Journal of NeuroVirology, 10(suppl. 1): 118–124, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490270798

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Role of interleukin-4 and monocyte chemoattractant protein-1 in the neuropathogenesis of X4 simian human immunodeficiency virus infection in macaques

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Recent studies on the coreceptor usage of human immunodeficiency virus (HIV) strains associated with acquired immunodeficiency syndrome (AIDS) dementia have shown that both X4 and R5 viruses are involved in the process. The disease is associated with enhanced virus replication and monocyte chemoattractant protein (MCP)-1 production in macrophages in the brain. Using the macaque model of the disease, the authors show here that X4, macrophage-tropic simian human immunodeficiency virus (SHIV) required the enhancing effect of interleukin (IL)-4 to achieve equivalent concentrations of virus and MCP-1 that are produced in macrophages infected with R5 viruses alone. Confocal microscopy showed that macrophages in the encephalitic brains were the major producers of MCP-1. The authors surmise, therefore, that whereas R5 viruses maybe capable of causing the disease as a primary pathogen, X4 viruses may require IL-4, induced by opportunistic pathogens, for induction of the neuropathological syndrome. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 118–124.

Keywords: chemokine; macrophages; SHIV encephalitis

Introduction

Animal models of simian immunodeficiency virus (SIV) and simian human immunodeficiency virus (SHIV) infection in macaques have provided excellent analogs of human immunodeficiency virus (HIV) encephalopathy and facilitate longitudinal studies on the pathogenesis (Lackner *et al*, 1991; Ringler *et al*, 1986; Sharer *et al*, 1988). Macrophages are the chief target cells of the virus in the brain, and although CCR5 is the major coreceptor for HIV-1 infection in these cells (Albright *et al*, 1999; Gabuzda and Wang, 1999; Ghorpade *et al*, 1998; He *et al*, 1997), recent studies by Gabuzda *et al* (1998) have shown that CXCR4 can also mediate efficient virus entry into microglia and suggest that X4 viruses in brain may be more prevalent than originally perceived (Gorry *et al*, 2001). Thus, macrophage tropism rather than coreceptor usage may be more important for causing the disease,

Histologically, characteristic pathological findings of lentiviral encephalitis in primates consist of microglial foci, dense infiltration of mononuclear cells (including monocyte/macrophages) in perivascular sites and in the brain parenchyma, and formation of syncytia comprising mainly of infected, fused cells bearing the macrophage marker (Bell, 1998;

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The work reported here was supported by grants MH62969 (SJB), RR16443-02 (SB and ON), NS-32203, AI-138492, RR-06753, and RR-13152 (ON) from the National Institutes of Health.

Received 14 February 2003; revised 18 March 2003; accepted 7 April 2003.

Gendelman *et al*, 1994; Nath, 1999). Although it is well established that the virus invades the brain during the early stages of the systemic infection, development of HIV encephalitis (HIV-E) is usually a late event when there is substantial viral replication in the central nervous system (CNS) (Sanders *et al*, 1998; Wiley *et al*, 1999). The factors leading to the productive virus replication and associated pathological changes in the brain during HIV-E remain largely elusive.

Whereas R5 viruses seem capable of causing primary encephalitis, our earlier studies showed that encephalitis associated with macrophage-tropic X4 viruses was invariably associated with coinfection in the brain with opportunistic pathogens such as cytomegalovirus (CMV), *Toxoplasma*, and simian virus 40 (SV40). Furthermore, X4 virus encephalitis was usually associated with expression of interleukin (IL)-4. Using *Schistosoma mansoni* eggs as an experimental surrogate of opportunistic pathogens, we showed that X4 virus infection, combined with inoculation of the animals with *S. mansoni* eggs, resulted in the development of lentiviral lesions in the CNS and lungs (Buch *et al*, 2001). Lesions in both organs were associated with increased virus replication in macrophages and in a milieu rich in the Th2 cytokines, IL-4 and IL-10. This suggested that the neuropathogenesis of X4 virus infections may be contingent on the presence of Th2 cyokines in the brain. Experimentally, IL-4 causes enhanced replication of X4 virus in macrophages by mechanisms that include enhanced expression of the X4 RNA (Hicks *et al*, 2002). The cytokine also causes enhanced expression of the secretory CC chemokine, monocyte chemoattractant protein (MCP)-1, in infected macrophage cultures (Hicks *et al*, 2002). MCP-1 has been shown to be the most potent among other monocytic chemoattractants, such as RANTES, macrophage inhibitory protein (MIP)-1α, MIP-1β, MCP-2, and MCP-3, at inducing the transmigration of monocytes across a model of the blood brain barrier (Uguccioni *et al*, 1995).

Selective accumulation of MCP-1, but not other chemokines, has been demonstrated in the cerebrospinal fluid (CSF) and brain parenchyma of patients with acquired immunodeficiency syndrome (AIDS) dementia (Conant *et al*, 1998), in HIV-E (Sanders *et al*, 1998), in SHIV encephalitis (SHIV-E) (Hicks *et al*, 2002), and also in the CSF of macaques with SIV encephalitis (SIV-E) (Zink *et al*, 2001). In the present study, we examined the identity of cells in the brain of macaques with SHIV-E that contributed to the accumulation of MCP-1.

Results

Replication of X4 and R5 viruses in rhesus macrophages in the presence and absence of recombinant macaque (rm) IL-4

Because X4 strains of HIV have also been identified in HIV-E, we used macrophage tropic strains of SHIV that use the X4 coreceptor to explore mechanisms of neuropathogenesis of the infection in macaques. Our earlier studies showed that the neuropathogenic potential of X4 SHIVs was contingent upon concurrent coinfection in the brain with opportunistic pathogen(s) (Buch *et al*, 2002). The neuropathological changes in these brains were indistinguishable from those caused by R5 agents, suggesting that the role of opportunistic pathogens in X4-mediated CNS disease may have been to promote replication of lentivirus in macrophages in the brain. IL-4 induced by opportunistic pathogens is a likely candidate that promoted virus replication in the brain.

The role of IL-4 in promoting X4 virus replication in macrophages was first reported by Valentin *et al* (1998) and confirmed later in our laboratory in studies using *S mansoni* eggs (powerful inducers of Th2 cytokines) as a surrogate of an opportunistic pathogen (Buch *et al*, 2001).

Based on the studies described earlier, we then explored the effect of exogenous IL-4 on the replicative capacity of both X4 and R5 viruses in primary macrophage cultures from rhesus macaques. Monocyte-derived macrophages (MDMs) were inoculated with X4 ($SHIV_{KU-2}$ and $SHIV_{89.6}P$) and R5 ($SIV_{smm}PGM$) viruses at a multiplicity of 0.1 in the presence or absence of rm IL-4, and supernatant fluids examined for virus content at various times after inoculation. As demonstrated earlier (Hicks *et al*, 2002) and as seen in Figure 1A and B, the X4 viruses replicated efficiently and addition of IL-4 caused a further enhancement of replication of the X4 viruses in the macrophage cultures. IL-4 had minimal effects on the replication of the R5 virus (Figure 1B).

Effect of virus infection in the presence and absence of rm IL-4 on MCP-1 secretion by rhesus MDMs

To investigate whether virus infection in macrophages would result in stimulation of the cells to produce MCP-1 and whether the amount of MCP-1 produced varied with the virus variant, we inoculated MDMs with X4 ($SHIV_{KU-2}$ and $SHIV_{89.6}P$) and R5 ($SIV_{smm}PGM$ and $SIV_{mac}251$) viruses in the presence and absence of IL-4 and measured production of MCP-1 in the cultures. As seen in Figure 1C, MCP-1 was constitutively expressed by uninfected MDMs but infection with X4 and R5 viruses led to an increase in the secretion of the chemokine. R5 viruses were better inducers of MCP-1 than X4 viruses. Although addition of IL-4 to uninfected cultures also enhanced secretion of the chemokine, the effect of the cyokine was more pronounced in rhesus MDMs infected with the X4 viruses. MCP-1 levels in cultures infected with R5 virus and treated with the cytokine showed no increase in MCP-1 secretion compared to that in R5-infected cells without the cytokine (Figure 1D). Thus, the effect of IL-4 on replication of macrophage-tropic X4 viruses was to achieve equivalent levels of virus and MCP-1

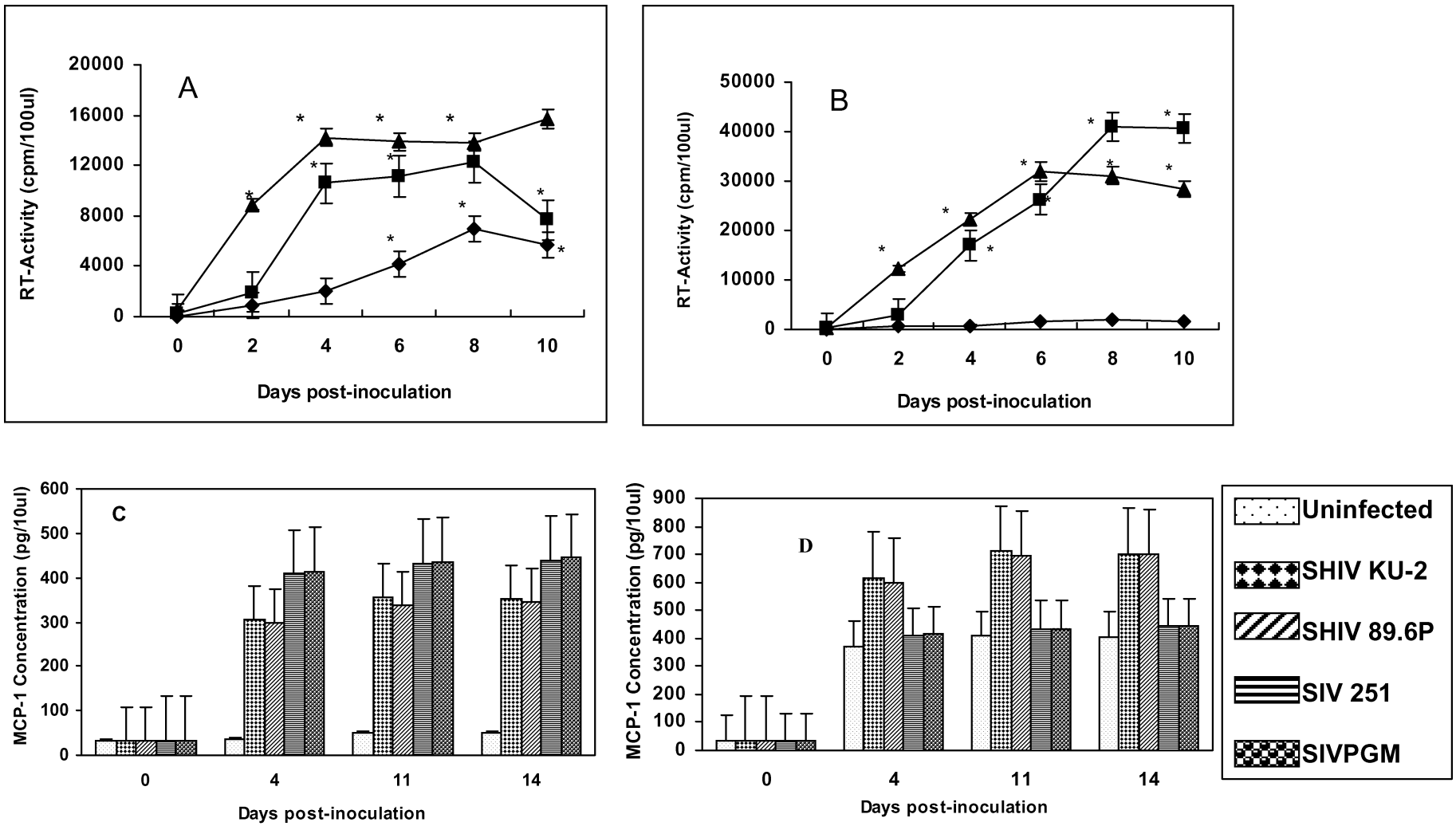

Figure 1 (**A** and **B**) Reverse transcriptase activity in supernatant fluids of macrophage cultures from rhesus macaques inoculated with $SHIV_{KU-2}$ (▲), $SHIV_{89.6}P$ (■), and SIVsmmPGM (♦) in the absence (**A**) or in the presence (**B**) of rm IL-4. Macrophage cultures from three animals were inoculated with each of the three viruses and analyzed in triplicate. Error bars represent standard deviations. *, Highly significant levels ($p < 0.05$, one-way analysis) compared to day 0 cultures. (**C** and **D**) MCP-1 ELISA analysis of supernatant fluids from uninfected macrophages and from $SHIV_{KU-2}$, $SHIV_{89.6}P$, $SIV_{mac}251$, and SIVsmmPGM-inoculated MDMs in the absence (**C**) or in the presence (**D**) of rm IL-4. Samples were collected on day 4, 11, 14 after inoculation. Data are shown as mean ± SE for three replicates. Figure 1 A–D were reproduced from Hicks *et al* Amer. J. Pathol. (2002) 161: 813–822 with permission of the publishers. (**E**) Samples of brain RNA from basal ganglia region from two uninfected animals (1 and 2), two SHIV-infected but non-encephalitic animals (3 and 4) and two SHIV-infected encephalitic animals (5 and 6) were subjected to RT-PCR analysis using macaque specific MCP-1 primers. The primer set MCP-1 was 5′-CTC GCT CAG CCA GAT GCA ATC -3′ (position 117–137) and 5′-GGG GTA GAA CTG TGG TTC AAG AGG-3′ (position 569–592), yielding a product of 475 bp.

in the cells as those caused by macrophage-tropic R5 viruses alone.

Increased MCP-1 RNA in the brains of macaques with SHIV-E

MCP-1 has recently been identified as one of the chemokines that is present in increasing amounts in the brains and CSF of patients with lentiviral encephalitis (Conant *et al*, 1998; Zink *et al*, 2001), and is known to be secreted by a variety of cells in the brain. The *in vitro* findings of lentiviral infection leading to enhanced MCP-1 secretion correlate well with our studies on SHIV-E caused by X4 viruses. Increased expression of MCP-1 RNA by reverse transcriptase–polymerase chain reaction (RT-PCR) was clearly demonstrated in the brains of macaques with SHIV-E as compared to SHIV-infected macaques without encephalitis (Figure 1E). SHIV-infected macaques without encephalitis did express low levels of MCP-1. In contrast, uninfected normal macaques lacked MCP-1 RNA.

Macrophages as the source of increased MCP-1 in the brains of macaques with SHIV-E

To investigate the source of increasing levels of MCP-1, we examined five brains from SHIV-infected macaques that succumbed to AIDS. Two of the four animals had developed CNS disease and had lentiviral encephalitis associated with opportunistic infections. The other two animals that died with AIDS had neither any CNS lesions nor infections with opportunistic pathogens. We used sections of brains from these animals to investigate whether MCP-1 production was enhanced only in the encephalitic brains, and if so, to identify the cells responsible for the enhanced production of the chemokine. Using indirect immunofluorescence with antibodies to MCP-1, we found that MCP-1 antigen was expressed only in the encephalitic brains. Confocal microscopy of these sections showed that perivascular macrophages that stained positively with macrophage specific Ham56 antibodies also were positive for the MCP-1 antigen (Figure 2A). In addition to perivascular macrophages, macrophages in the microglial nodules also stained positively MCP-1 antigen (data not shown). Astrocytes surrounding the blood vessels were negative for MCP-1 (data not shown). Because virus infection is known to cause an upregulation of MCP-1 expression in rhesus macrophage cultures, it was of interest to assess whether MCP-1–expressing perivascular macrophages were also expressing the virus protein. To answer this question, confocal microscopy of the brain sections was performed and, as shown in Figure 2B, perivascular macrophages that stained positively with MCP-1 antigen also stained positively for the virus p27 antigen.

Discussion

MCP-1 has recently been identified as one of the chemokines that is present at very high levels in the brains and CSF of individuals with lentiviral encephalopathy (Conant *et al*, 1998; Zink *et al*, 2001). The role of MCP-1 in monocytic infiltration *in vivo* is substantiated by several studies (Lahrtz *et al*, 1997; Fuentes *et al*, 1995; Bell *et al*, 1996). Earlier findings from our laboratory have demonstrated that the development of X4 virus–mediated encephalitis in rhesus macaques was contingent upon two factors: productive virus replication in brain macrophages and an optimal threshold of MCP-1 concentration in the brain. R5 viruses that cause primary encephalitis achieve this threshold by virtue of infection. In contrast, SHIV-E is usually associated with coinfection with opportunistic pathogen(s) and expression of IL-4 (Buch *et al*, 2002), a factor that enhances MCP-1 expression in X4-infected but not R5-infected macrophages (Hicks *et al*, 2002). These findings therefore led us to surmise that the attainment of optimal MCP-1 concentrations needed to initiate neuropathogenesis may have required virus replication and IL-4 help. Whether development of HIV-E by X4 viruses is also contingent upon IL-4 help remains poorly understood. In an earlier report examining cytokine gene expression in HIV-E, Wesselingh *et al* (1993) had shown decreased levels of IL-4 in the brain. However, in this latter report, HIV-E cases that were coinfected with opportunistic coinfections were not included in the study and this may, in part, explain the discrepancy between the two findings. It is possible that only cases with R5 variants (that do not need coinfection for manifestation of encephalitis) in the brains were evaluated in this human study. Furthermore, the fact that R5 variants are known to be more prevalent in the brains of patients with HIV-E than the X4 variants could, in part, explain the lack of IL-4 in the brains of patients with HIV-E.

In order to understand the source of MCP-1in the brain of macaques with SHIV-E, we had performed *in situ* hybridization and immunohistochemistry and had demonstrated that MCP-1–producing cells in the brains mainly confined to microglial nodules and that the majority of the cells in these nodules were productively infected macrophages (Hicks *et al*, 2002). The present report demonstrates expression of MCP-1 protein in the perivascular macrophages of the brains of macaques with SHIV-E. Accumulation of the maturing monocytes in perivascular locations at their point of entry into the brain is perhaps the earliest morphological evidence of the recruitment process and is followed later by accumulations of macrophages close to the blood vessel in the brain parenchyma as microlgial nodules. One of plausible explanations for the neuropathogenic potential of X4 SHIVs could be the enhanced expression of MCP-1 following X4 SHIV infection and

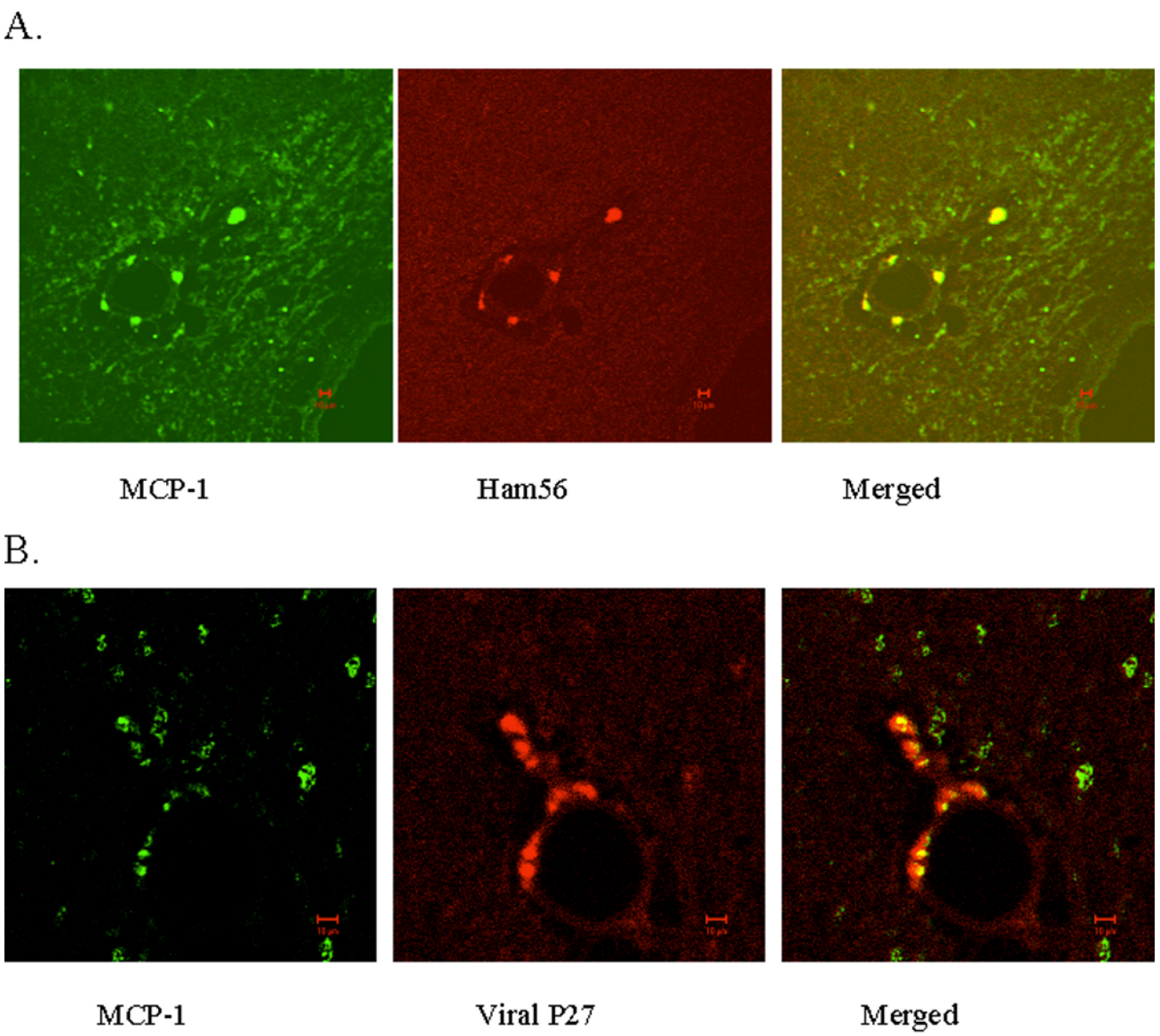

Figure 2 (**A**), Colocalization of macrophage- and MCP-1–specific antibodies, in the brain of macaque with SHIV-E. Brain sections were first stained with primary antibody, C17, goat anti-human MCP-1 antibody, followed by treatment with Alexa Fluor 488 donkey anti-goat secondary antibody. The sections were then stained with another primary antibody, Ham56, a mouse anti-human macrophage-specific antibody, followed by treatment with Alexa Fluor 594 goat anti-mouse antibody. Stained sections were examined under a fluorescence microscope with appropriate filters. (**B**), Colocalization of MCP-1–specific antibody, C17, and viral p27 protein in the perivascular cuff in the brain of macaque with SHIV-E. Brain sections were first stained with primary antibody, C17, goat anti-human MCP-1 antibody, followed by treatment with Alexa Fluor 488 donkey anti-goat secondary antibody. The sections were then stained with another primary antibody, viral p27 antibody, followed by treatment with Alexa Fluor 594 goat anti-mouse antibody. Stained sections were examined under a fluorescence microscope with appropriate filters.

IL-4 expression and this, in turn, could lead to enhanced recruitment of macrophages during SHIV-E. Corroboration of the role of MCP-1 in SHIV-E comes from findings of Conant *et al* (1998) demonstrating an up-regulation of MCP-1 in the brains of patients with HIV-1–associated dementia (Conant *et al*, 1998). However, the source of MCP-1 in this study were the astrocytes and the neurons and not macrophages. Unlike the human brain though, studies reported here suggest that macaque astrocytes around the blood vessels were not a source of MCP-1 in SHIV-E. Although it can be argued that $CD4^+$ T cells that are capable of producing MCP-1 and that are permissive for X4 viruses could also be contributing to the MCP-1 pool during SHIV-E, the fact that these cells are usually absent during the onset of encephalitis (Joag *et al*, 1997) rules them out as a source of MCP-1 in the brain. Support of this comes from our findings demonstrating lack of $CD4^+$ T cells in the brains of macaques with SHIV-E (Buch *et al*, unpublished data). Interestingly, however, the X4 viruses are permissive for rhesus macrophages

(Hicks *et al*, 2002). This supports a basic tenant that macrophage-tropism, irrespective of coreceptor usage, predicts HIV-1 neurotropism (Gorry *et al*, 2001).

Taken together, the studies presented here strongly suggest that IL-4 has a major role in promoting neuropathogenesis of macropahge-tropic X4 viruses. By virtue of its ability to enhance virus replication and MCP-1 production in macrophages, IL-4 may have contributed to optimal virus and chemokine concentrations in the brain necessary for causing the CNS disease.

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