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MCP-3/CCL7 production by astrocytes: implications for SIV neuroinvasion and AIDS encephalitis

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Abstract Monocyte/macrophages and activated lymphocytes traffic through normal brain, and this trafficking is increased in inflammatory conditions such as HIV encephalitis (HIVE). HIVE is characterized in part by perivascular accumulations of macrophages. The earliest events in this process are poorly understood and difficult or impossible to address in humans. The SIV-infected macaque model of neuroAIDS has demonstrated migration of monocytes into the brain early in disease, coincident with peak SIV viremia. The chemotactic signals that initiate the increased emigration of mononuclear cells into the CNS have not been described. Here, we describe astrocytes as a primary source of chemokines to facilitate basal levels of monocyte trafficking to CNS and that increased chemokine (C-C motif) ligand 7 (CCL7) production may be responsible for initiating the increased trafficking in neuroAIDS. We have previously published complementary in vivo work demonstrating the presence of monocyte chemoattractant protein 3 (MCP-3)/CCL7 within the brain of SIV-infected macaques. Here, we demonstrate that MCP-3/CCL7 is a significant chemokine produced by astrocytes, that basal monocyte migration may be facilitated by astrocyte-derived CCL7, that production of CCL7 is rapidly increased by TNF-α and thus likely plays a critical role in initiating neuroinvasion by SIV/HIV.

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Introduction

Astrocytes and other glial elements are known to modulate monocyte/macrophage migration into the brain. The glia limitans, formed predominantly by astrocyte foot processes, lines the basement membrane of microvascular brain endothelia. Thus, these cells are optimally situated to provide chemotactic signaling for emigration of monocytes/macrophages into the brain. Bone marrow-derived perivascular macrophages are the predominant cell type productively infected by HIV and SIV (Williams et al. 2001). Perivascular macrophages have relatively short half-lives and are replenished by circulating monocytes that traffic to the CNS. These cells are also known to traffic through brain (Williams and Hickey 1995). NeuroAIDS associated with SIV or HIV infection of macaques and humans, respectively, is characterized by perivascular accumulations of macrophages many of which are infected. Whether these cells are actively recruited or selectively retained in the brain is unclear. In late-stage disease, abundant expression of β-chemokines in brain parenchyma has been described which would be expected to contribute to increased recruitment of monocytes/macrophages (Gonzalez-Scarano and Martin-Garcia 2005). The signals involved in the initial recruitment/neuroinvasion of SIV-/HIV-infected monocytes/macrophages across the blood–brain barrier (BBB) are unknown. It is known, however, that viral infection of monocytes does not alter their chemotaxis (Persidsky et al. 1997), and that monocytes/macrophages, microglia (McManus et al. 1998), and astrocytes (Oh et al.

1999) can all secrete proinflammatory cytokines, and chemokines, to which monocyte/macrophages respond. It has been proposed that the initial neuroinvasion is mediated at least in part by astrocyte production of monocyte chemotactic protein-1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2; Weiss et al. 1998). However, in vivo data examining MCP-1/CCL2 production in the brain parenchyma of SIV-/HIV-infected individuals are conflicting (McManus et al. 2000; Sasseville et al. 1996). Furthermore, MCP-1/CCL2 would also be expected to cause large numbers of lymphocytes to migrate into the CNS—an event rarely observed with either HIV or SIV infection or SIVE/HIVE.

It is difficult or impossible to examine these early events in vivo in humans, to confirm that the results have biological relevance. The premier in vivo model of HIV CNS infection is the SIV-infected rhesus macaque (Klein et al. 1999; Persidsky and Gendelman 1997; Sasseville et al. 1996; Veazey et al. 2000; Westmoreland et al. 1999; Williams et al. 2001). In vitro models have been developed to approximate early events of HIV-induced neurological disease (Persidsky et al. 1997; Weiss et al. 1998). We hypothesized that the initiation of neuroinvasion by monocyte/macrohpages is likely an exaggeration of the normal trafficking patterns to replace perivascular macrophages. We further hypothesized that chemokines secreted by astrocytes may be responsible for this basal level of monocyte turnover in the CNS based in part on observations that astrocytes under resting conditions produce chemokines capable of inducing monocyte migration which is enhanced by tumor necrosis factor-alpha (TNF-α; Oh et al. 1999). Until now, this basal migration induced by astrocyte-conditioned media has been regarded as a nuisance for chemotactic studies, something that can be reduced by replacing the medium immediately before starting the chemotaxis assays (Stanimirovic et al. 2001).

In this study, we demonstrate that this basal level monocyte migration is largely due to chemokine (C-C motif) ligand 7 (CCL7) released by astrocytes and that the production rapidly increases upon TNF-α stimulation. Furthermore, baseline production of CCL7 at the glia limitans is observed in vivo (Sasseville et al. 1996) and is likely responsible for the trafficking of mononuclear cells through the brain as described by Williams and Hickey (1995). In addition to CCL7, we also demonstrate that stimulated astrocytes produce numerous chemokines specific for monocytes/macrophages and thus are likely to play a major role in augmenting monocyte recruitment initiated by CCL7.

Materials and methods

Culture of astrocytes Astrocytes were cultured as described previously (MacLean et al. 2002). In brief, frontal cortices from normal macaques were obtained at necropsy. Pooled supernatants from multiple astrocyte preparations were used in these studies to eradicate any variability between cultures. Meninges were removed and the tissue finely diced before incubation with PBS containing 0.25% trypsin (Invitrogen, Carlsbad, CA) and DNAse (4 U/ml) for 45 min at 37°C. Trypsin was inhibited with calf serum, and the resulting slurry filtered with 110-μm pore filters. Filtrate was washed twice with culture media (M199 containing 5% glucose, 5% fetal calf serum, Penicillin, Streptomycin, and fungizone) and plated in T25 flasks. Contaminating microglia were shaken free after 10–12 days. Residual microglia were killed using L-leucine methyl ester (10 mM for 1 h). Astrocyte cultures were routinely >95% astrocytes as determined by glial fibrillary acidic protein (GFAP) staining.

Astrocyte supernatants Astrocytes were cultured to near confluence in 75-cm^2 flasks (Corning) before incubation with 50, 100, or 500 U/ml TNF-α for 48 h. This time was determined to induce maximal responses. Supernatants were decanted, centrifuged to remove cellular debris, and frozen at −80°C until use. Astrocytes were removed from flasks by trypsinization and prepared for molecular analysis.

Peripheral blood mononuclear cell migration induced by astrocyte supernatant Frozen astrocyte supernatants were warmed to 37°C and used for migration assays. Control or TNF-α-treated astrocyte-conditioned medium (600 μl) was added to lower compartments of 3-μm pore multiwell insert filters (Becton Dickinson, Franklin Lakes, NJ). Freshly isolated PBMCs (100 μl) at 10^6/ml from normal rhesus macaques were added to the upper compartment and allowed to transmigrate for 2 h (to allow monocyte migration; Weiss et al. 1998) before preparation for flow cytometric analysis as described previously (Veazey et al. 2000; Weiss et al. 1998). To determine the cell type and number of transmigrated cells, samples were labeled with CD11b (clone Bear1, Immunotech), CD14 (clone M5E2, BD Pharmingen), and CD3 (clone FN18, Biosource). This combination of antibodies in conjunction with forward vs. side scatter allows differentiation of T cells from monocytes. Samples of input cells were stained using the same antibodies to determine ratios of monocytes: T cells added to filters.

Gene array analysis for chemokine mRNA Astrocytes were cultured to near confluence in 75-cm^2 flasks (Corning) before incubation with 50, 100, or 500 U/ml TNF-α for 48 h. Messenger RNA (mRNA) was extracted from astrocytes as described previously (Orandle et al. 2001). Superarray™ GE chemokine kits were purchased and used according to manufacturer's instructions. All samples were corrected to internal controls and graphed as relative units.

A value of 2 or greater (relative to housekeeping genes) was determined to be significant.

Detection of CCL7 protein by fluorescence microscopy Astrocytes were cultured in 75-cm^2 flasks as described above. After trypsinization, astrocytes were plated on eight-well coverslips and allowed to recover for 48 h. Astrocytes were incubated in culture medium with or without TNF-α (Pharmingen, 100 U/ml) for 48 h. To inhibit secretion of chemokines, brefeldin A (10 μg/ml) was added to the media for the last 4 h before fixation. Cells were fixed with 2% paraformaldehyde for 10 min and stained as described elsewhere (MacLean et al. 2001). Numbers of CCL7 immunopositive vesicles were quantified using Image J. Statistical analyses between treatment groups were performed using the Kruzkal–Wallis test in InStat (La Jolla, CA).

Ex vivo studies

Fresh brain tissues were obtained from normal macaques immediately postmortem at necropsy, sliced into 2-mm thick sections using a custom-fabricated brain slicer (Ted Pella Inc.) and maintained on filters in six-well culture dishes (BD, Franklin Lakes, NJ). Macrophages were cultured from previously obtained bone marrow obtained from control macaques at necropsy as described (Ivey et al. 2009). SIVmac251-infected (or control) macrophages were incubated with the brain slices for 4 h either with or without Brefeldin A to block protein transport and secretion (Jackson 2000). Following fixation in 2% paraformaldehyde and sucrose protection, the brain slices were embedded in OCT and sectioned at 50 μm. Sections were then labeled for CCL7 and GFAP to detect astrocytes.

Results

Induction of mononuclear cell migration Astrocyte supernatants added to the lower compartment of 3-μm pore multiwell insert filters were noted to induce chemotaxis of monocytes from PBMCs added to the upper chamber. To determine if this chemotaxis was dose-dependent, astrocyte-conditioned supernatants were diluted with fresh medium. Figure 1a demonstrates that the induced chemotaxis was, indeed, dose-dependent, and not an artifact of this system. The transmigrated cells were determined to be monocytes by flow cytometry (Fig. 1b, c) Cells added to the upper compartment were determined to contain approximately 35% lymphocytes and 14% monocytes (Fig. 1b). Following migration towards astrocyte-conditioned medium, it was determined that the cells comprised 70% monocytes and less than 3% lymphocytes, as determined by CD14 and CD3 staining, respectively (Fig. 1c).

We next sought to determine if exposure of astrocytes to TNF-α, the primary cytokine known to be elevated by the presence of HIV or SIV within the brain, would result in release of increased chemokine secretion. Treatment of astrocytes, cultured in 75-cm^2 flasks with 50, 100, or 500 U/ml of TNF-α, for 48 h led to a significant increase in the number of migrating monocytes/macrophages (Fig. 1d, black bars). Migrated cells comprised 76% monocytes and 3% lymphocytes (data not shown). Within the concentration range used, there was no significant dose dependency. To ensure that the observed induction of chemotaxis was not induced directly by TNF-α, we performed control experiments whereby TNF-α was added to lower wells of 3-μm pore multiwell inserts (Fig. 1d, gray bars).

Gene array analysis for chemokine mRNA To determine which chemokines were being produced by resting astrocytes in culture, we performed gene array analysis of chemokine mRNA. Analyses of data revealed that resting astrocytes in culture were transcribing large quantities of CCL7 mRNA, considerably smaller levels of chemokine (C-C motif) ligand 11 (CCL11) and chemokine (C-C motif) ligand 4 (CCL4), and insignificant levels of any other chemokine tested (Fig. 2, black bars).

Considerably higher levels of CCL7 mRNA were produced by purified astrocytes in response to 50, 100, and 500 U/ml TNF-α than any other chemokine analyzed (Fig. 2). Twofold or greater elevations of target mRNA levels relative to control levels were considered to be significant. There were also substantial increases in levels of CCL2, CCL11, and chemokine (C-C motif) ligand 5 (CCL5) mRNAs following incubation with TNF-α compared to resting astrocytes. However, measured as proportion increase following TNF-α incubation, CCL2 was, by far, the most increased chemokine mRNA at 27-fold (Table 1). Other chemokines with notable increases were: CCL5 (eightfold), CCL4 (2.4-fold), and CCL7 (7.3-fold).

Localization of CCL7 production within astrocytes To examine the presence of CCL7 protein in astrocytes, we performed immunofluorescence for CCL7 and the astrocyte-specific protein glial fibrillary acidic protein (GFAP). Resting astrocytes had a low level of CCL7 staining (Fig. 3a). The number of CCL7 immunopositive vesicles was increased slightly following stimulation with TNF-α (100 U/ml for 48 h) although this increase was not significant (Fig. 3b). We suspect that this is due to secretion of the chemokine. Numerous small vesicles were observed over much of the cell area. Astrocytes stimulated with TNF-α and brefeldin A (Fig. 3d) had significantly increased numbers of vesicles per cell compared with the brefeldin A control

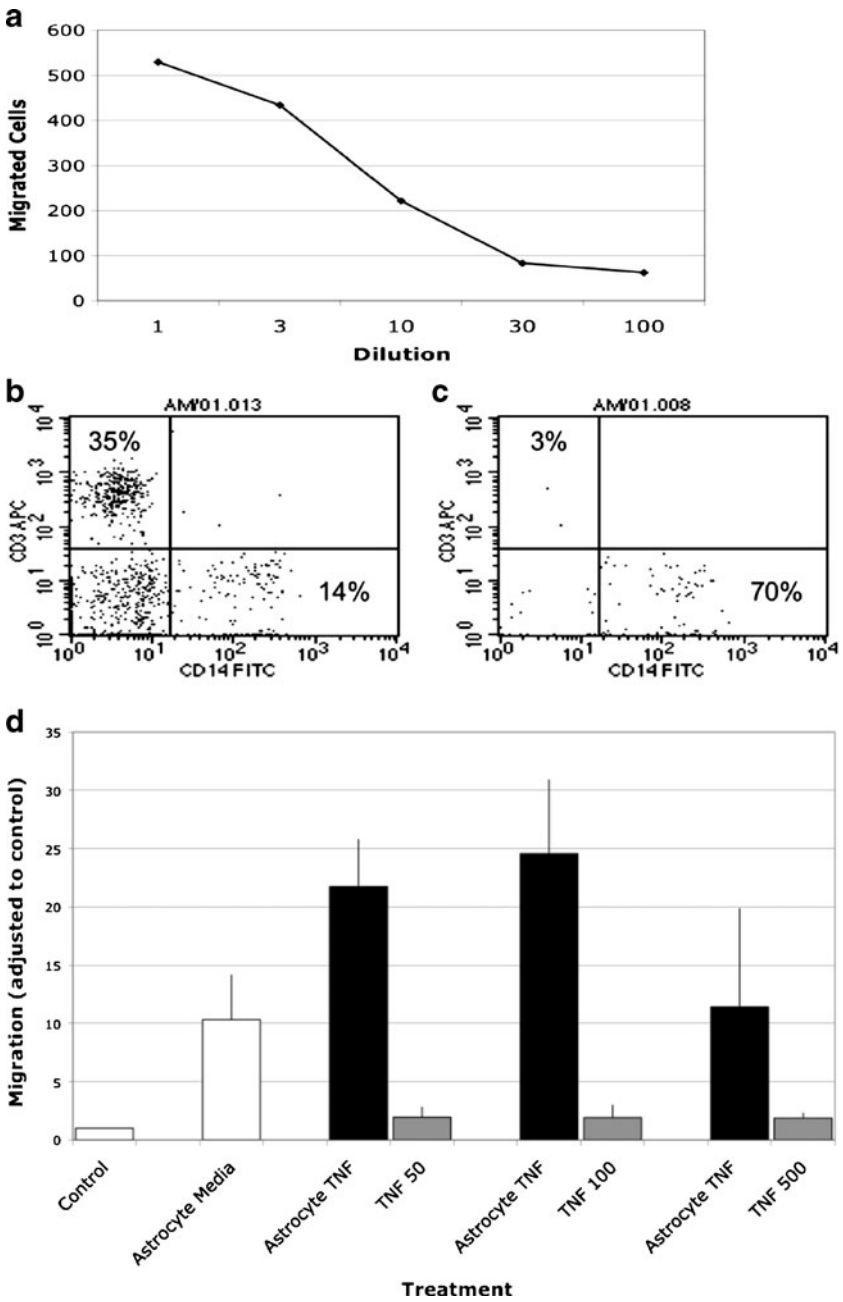

Fig. 1 Induction of monocyte/macrophage chemotaxis by resting astrocyte supernatants. Monocytes/macrophages were observed to have increased chemotaxis in response to supernatants from astrocytes in culture. This was dose-dependent and could be diluted out with astrocyte culture media (**a**). Migrated cells were determined to be macrophages by using forward and side scatter gating, followed by gating through CD11 (**b**). Cells added to the upper compartment contained approximately 35% lymphocytes and 14% monocytes. Cells that had migrated to astrocyte supernatant contained approximately 70% monocytes and less than 3% lymphocytes (**c**). Preincubation of astrocytes for 48 h with TNF-α (**d**, *gray bars*) led to increased migration of PBMCs through the blood–brain barrier model over migration to cytokine alone (*black bars*) with migrated cells comprising 76% monocytes and 3% lymphocytes

(Fig. 3c; $p<.001$), demonstrating that the chemokine is normally secreted by astrocytes. These data are presented graphically in Fig. 3e.

CCL7 production in situ To examine the production of CCL7 in tissues in response to SIV-infected macrophages, we performed ex vivo studies. Expression of CCL7 (green, open arrows) is present in normal frontal cortex predominantly in cells morphologically consistent with endothelial cells (Fig. 4a, closed arrow) rather than in parenchymal astrocytes (red). Figure 4b demonstrates colocalization of CCL7 (green) with von Willebrand's Factor (blue), indicating that CCL7 is expressed in endothelial cells. Colocalization of blue and green (turquoise) is marked with a closed arrow. Following 4-h incubation with SIVmac251-infected macrophages at (10^6/ml), increased CCL7 expression was evident in astrocytes (appearing yellow due to colocalization). Brefeldin A blocking forced the CCL7 to remain in vesicles along the length of astrocyte processes (Fig. 4c).

Taken together, these results demonstrate that CCL7 is an important chemokine actively produced and secreted by astrocytes, and that this production can be increased by SIV-infected macrophages and cytokines found within the AIDS encephalitic brain.

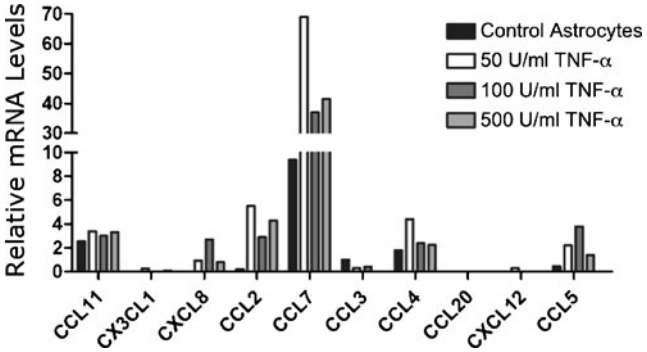

Fig. 2 mRNA analysis of chemokine genes produced by astrocytes in culture. Gene array analyses demonstrated that of the 23 chemokines tested, mRNA for CCL7 was produced in quantities significantly above all other genes tested by resting astrocytes (*black bars*). Following incubation with 50, 100, or 500 U/ml TNF-α, CCL7 was still produced at higher levels than any other chemokine on the array. Other monokines were expressed at significant levels, notably CCL2, CCL4, CCL5, and CCL11. Relative mRNA levels (compared with housekeeping genes) of genes of interest are shown in Table 1

Discussion

Our data indicate that CCL7 secretion by astrocytes increases in response to TNF-α stimulation (in vitro) or exposure to SIV-infected macrophages (ex vivo), and that CCL7 actively recruits macrophage transmigration across 3-μm filters and potentially across the blood–brain barrier.

Multiple lines of evidence implicate trafficking of monocyte/macrophages from the periphery to brain in neuroinvasion by HIV and SIV (McManus et al. 2000; Persidsky and Gendelman 1997; Persidsky et al. 1997; Sasseville et al. 1995, 1996; Weiss et al. 1999; Williams et al. 2001). How this process is initiated is however unclear. In this study, we have begun to address this by examining chemokine production by resting astrocytes in the hope of elucidating processes that initiate infiltration by HIV-/SIV-

Table 1 Chemokines with increased expression following TNF-α incubation

Chemokine	Control astrocytes	50 U/ml TNF-α	100 U/ml TNF-α	500 U/ml TNF-α	Maximum fold change
CCL11	2.55	3.4	3	3.3	1.3
CX3CL1	0	0.27	0	0.1	0.27
CXCL8	0	0.93	2.7	0.8	2.7
CCL2	0.2	5.53	2.9	4.3	27.65
CCL7	9.4	69	37	41.5	7.34
CCL3	1	0.32	0.43	0	−3.125
CCL4	1.8	4.4	2.38	2.25	2.2
CCL20	0	0	0	0	0
CXCL12	0	0.3	0	0	0.3
CCL5	0.46	2.2	3.8	1.4	8.25

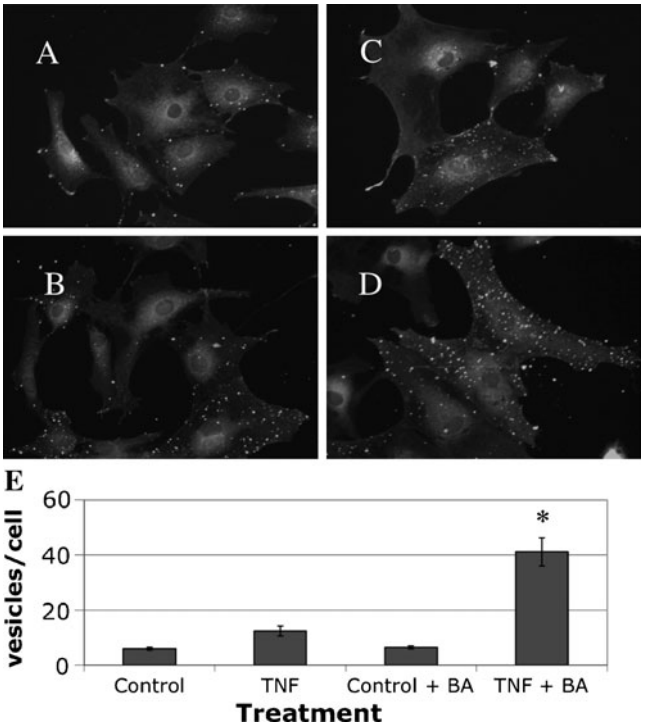

Fig. 3 Visualization of CCL7 protein within astrocytes in culture. CCL7 expression was visible weakly in many astrocytes in culture (**a**). Stimulation with TNF-α increased the amounts of CCL7 detected slightly, with a characteristic punctate vacuolar distribution (**b**). This was augmented further when the cells were incubated with brefeldin A (**c**) or with TNF-α and brefeldin A (**d**), indicating that CCL7 is constitutively produced and released from cells, but that the rate of production can be increased by proinflammatory stimulus (demonstrated in graph **e**)

infected cells into the brain. Our data implicate CCL7 production by resting astrocytes in this process with other mediators such as CCL2, CCL4, and CCL5 playing a much greater subsequent role as has been suggested previously (Hurwitz et al. 1995; McManus et al. 1998, 2000; Oh et al. 1999; Sasseville et al. 1996; Weiss et al. 1998, 1999). The data further suggest that CCL7 may play a major role in the basal level of trafficking of monocytes across the BBB.

These data generated from an in vitro model of monocyte chemotaxis complement existing knowledge of very early in vivo events in SIV-infected macaques (Sasseville et al. 1995). Of particular relevance, in vivo data from macaques infected with neurovirulent SIV demonstrate TNF-α expression in the brain concurrent with increased endothelial adhesion molecule expression, chemokine production, mononuclear cell infiltration into the brain, and SIV neuroinvasion (Sasseville et al. 1995, 1996; Tyor et al. 1992; Wesselingh et al. 1993). TNF-α has similarly been implicated in the neuropathogenesis of AIDS (Tyor et al. 1992; Wesselingh et al. 1993). In SIV- and HIV-associated encephalitis, and other encephalitides, the proinflammatory stimulus usually originates within the parenchyma of the

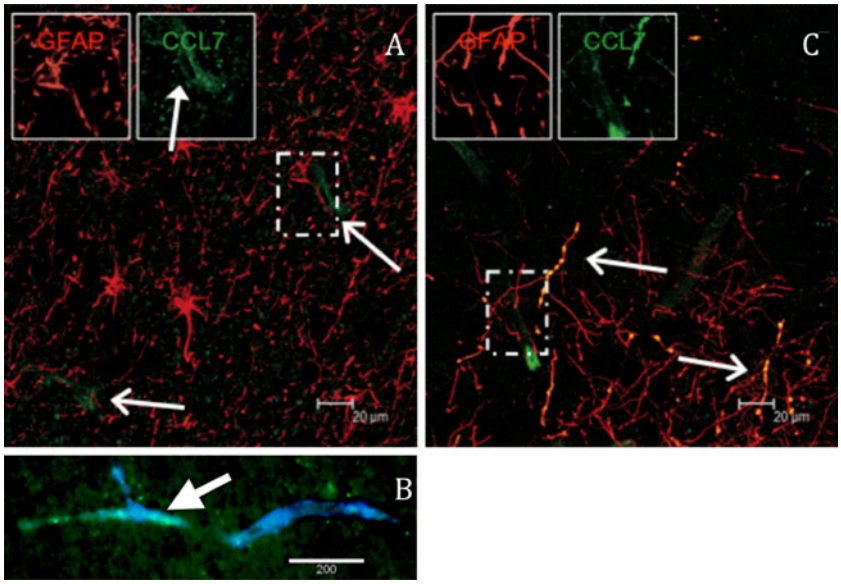

Fig. 4 CCL7 expression in situ: Brain slices were noted to have limited expression of CCL7 (*green*), predominately perivascular in localization (**a**). One such vessel double-labeled with Von Willebrand's Factor (*blue*) and CCL7 (*green*) is shown in (**b**). After 4-h incubation with SIVmac251-infected macrophages, there was marked increase in CCL7 expression in astrocytes (**b**, *red*). The beaded pattern is due to blocking secretion with brefeldin A for 4 h

brain, rather than in the circulation. Therefore, we added TNF-α to astrocytes, which are a key parenchymal component of the BBB.

The current study suggests that CCL7 is responsible for normal trafficking of monocytes/macrophages through the BBB (into the parenchyma), and, thus, for initiating SIV/HIV neuroinvasion. This interpretation is supported by the presence of CCL7 in normal brain tissues of both humans and rhesus macaques (Sasseville et al. 1996). Furthermore, molecular analysis of the chemokine mRNA produced by resting astrocytes in culture showed that CCL7 was the predominant chemokine produced. This mRNA was translated into protein, as supernatants of astrocytes in culture contained chemokines capable of inducing chemotaxis in resting monocytes (yet not in lymphocytes). Taken together, these data suggest that previously observed trafficking of hematogenous cells through brain may be due in very large measure to production of CCL7 by astrocytes, most probably at foot processes as the majority of these mononuclear cells are immediately adjacent to the BBB (Williams and Hickey 1995). Further evidence for this story is given by a demonstration by Chen and colleagues that CCR2, a receptor for CCL7, is required to be present on monocytes/macrophages for migration into virally infected brain (Chen et al. 2001).

We also investigated the role of CCL7 and other chemokines produced by astrocytes after stimulation by TNF-α. We focused on TNF-α because there is an initial increase in levels of TNF-α within brains of monkeys infected with SIVmac251 associated with SIV neuroinvasion (Orandle et al. 2002). Astrocytes are known to produce chemokines under the influence of proinflammatory cytokines such as TNF-α (Hurwitz et al. 1995; Oh et al. 1999; Weiss and Berman 1998; Weiss et al. 1998, 1999). Data from several groups indicate that HIV-infected monocyte/macrophages are activated and produce significant quantities of TNF-α (Veazey et al. 2000; Weiss et al. 1998). Thus, we expected to find an increase in chemokine production in astroctyes treated with TNF-α. This was observed, but the effect was not global, with striking increase in the mRNA levels of chemokines (such as CCL2, CCL7, CCL5, and CCL4) associated with monocyte recruitment. Elevations of each of these chemokines have previously been associated with neuroAIDS in humans or macaques (Conant et al. 1998; Persidsky et al. 1999; Sasseville et al. 1996; Schmidtmayerova et al. 1996). The probable synergistic, or at the very least, sequential release of these chemokines would be expected to increase numbers of monocytes/macrophages to migrate through the BBB, and thus, augment neuroinvasion and the formation of perivascular cuffs prominent in neuroAIDS.

In conclusion, we suggest that CCL7 may be responsible for initial entry of HIV-infected cells into the brain, probably during 'normal' turnover of perivascular macrophages (Hickey 1999; Williams and Hickey 1995). Subsequent production of TNF-α in the parenchyma by these infected, activated macrophages stimulates astrocytes to produce increased levels of CCL7 and additional chemokines, notably CCL2, CCL5, and CCL4 that are important for further recruitment of monocyte/macrophage to the CNS.

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