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Short Communication

Chemokine Receptor Expression on Resident and Inflammatory Cells in the Brain of Macaques with Simian Immunodeficiency Virus Encephalitis

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Although the mechanisms of human immunodeficiency virus (HIV) neuroinvasion, neuronal injury, and subsequent development of HIV-1-associated AIDS dementia complex are not fully understood, a correlation between monocyte/macrophage infiltrates in the brain and neurological disease exists. In light of the many potential roles that chemokines and chemokine receptors may play in HIV neuropathogenesis, we sought to describe their pattern of expression in the SIV-infected rhesus macaque model of HIV encephalitis. We previously demonstrated elevated expression of the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES, and interferon-inducible protein (IP)-10 in brain of macaque monkeys with SIV encephalitis. In this study, we demonstrate that the corresponding chemokine receptors CCR3, CCR5, CXCR3, and CXCR4 are expressed in perivascular infiltrates in these same tissues. In addition, we detected CCR3, CCR5, and CXCR4 on subpopulations of large hippocampal and neocortical pyramidal neurons and on glial cells in both normal and encephalitic brain. These findings suggest that multiple chemokines and their receptors contribute to monocyte and lymphocyte recruitment to the brain in SIV encephalitis. Furthermore, the expression of known HIV/SIV co-receptors on neurons suggests a possible mechanism whereby HIV or SIV can directly interact with these cells, disrupting their normal physiological function and contributing to the pathogenesis of AIDS dementia complex. ***(Am J Pathol 1998, 152:659–665)***

Human immunodeficiency (HIV)-1-associated AIDS dementia complex (ADC) is a debilitating disease seen in 25% of AIDS patients, which manifests as a triad of cognitive, behavioral, and motor deficits.[1,2] The severity of the clinical neurological disease has been correlated with several different pathological features, including the presence of virus in the brain,[3] dendritic alterations in cortical neurons,[4] the spatial pattern of neurons,[5] and the extent of neuronal cell loss.[6] Although it has been reported that all patients with HIV encephalitis (defined by the presence of perivascular cuffs of macrophages and multinucleated giant cells (MNGCs)), have ADC, not all patients with ADC have HIV encephalitis.[7] Subsequent studies have shown that the best histopathological correlate of ADC is the number of macrophages in the brain.[8] These studies suggest that even subtle increases in the number and activation state of macrophages in the brain are sufficient to cause neurological dysfunction and represent an early manifestation in the progression to fulminant MNGC encephalitis.[9] Hence, the brain macrophage is a key component of current theories concerning the neuropathogenesis of HIV infection.[8,10–12] Therefore, a better understanding of the mechanisms responsible for recruitment of leukocytes in general, and of monocyte/macrophages in particular, into the brain is critical for understanding both neuroinvasion by HIV and the neuropathogenesis of ADC.

Simian immunodeficiency virus (SIV) has extensive sequence homology and similar genomic organization, morphology, and biological properties to HIV-1 and HIV-2.[13] Similar to HIV-1, the targets for SIV infection *in vivo* and *in vitro* are monocyte/macrophages and lymphocytes.[13,14] Moreover, SIV-infected macaques develop an encephalitis virtually indistinguishable from that observed

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in HIV-infected people, particularly infants and children.[14,15] Thus, the SIV-infected macaque is the premier animal model for examining the neuropathogenesis of HIV.[2,9] Using this model, we have shown that neuroinvasion by SIV is associated with an increase in the number of perivascular macrophages in the brain.[16] Moreover, we have shown an association between the presence of elevated endothelial vascular cell adhesion molecule-1 (VCAM-1) and perivascular cellular infiltrates in encephalitic brain in SIV-infected macaques and HIV-1-infected humans.[10,17] Subsequently, we demonstrated elevated endothelial and/or perivascular macrophage expression of the chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β, RANTES (regulated on activation, normal T cell expression presumed secreted), and interferon-inducible protein (IP)-10 compared with that observed in SIV-infected, nonencephalitic animals.[18] To further characterize the mechanisms of leukocyte recruitment to the brain and the neuropathogenesis of HIV/SIV infection, we have examined the expression of chemokine receptors of MIP-1α, MIP-1β, and RANTES (CCR3 and CCR5) and IP-10 (CXCR3) in brain from SIV-infected rhesus macaques with and without SIV encephalitis and compared it with that in uninfected animals. In addition, we examined the expression of the chemokine receptor CXCR4, which is the receptor for stromal-cell-derived factor (SDF)-1.[19,20]

Expression of CCR3, CCR5, and CXCR4 was abundant on perivascular macrophages and MNGCs in brain from macaques with SIV encephalitis. In contrast, CXCR3 expression was limited to scattered small mononuclear cells morphologically compatible with lymphocytes. In addition to inflammatory cell expression of CCR3, CCR5, and CXCR4, these receptors were detected on neurons and glial cells in encephalitic and normal brain. We also observed that CCR3 and CCR5 expression was increased in microglia and astrocytes in encephalitic brain. The presence of chemokine receptors in perivascular infiltrates in brains of macaques, which were previously shown to express the corresponding chemokine ligands (MIP-1α, MIP-1β, RANTES, and IP-10) and elevated endothelial VCAM-1, supports the notion that virus-infected cells are actively recruited to the brain. Furthermore, these receptors can also function as co-receptors for HIV and SIV infection in microglia.[21] In addition, the expression of known HIV/SIV co-receptors CCR3, CCR5, and CXCR4 on neurons suggests a possible mechanism whereby HIV/SIV can directly interact with these cells through chemokine receptors, possibly disrupting normal physiological function and contributing to the pathogenesis of ADC.

Materials and Methods

Animals and Virus

Twenty-one macaque monkeys previously used to define chemokine expression in the brain[18] were used in this study. Sixteen of these twenty-one animals were SIV infected. Twelve of these sixteen animals had SIV encephalitis (SIVE), whereas four did not. Details of viral inoculum, route of infection, disease course, and histological analysis of tissues have been previously published.[17,18,22,23] All SIV-infected animals showed clinical signs of AIDS at death. An additional five uninfected animals were used as controls.

Immunohistochemical Analysis

During necropsy, brain sections were bisected and one-half preserved in 10% neutral buffered formalin and the other one-half embedded in O.C.T. Compound (Miles, Elkhart, IN) and snap-frozen by immersion in 2-methylbutane cooled in dry ice and stored at −70°C. The frozen brain sections taken adjacent to the formalin-fixed, paraffin-embedded brain sections used previously to examine chemokine expression in the brain of SIV-infected macaques with encephalitis[18] were used in this study. Monoclonal antibodies to CCR3, CCR5, and CXCR3 were provided by LeukoSite, Cambridge, MA. The generation and characterization of these antibodies have been previously described.[24–26] Monoclonal antibody to human fusin (CXCR4) was purchased from a commercial source (PharMingen, San Diego, CA). Immunohistochemical expression of chemokine receptors CCR3, CCR5, CXCR3, and CXCR4 was examined using avidin-biotin-alkaline phosphatase complexes (Biogenex, San Ramon, CA) with Fast Red chromogen (Biogenex) and Mayer's hematoxylin counterstain as previously described.[27] Corresponding irrelevant monoclonal antibodies were used as negative controls for each section.

Results

CCR3, CCR5, CXCR3, and CXCR4 Are Expressed in Cellular Infiltrates in SIV Encephalitis

In all 12 animals with SIV encephalitis, the perivascular lesions (composed primarily of macrophages and MNGCs with lesser numbers of lymphocytes) expressed CCR3, CCR5, and CXCR4 (Figure 1). Most of the cells in each of the perivascular lesions had cytoplasmic immunostaining for these antigens. These CCR3, CCR5, and CXCR4 immunopositive cells were large with abundant cytoplasm and often had multiple nuclei compatible with macrophages and MNGCs. Scattered small cells compatible with lymphocytes were also intensely immunopositive for CCR3, CCR5, and CXCR4. In addition, CXCR3 expression was limited to these small cells. Previously, we demonstrated that perivascular lesions in macaques with SIVE contain 5 to 10% CD8-positive lymphocytes.[14] This is consistent with the observation that CXCR3 is expressed predominantly on activated $CD8^+$ lymphocytes.[25] The morphology, distribution, and number of CXCR3-expressing cells within the encephalitic lesions are comparable to the morphology, distribution, and number of $CD8^+$ lymphocytes. The distribution and expression of these chemokine receptors within the

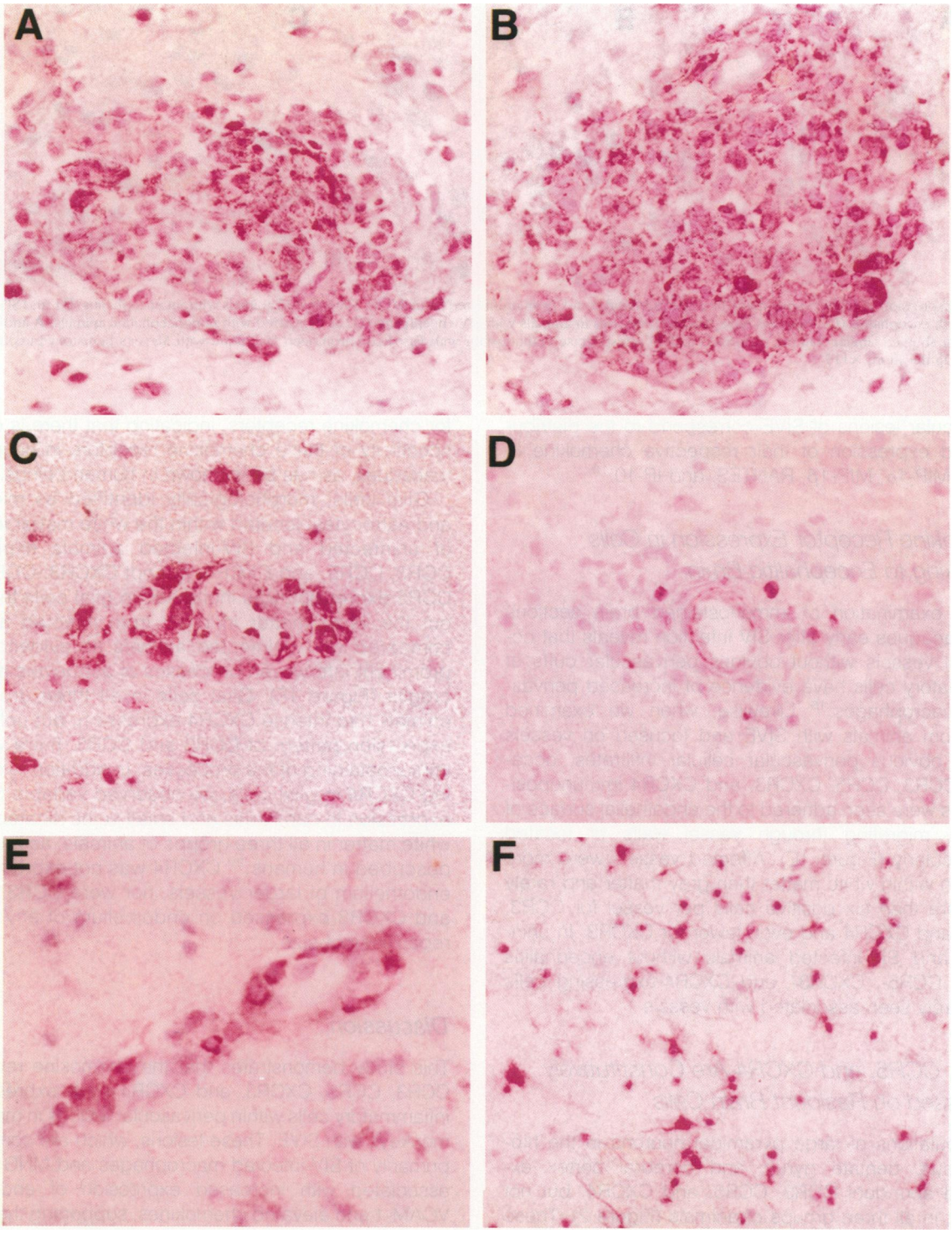

Figure 1. Immunohistochemical expression of chemokine receptors in animals with SIV encephalitis. Macrophages and MNGCs in perivascular lesions are intensely immunopositive for CCR3 (**A**), CCR5 (**B**), and CXCR4 (**C**). CXCR3 expression was restricted to small mononuclear cells compatible with lymphocytes (**D**). Occasionally, vessels without distinct perivascular cellular infiltrates had CCR5-immunopositive inflammatory cells in the perivascular space (**E**). Glial cell expression of CCR3 was increased in SIV-infected animals (**F**) compared with uninfected normal brain. Alkaline phosphatase technique with Fast Red chromogen and Mayer's hematoxylin counterstain; original magnification, ×115 (**A**, **C**, and **F**) and ×140 (**B**, **D**, and **E**).

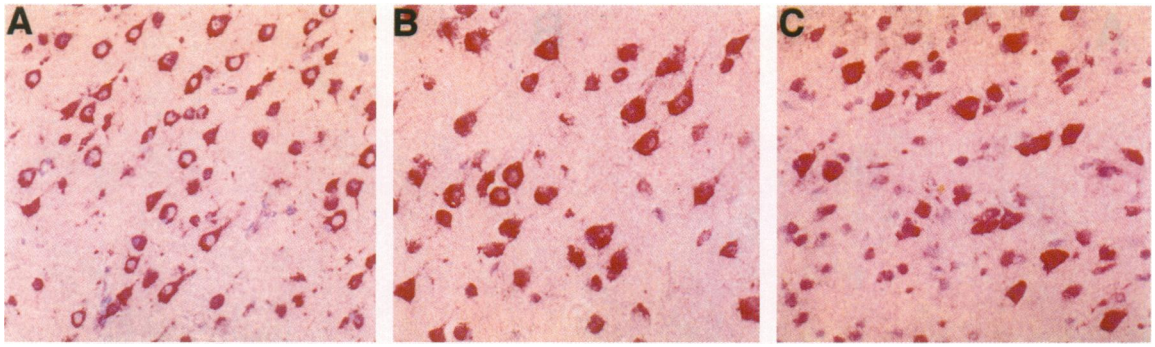

Figure 2. Expression of CCR5 on pyramidal neurons in frontal cortex from uninfected animals (**A**), SIV-infected animals without encephalitis (**B**), and SIV-infected animals with encephalitis (**C**). Neurons have blunted processes in animals with SIV encephalitis (**C**) as compared with nonencephalitic animals (**A** and **B**); CCR3 and CXCR4 showed similar staining patterns. Alkaline phosphatase immunostain with Fast Red chromogen technique with Mayer's hematoxylin counterstain; original magnification, ×115.

perivascular lesions of SIVE correspond to the distribution and expression of their respective chemokine ligands, MIP-1α, MIP-1β, RANTES, and IP-10.[18]

Chemokine Receptor Expression in Cells Trafficking to Encephalitic Brain

Careful examination of immunostained brain sections from macaques early after SIV infection reveals that occasional vessels without obvious perivascular cuffs of inflammatory cells have evidence of increased perivascular macrophages.[16] Likewise, when we examined brain from animals with SIVE and focused on vessels without obvious perivascular cellular infiltrates, occasional CCR3, CCR5, CXCR3, and CXCR4 immunopositive cells were seen adhered to the endothelial surface of vessels, migrating through vessel walls or located perivascularly (Figure 1E). Affected vessels were more common within white matter than gray matter and rarely had more than six positive cells per vessel for CCR3, CCR5, and CXCR4 and even fewer for CXCR3. In uninfected and SIV-infected animals without encephalitis CCR3-, CCR5-, CXCR3-, and CXCR4-expressing cells were rarely seen associated with vessels.

CCR3, CCR5, and CXCR4 Are Constitutively Expressed on Resident Brain Cells

Subpopulations of large pyramidal neurons in the hippocampus, dentate gyrus, and cerebral cortex expressed abundant CCR3, CCR5, and CXCR4, but not CXCR3, in all three groups of animals (Figure 2). These neurons exhibited intense and diffuse cytoplasmic staining of the cell bodies and their processes. However, subtle differences were noted in cytoplasmic staining of CCR3, CCR5, and CXCR4 between animals with SIVE and animals without encephalitis. Staining for CCR3, CCR5, and CXCR4 in brain of uninfected and SIV-infected macaques without encephalitis showed that large pyramidal neurons exhibited discernible dendritic processes, whereas the same neurons in encephalitic brain had poorly defined dendritic processes (Figure 2, A and C). Small interneurons in the same regions did not label for chemokine receptors, indicating that there is cellular specificity of these chemokine receptors in neurons in macaques, as has been shown in human beings.[28]

Glial cells, morphologically identified as microglia and astrocytes, located within the white matter in brain of uninfected and SIV-infected animals expressed CCR3, CCR5, and CXCR4, but not CXCR3. CCR3 and CCR5 expression was increased in glial cells throughout the white matter in brain from infected animals compared with uninfected animals as evidenced by prominent interwoven processes of astrocytes and microglia (Figure 1F). Glial cells in all three groups of animals had intense CXCR4 expression, but unlike the expression pattern of CCR3 and CCR5, there was no immunostaining of the processes. In addition to detecting CXCR4 in glial cells, we observed variable CXCR4 expression in capillaries and small veins located in the white matter in all three groups of animals, as has been described in humans.[29] CXCR4 was not expressed on endothelium of larger vessels, nor were CCR3, CCR5 and CXCR3 expressed on endothelium of any vessel regardless of size.

Discussion

This study demonstrates that the chemokine receptors CCR3, CCR5, CXCR3, and CXCR4 were expressed in inflammatory cells within perivascular lesions in macaque monkeys with SIVE. These lesions, which are composed primarily of SIV-infected macrophages and MNGCs, are associated with increased expression of endothelial VCAM-1 and elevated chemokines, supporting the notion that these monocytes and lymphocytes are actively trafficking to brain in animals with SIVE.[9,17,18] Similar studies in patients with HIV encephalitis demonstrated elevated cytokines,[30] chemokines (MIP-1α and MIP-1β),[12] and adhesion molecules[10] in the brain. Of these, the CC chemokines are potent chemoattractants for monocytes and lymphocytes[31] and, in conjunction with cytokine-induced up-regulation of adhesion molecules, provide a likely mechanism for monocyte and lymphocyte recruitment to the brain in HIV-infected patients. Our demonstration of the CC chemokine receptors (CCR3 and CCR5) for

RANTES, MIP-1α, and MIP-1β within encephalitic brain is additional evidence for the theory of selective trafficking of monocytes and lymphocytes to the brain in HIV/SIV encephalitis. In addition to the CC chemokines, these same lesions were previously shown to contain abundant immunohistochemical expression of the CXC chemokine IP-10.[18] IP-10 is unique among the CXC chemokines in that it attracts monocytes and T lymphocytes but not neutrophils.[32,33] CXCR3, the chemokine receptor for IP-10 and MIG,[32] was demonstrated within these lesions on small mononuclear cells morphologically compatible with lymphocytes. The CXCR3-positive cells are most likely CD8-positive T lymphocytes because CXCR3 is expressed predominantly on activated CD8-positive T lymphocytes,[25] and perivascular lesions in macaques with SIVE contain between 5 and 10% CD8-positive T lymphocytes.[14] These findings suggest that cytotoxic CD8-positive T lymphocytes are selectively recruited to the brain in response to IP-10 expression by activated perivascular inflammatory cells. IP-10 has also been implicated in other T-lymphocyte-mediated responses in the brain, such as experimental autoimmune encephalomyelitis.[34] Thus, together with our previous findings of increased adhesion molecule and chemokine expression in brain from SIV-infected macaques with encephalitis, expression of the corresponding chemokine receptors on perivascular inflammatory cells suggests that one or all of these chemokines and their receptors contribute to leukocyte recruitment to the brain in SIVE.

It is increasingly apparent that expression of chemokine receptors is not limited to cells of the immune system, but includes a wide distribution of cell types.[27–29,35] In addition to chemokine receptor expression in inflammatory cells, we found abundant constitutive expression of CCR3, CCR5, and CXCR4 in subpopulations of large pyramidal hippocampal and cortical neurons in infected and uninfected animals. We also found that glial cells, morphologically compatible with parenchymal microglia and astrocytes, in brain from infected and uninfected animals were strongly immunopositive for CCR3, CXCR4, and CCR5. Our observation that CCR3, CCR5, and CXCR4 are expressed in pyramidal neurons and glial cells in normal macaque brain contributes to our understanding of chemokine receptor distribution in normal tissues. Moreover, the observation that CCR3, CCR5, and CXCR4 expression is limited to a subpopulation of large neurons in macaques is consistent with other studies showing that subsets of projection neurons in diverse regions of human brain and spinal cord express the chemokine receptors CCR1, CXCR2, and DARC.[28] These findings indicate that there is cellular specificity of chemokine receptor expression in subpopulations of neurons.[28] Detection of CCR3, CCR5, and CXCR4 on glial cells in our study is not unique to macaque monkeys. Human microglia have been shown to express CCR3,[21] and human microglia and astrocytes express CXCR4[29] and CCR5.[27] It has been postulated that chemokines secreted by glial cells interact with their corresponding chemokine receptors to maintain normal cellular function and/or mediate communication between glial cells and specific subpopulations of neurons.[28] A functional role for CXCR4 in normal brain cells is supported by the findings that SDF-1 induces calcium mobilization in mouse microglia and astrocytes[36] and that chemokines induce chemotaxis of hNT neural cells through chemokine receptors including CXCR4.[35] Clearly, additional research is required to fully understand the roles that chemokines and their receptors play in normal brain cells.

In addition to serving as mediators of inflammation, multiple chemokine receptors are used by HIV-1, HIV-2, and SIV for entry of CD4-positive cells.[37–45] Of these chemokine receptors, among the most important is CCR5, which is used by SIV, macrophage-tropic, nonsyncytial inducing HIV-1, and HIV-2.[40–42,44–47] In this study we demonstrate abundant CCR5 expression in the SIV-infected perivascular macrophages and MNGCs in the animals with SIVE. We postulate that activated perivascular cells, which have increased CCR5 expression, are attractive targets for SIV infection and replication. Although CCR3 and CCR5 have been shown to function as co-receptors for HIV-1 infection of microglia *in vitro*,[21] their *in vivo* role as co-receptors has not been determined. In brain from macaques with SIVE, expression of CCR3 and CCR5 was markedly increased in parenchymal microglia, but whether or not these cells were SIV infected was difficult to discern as most virus-positive cells were concentrated in perivascular foci. There is also *in vitro* evidence that CD4-negative brain capillary endothelial cells are permissive for HIV[46] and SIV.[47] Recent *in vitro* data suggest that SIV can use CCR5 in the absence of CD4 for infection of brain capillary endothelial cells,[48] but the importance of endothelial infection *in vivo* remains controversial. In this study we did not observe any appreciable expression of CCR5 on endothelium in any brain sections.

Taken together, these observations suggest several possible mechanisms whereby chemokine receptors contribute to the pathogenesis of HIVE and SIVE. First, chemokine receptors could facilitate trafficking of monocyte/macrophages across the blood-brain barrier increasing the likelihood of HIV/SIV-infected cells being able to enter the brain. Second, these activated macrophages trafficking to the brain have increased CCR5, CCR3, and CXCR4 expression, making them vulnerable to HIV/SIV infection. Third, activated perivascular macrophages in the brain may release neurotoxic cytokines, perpetuating a cycle of neuronal injury and loss. Fourth, chemokine receptors may allow direct interaction of HIV and SIV or viral proteins, such as gp120, with neurons, which may alter cell signaling and interrupt normal cellular functions. These observations contribute to our understanding of the possible roles chemokine receptors have in normal brain and in the pathogenesis of HIV/SIV encephalitis.

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