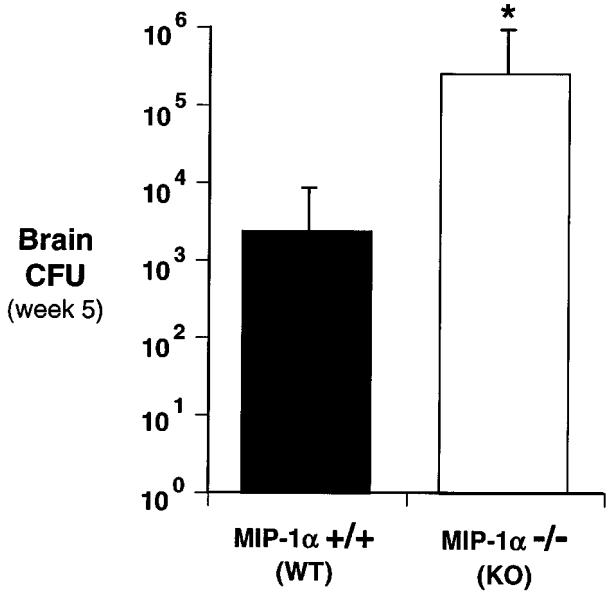

Figure 5 Brain c.f.u. in MIP-1α knockout (MIP-1α−/−, KO) and wild-type mice (MIP-1α+/+, WT) at week 5 post-infection. Mice were inoculated intratracheally with 10^4 c.f.u. of *C. neoformans* strain 145 on day 0. c.f.u. are expressed as the mean ± s.e.m. c.f.u. per whole brain. n=18–19 mice per group. $^*P<0.02$ compared to WT mice.

cyte recruitment into the CNS (Aguirre *et al*, 1995). Finally, the production and shedding of polysaccharide capsule by *C. neoformans* ultimately destroys the osmotic balance in the brain and leads to intracranial swelling (Lee *et al*, 1996). Changes in the osmotic gradient in the brain can also be a signal for chemokine production in the brain (Koike *et al*, 1997). Clearly, multiple pathways exist for induction of chemokine expression in the brain during *C. neoformans* infection.

C. neoformans infection of MIP-1α and CCR5 knockout mice has highlighted the complex role that some chemokines may play in different organs. MIP-1α is a CC chemokine that is important in monocyte and neutrophil recruitment into the lungs of *C. neoformans*-infected mice and into sites of DTH reactions against cryptococcal antigen (Doyle and Murphy, 1997; Huffnagle *et al*, 1997). MIP-1α knockout mice have decreased survival following intratracheal inoculation of high virulence *C. neoformans* strain 145 (Huffnagle *et al*, submitted). These mice develop a chronic pneumonia but die from unrestricted growth of *C. neoformans* in the brain (Figure 4 and Huffnagle *et al*, submitted). Brain c.f.u. were over 100-fold higher in MIP-1α knockout mice compared to wild-type mice at week

5 (Figure 5). By week 10, the infectious load of *C. neoformans* (organisms plus extracellular polysaccharide capsule) in the brain was strikingly different between wild-type and knockout mice, as demonstrated in histological sections (Figure 4). MIP-1α knockout mice clearly also have a defect in the ability to recruit leukocytes into the brain (Figure 4). However, the T cell response in the lungs of these mice also resembles a Th2 response (Huffnagle *et al*, submitted) and the development of a Th2 response to *C. neoformans* also prevents clearance of the organism from the CNS (Figure 3). Thus, MIP-1α may play a role as a recruitment molecule in the CNS and/or as a 'switch factor' early in the response for promoting Th1 over Th2 immunity.

The most intriguing data on the complex role of chemokines in the CNS has come from studies of the role of the CC chemokine receptor CCR5 in *C. neoformans* infections. CCR5 is a receptor for MIP-1α, MIP-1β, and RANTES and a co-receptor for monotropic strains of HIV (Alkhatib *et al*, 1996; Deng *et al*, 1996; Dragic *et al*, 1996; Samson *et al*, 1996). Surprisingly, intratracheal inoculation of *C. neoformans* into CCR5 knockout mice appears to result in a pulmonary infection that is cleared by an exaggerated immune response in the lungs but a central nervous system infection that is not cleared due to a lack of leukocyte recruitment into the brain (Huffnagle *et al*, submitted). Memory Th1 cells appear to preferentially express CCR5 (compared to naive or memory Th2 cells) (Bonecchi *et al*, 1998; Loetscher *et al*, 1998; Qin *et al*, 1998). Since the brain is a secondary site of cryptococcal infection (the lungs are the primary site), CCR5 may play a critical role in Th1 cell trafficking into the CNS. While the pathology appears to suggest that CCR5 is required for leukocyte trafficking into the CNS of *C. neoformans*-infected mice (Huffnagle *et al*, submitted), it is not clear what role CCR5 plays on other cells in the CNS such as microglial cells and endothelial cells (Rottman *et al*, 1997).

Research is beginning to define the roles of specific chemokines in immunity and leukocyte recruitment into the central nervous system during *C. neoformans* infection. It is now becoming clear that inflammatory responses in the CNS during *C. neoformans* infection are protective and function to clear disseminated infection. Chemokines will be major mediators of leukocyte recruitment into the CNS. However, as demonstrated by the studies of MIP-1α and CCR5 knockout mice, many chemokines will likely be pleiotropic and their function determined by (1) the timing of expression, (2) the cytokine milieu at the time of expression, and (3) the organ in which they are expressed.

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