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Reconstitution of human immunodeficiency virus–induced neurodegeneration using isolated populations of human neurons, astrocytes, and microglia and neuroprotection mediated by insulin-like growth factors

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Primary human neuron cultures are an important *in vitro* model system for studies on mechanisms involved in human immunodeficiency virus (HIV)-associated dementia (HAD) and other neurological disorders. Here, more than 80 cell surface antigens were screened to identify a marker that could readily distinguish between neurons and astrocytes and found that neurons lack CD44 surface expression, whereas astrocytes and other cell types in brain are CD44$^+$. Neurons and astrocytes were isolated from human fetal brain based on differential expression of CD44. Using purified neurons cocultured with astrocytes and/or microglia, it was demonstrated that HIV infection of microglia induces cellular activation and production of soluble factors that activate uninfected microglia and astrocytes and induce neuronal cell death. Activated astrocytes promoted HIV replication in microglia, thereby amplifying HIV-induced neurotoxicity. A screen for 120 cytokine/proteins detected upregulation of insulin-like growth factor (IGF)-binding protein (IGFBP)-2, interleukin (IL)-6, and CCL8/MCP-2 (monocyte chemoattractant protein 2) in supernatants of HIV-infected brain cell cultures. IGF-1 and -2 increased neuronal survival in HIV-infected brain cell cultures, whereas IGFBP-2 inhibited prosurvival effects of these growth factors. These findings identify CD44 as a marker that can be used to sort neurons from other cell types in brain, suggest the importance of microglia-astrocyte interactions in neurodegenerative mechanisms associated with HIV infection, and indicate a role for insulin-like growth factors in neuroprotection from HIV-induced neurodegeneration. The ability to reconstitute brain cultures using isolated populations of neurons, astrocytes, and microglia will be valuable for studies on pathogenic mechanisms in HAD and other neurological disorders, and will also facilitate neuroactive drug discovery. *Journal of NeuroVirology* (2006) **12,** 472–491.

Keywords: astrocyte; HIV; IGF; microglia; neurodegeneration; neuron

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Introduction

Human immunodeficiency virus type 1 (HIV-1) infects the central nervous system (CNS) and frequently causes dementia and other neurological disorders (Gartner, 2000; McArthur *et al*, 2003; Price, 1996). HIV enters the CNS in the early stages of infection by trafficking across the blood-brain barrier within infected monocytes and possibly lymphocytes (Gartner, 2000; Kaul *et al*, 2001). Brain macrophages and microglia are the main cellular reservoir for HIV infection and persistence in the CNS, whereas neurons and astrocytes are rarely infected (Gonzalez-Scarano and Martin-Garcia, 2005; Kaul *et al*, 2001). Neuropathological abnormalities in the brain of patients with HIV encephalitis include brain atrophy, reactive astrocytosis, myelin pallor, microglia nodules, perivascular inflammation, multinucleated giant cells, abnormal blood-brain barrier permeability, and neuronal loss (McArthur *et al*, 2003; Price, 1996). Diffuse proliferation and immune activation of macrophages and microglia in the brain correlate with the severity of clinical dementia, suggesting that macrophages and microglia play a pivotal role in disease pathogenesis (Gartner, 2000; Gonzalez-Scarano and Baltuch, 1999; Price, 1996).

HIV infection of target cells is initiated by binding of the viral envelope glycoprotein gp120 to a chemokine coreceptor in conjunction with CD4 (Berger *et al*, 1999; Davis and Doms, 2004). CCR5 is the major coreceptor for HIV infection of macrophages and microglia (Albright *et al*, 1999; Ghorpade *et al*, 1998; He *et al*, 1997). CXCR4 is also expressed on macrophages and microglia and supports efficient replication by a subset of CXCR4-tropic (X4) HIV strains (Ohagen *et al*, 1999; Verani *et al*, 1998). CCR5-tropic (R5) HIV strains initiate primary infection and are present during all stages of HIV disease, whereas X4 and dual-tropic (R5X4) HIV strains emerge at later stages of disease (Connor *et al*, 1997). Entry and postentry restrictions to HIV infection in primates and other nonhuman species are major obstacles for the establishment of good *in vivo* small animal models to study HIV pathogenesis (Haigwood, 2004; van Maanen and Sutton, 2003). Therefore, *in vitro* models using cultured primary human cells are critical tools to study mechanisms of HIV pathogenesis.

Primary human fetal brain cultures are an important *in vitro* model system to study mechanisms involved in HIV-associated dementia (HAD) as well as other neurological disorders such as Alzheimer's disease and Parkinson's disease (Busciglio *et al*, 1995; Shi *et al*, 1996; Xu *et al*, 2002; Zhang *et al*, 2004). Such studies have been limited by the lack of a method for isolation of highly pure populations of primary human fetal neurons. Cell isolation based on expression of cell type–specific surface markers is the gold standard for purification of single cell populations. However, a cell surface antigen suitable for isolation of neurons has not yet been identified. Neuron-enriched cultures are frequently prepared using antimitotic reagents such as cytosine arabinoside, mitomycin C, or 5-fluorouracil to eliminate proliferating astrocytes. However, these reagents can be neurotoxic (Anderson and Tolkovsky, 1999; Courtney and Coffey, 1999). Furthermore, nonproliferating astrocytes are not sensitive to these reagents. A recent study utilized binding of tetanus toxin C fragment (TTC) to cell-surface gangliosides to isolate primary neurons from adult human brains (Konishi *et al*, 2002). However, cells prepared by this method contained only 80% neurons. Furthermore, neurite outgrowth was rarely observed after 2 weeks in culture. Because this method uses positive selection, a further disadvantage is the concern that neuronal functions may be altered by binding of the selection marker (Konishi *et al*, 2002). Transgenes such as green fluorescence protein have been introduced into animals in a cell type–restricted manner to facilitate isolation of neurons (Keyoung *et al*, 2001; Sawamoto *et al*, 2001). However, the introduction of transgenes may alter normal neuronal functions. Moreover, the efficiency of gene transfer into cultured primary neurons is usually low.

In this study, we screened more than 80 cell surface antigens to identify a marker that could readily distinguish between neurons and astrocytes, and used these findings to develop a novel method for isolation of primary human neurons and astrocytes based on differential expression of CD44. Using reconstituted brain cell cultures, we demonstrated that HIV infection of microglia induces cellular activation and production of soluble neurotoxic factors that act directly on neurons or indirectly through stimulation of uninfected microglia and astrocytes. Activated astrocytes promoted HIV replication in microglia, thereby amplifying HIV-induced neurotoxicity and implying the importance of cooperative microglia-astrocyte interactions in neurodegenerative mechanisms associated with HIV infection. We screened 120 cytokines and found upregulation of insulin-like growth factor (IGF)-binding protein 2 (IGFBP-2), interleukin (IL)-6, and CCL8/MCP-2 (monocyte chemoattractant protein 2) in supernatants of HIV-infected cultures. Our studies suggest that IGFBP-2 decreases neuronal survival by blocking neurotrophic effects of IGF-1 and IGF-2 in HIV-infected brain cell cultures. These findings reveal a novel pathway that may contribute to HIV-induced neurodegeneration, and suggest that insulin-like growth factors are potential neuroprotective factors in the pathogenesis of HAD.

Results

Identification of CD44 as a candidate marker for isolation of neurons by negative selection

Primary human fetal brain cell cultures (HBCs, 1–10 $\times$ 10^8 cells per tissue sample) were prepared from fetal brain tissues at gestational ages spanning

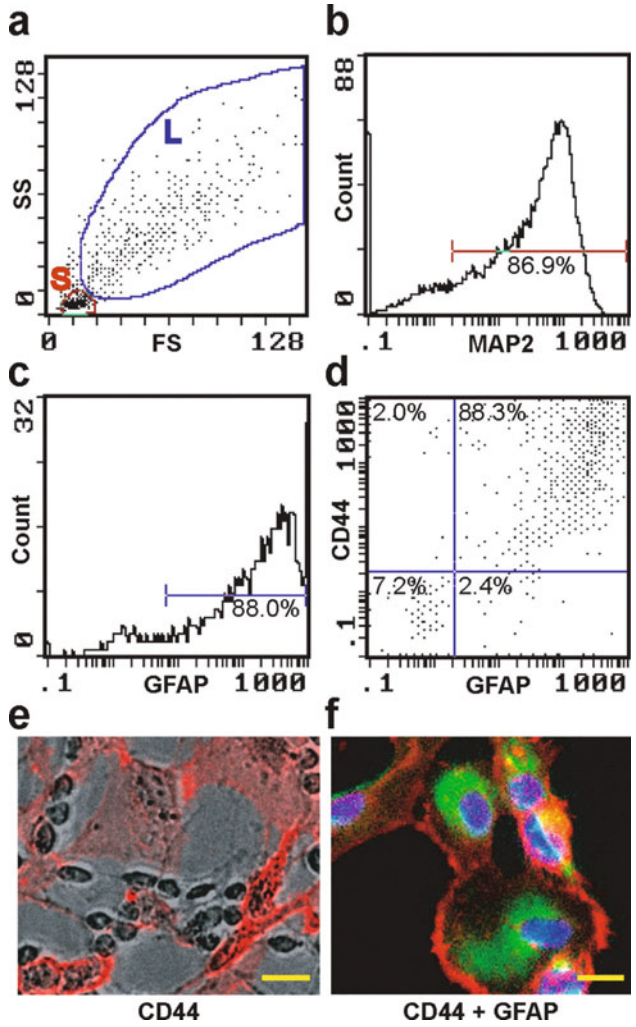

Figure 1 Expression of CD44 on a subpopulation of human brain cells. (a–d) Single-cell suspensions of primary human fetal brain cell cultures (HBCs) were stained with the indicated mAbs against cell surface and/or intracellular antigens and subjected to flow cytometric analysis. HBC were gated into small-size (S) and large-size (L) populations based on FS/SS characteristics (**a**). Gated S and L cells were analyzed for intracellular MAP2 (**b**) and GFAP (**c**) expression, respectively. Gated L cells analyzed for surface CD44 and intracellular GFAP expression (**d**). (**e**, **f**) For immunocytochemical analysis, HBCs were incubated with PE-conjugated anti-CD44 mAb (*red*) and Hoechst 33342 (*blue*), and then fixed, permeabilized, and stained with FITC-conjugated anti-GFAP mAb (*green*) (**f**). Data were acquired with a CCD camera connected to a Nikon TE300 microscope. Scale bar represents 20 μm for (**e**) and 5 μm for (**f**). Results shown in **a** to **f** are representative of three to seven experiments.

16 to 22 weeks. HBCs were composed of a small-size cell population (S cells) and a more heterogeneous large-size cell population (L cells) according to size and granularity detected by light scatter detectors (FS/SS) in flow cytometric analysis (Figure 1a). Nearly 90% of the S cells expressed microtubule-associated protein 2 (MAP2), a neuron-specific intracellular antigen, whereas nearly 90% of the L cells expressed glial fibrillary acidic protein (GFAP), an astrocyte-specific intracellular antigen (Figure 1b, c). These results suggest that nearly 90% of the S and L cells are neurons and astrocytes, respectively. Brain macrophages/microglia, which express CD45 and myeloid-specific antigens such as CD11b, CD11c, CD14, and CD68 (Wang *et al*, 2002), constituted less than 5% of the total cells and were excluded from the analysis based on anti-CD45 mAb staining. Less than 1% of the cells were oligodendrocytes, endothelial cells, and fibroblasts based on staining with anti-galactocerebroside (GalC), anti-von Willebrand Factor (VWF) (Sigma), and anti-fibroblast (Dako) antibodies (Abs), respectively.

To identify either a neuron-specific cell surface antigen to use for positive selection or an astrocyte-specific antigen for negative selection, we screened more than 80 monoclonal antibodies against cell surface antigens by flow cytometric analysis. Single-cell suspensions were prepared from HBCs by trypsin digestion, because preliminary experiments indicated that dissociation of HBCs with trypsin is more efficient and yields better cell viability than dissociation with other enzymes such as collagenase, papain, and hyaluronidase. To confirm that negative staining was not due to the trypsin treatment, the expression of cell surface antigens was also examined by immunocytochemical staining. Alternatively, expression of selected cell surface antigens was tested by flow cytometric analysis using single-cell suspensions prepared by mechanical dissociation (i.e., without trypsin). S and L cells were defined by flow cytometric analysis after gating out $CD45^+$ cells. The majority of cell surface antigens tested were not detected, or were detected on less than 5% of S or L cells (Table 1). A second group of antigens including CD24, CD56, CD57, CD81, CD90, A2B5, A-CAM, E-NCAM, MHC-I, and CXCR4 were expressed on both S and L cells. Among the antigens tested, only NCAM-L1 was detected more frequently on S (47%) than L (13%) cells. As expected, immunocytochemical staining demonstrated that NCAM-L1 was expressed on neurons but not astrocytes (data not shown). However, NCAM-L1 was not a suitable cell surface marker for isolation of human neurons because NCAM-L1 staining was sensitive to trypsin treatment (data not shown). Furthermore, NCAM-L1 expression was detected on only 47% of S cells by flow cytometry using single-cell suspensions prepared by mechanical dissociation (i.e., without trypsin). A subset of other surface antigens such as A-CAM and E-NCAM were also sensitive to trypsin treatment. Therefore, expression of these antigens was also tested by flow cytometric analysis using single cell suspensions prepared by mechanical force (Table 1). A third group of antigens including CD9, CD44, CD54, CD58, CD95, AC133, $\alpha v\beta 5$, and MHCII were detected on about 90% of L cells for CD9, CD44, and CD95 and 28% to 80% of L cells for the other antigens, but on less than 11% of S cells. Among these, the cell surface expression level of CD44 was the highest based on the

Table 1 Expression of cell surface antigens on primary human fetal brain cell cultures

		Expression (%)				Expression (%)	
Antibody	*Source*	*S*	*L*	*Antibody*	*Source*	*S*	*L*
CD1a	BD Phar[a]	—[*]	—	CD57	Sigma	24	71
CD1c	Coulter	—	—	CD58	Coulter	8	80
CD2	Coulter	—	—	CDw60	Sigma	—	21
CD3	Coulter	—	—	CD62L	Coulter	—	—
CD4	BD Phar	—	—	CD64	BD Phar	—	—
CD8	BD Phar	—	—	CD69	BD Phar	—	—
CD9	BD Phar	11	90	CD74	BD Phar	—	—
CD11b	Coulter	—	—	CD80	Caltag	—	—
CD11c	Dako	—	—	CD81	BD Phar	51	86
CD14	BD Phar	—	—	CD83	BD Phar	—	—
CD15	BD Phar	—	15	CD86	BD Phar	—	—
CD16	Coulter	—	—	CD90	BD Phar	88	95
CD18	BD Phar	—	—	CD95	BD Phar	8	90
CD23	Coulter	—	—	CD100	BD Phar	—	—
CD24	BD Phar	83	30	CD106	BD Phar	—	24
CD25	BD Phar	—	—	CD116	BD Phar	—	—
CD27	BD Phar	—	—	CD119	Caltag	—	—
CD28	BD Phar	—	—	CDw123	BD Phar	—	—
CD31	BD Phar	—	—				
CD32	Coulter	—	—	A2B5	Chem[b]	56	59
CD33	Coulter	—	—	AC133	Miltenyi[c]	10	59
CD35	Coulter	—	—	A-CAM[¶]	Sigma	68	92
CD36	BD Phar	—	—	$\alpha v\beta 5$	BD Phar	8	47
CD38	BD Phar	—	44	E-NCAM	BD Phar	84	44
CD40	Caltag	—	—	FasL	BD Phar	—	—
CD44	BD Phar	10	89	KDR1	Sigma	—	—
CD45RA	Caltag	—	—	MHC-I	BD Phar	35	83
CD45RO	Caltag	—	—	MHC-II	BD Phar	—	30
CD49d	BD Phar	—	10	NCAM-L1	BD Phar	47	13
CD50	Coulter	—	—	Syntaxin	Chem	—	—
CD54	Coulter	—	28	CXCR4	BD Phar	23	76
CD56	BD Phar	98	97				

Note. Single-cell suspensions were prepared from primary human fetal brain cell cultures after 4 to 14 days in culture. Cells were then stained with the indicated antibodies and subjected to flow cytometric analysis. Cells were gated into small-size (S) and large-size (L) populations based on FS/SS characteristics. Brain macrophages/microglia were excluded from the analysis based on anti-CD45 mAb staining. Data shown are the median of specific antibody staining from 3 to 10 independent experiments using cells derived from different donors. Specific antibody staining was calculated as (% of cells stained with indicated mAb) − (% of cells stained with isotype control mAb).

[*]—indicates that specific staining is less than 5%.

[¶]Single-cell suspensions prepared by mechanical dissociation (without trypsin) were used for staining.

[a]BD Phar: BD PharMingen; [b]Chem: Chemicon International; [c]Miltenyi: Miltenyi Biotec.

mean fluorescence intensity of stained cells. Furthermore, CD44 was resistant to trypsin treatment (data not shown). Thus, CD44 was considered to be the best candidate marker to isolate neurons by negative selection.

Differential cell surface expression of CD44 on neurons and astrocytes

CD44 is a transmembrane glycoprotein that acts as a cell adhesion and signaling molecule (Cichy and Pure, 2003; Ponta *et al*, 2003; Sy *et al*, 1997). CD44 is encoded by a single gene, but multiple isoforms are generated by alternative RNA splicing and post-translational modifications (Mackay *et al*, 1994). The anti-CD44 mAb used in the preceding experiments was raised against the most common form of CD44, the standard isoform (CD44s). In dual-color flow cytometric analysis, staining of HBCs with anti-CD44 mAb correlated with that of anti-GFAP mAb (Figure 1d). Immunocytochemical analysis revealed that anti-CD44 mAb specifically stained large, flat adherent cells, but not small, uni- or bipolar cells (Figure 1e). Dual immunocytochemical staining further demonstrated that CD44 was expressed on the surface of astrocytes identified by cytoplasmic staining with anti-GFAP mAb (Figure 1f). These results suggest that CD44 is expressed on the cell surface of astrocytes but not neurons. CD44 was also expressed on brain macrophages/microglia and oligodendrocytes (data not shown). Endothelial cells and fibroblasts, which are rarely detected in HBCs, also express CD44 on the cell surface (Griffioen *et al*, 1997; Messadi and Bertolami, 1993). Therefore, the lack of surface expression of CD44 was unique to neurons in HBCs and thus could be utilized to isolate neurons by negative selection.

Characterization of isolated CD44$^-$ and CD44$^+$ cells in culture

To isolate neurons, trypsin-dissociated HBCs were first depleted of CD45$^+$ brain macrophages/microglia by automated magnetic cell sorting (autoMACS) after incubation of cells with anti-CD45–conjugated microbeads (Miltenyi Biotec, Auburn, CA). CD45$^-$ cells were then incubated with anti-CD44 monoclonal AB (mAb) preincubated with anti-mIgG coupled to Dynabeads through a DNA linker, followed by magnetic isolation and DNase treatment to obtain bead-free CD44$^+$ cells. CD44$^-$ cells were further depleted of CD44$^+$ cells by autoMACS as described in Materials and Methods to obtain highly purified CD44$^-$ cells (more than 98% pure). CD44$^-$ cells were obtained at similar purity by cell sorting using a flow cytometer (data not shown). Freshly isolated CD44$^-$ and CD44$^+$ cells were tested for immunophenotype. Isolated CD44$^-$ cells, but not CD44$^+$ cells, expressed MAP2 intracellularly. In contrast, isolated CD44$^+$ cells expressed GFAP, but not MAP2 (Figure 2a). Up to 50% of isolated CD44$^+$ cells expressed the cell proliferation antigen Ki67, whereas isolated CD44$^-$ cells did not show significant expression (Figure 2a). Thus, CD44$^-$ cells are postmitotic, whereas a subpopulation of CD44$^+$ cells is mitotically active.

Percentages of each cell type before and after magnetic selection from HBCs are summarized in Table 2. After 1 to 2 weeks in culture, CD44 was expressed on 49% ± 13% ($n = 7$) of HBCs comprised of 43% ± 10% ($n = 4$) MAP2$^+$ neurons and 54% ± 7% ($n = 4$) GFAP$^+$ astrocytes. Floating microglia (1% to 2% of total cells) were removed before dissociation of adherent cells and were not included in the analysis. Residual CD45$^+$ macrophages/microglia comprised only 0.7% ± 0.5% ($n = 7$) of HBCs. After magnetic selection based on CD44 expression, neuron preparations were isolated to high purity (98% ± 1% CD44$^-$, $n = 7$), 96% ($n = 3$) of which were MAP2$^+$ neurons. Astrocyte preparations (98% ± 2% CD44$^+$, $n = 7$) contained 97% ± 2% ($n = 3$) GFAP$^+$ astrocytes. No CD45$^+$macrophages/microglia were detected in neuron preparations ($n = 4$) or in three of four isolated astrocyte preparations. One astrocyte preparation contained a minor population (0.3%) of CD45$^+$ macrophages/microglia. These results suggest that neurons can be isolated to high purity by negative selection based on the lack of surface CD44 expression.

Isolated CD44$^-$ and CD44$^+$ cells exhibited distinct morphological characteristics. Compared to CD44$^+$ cells, freshly isolated CD44$^-$ cells were smaller (Figure 2b). After 3 days in culture, outgrowth of neurites from the cell bodies of CD44$^-$ cells was already prominent, and some cells had made physical contact with one another through neurites. After 7 days in culture, most cells were connected with one another through the extension of neurites. Isolated CD44$^+$ cells firmly attached to plastic within 2 to 12 h in culture. Most cells exhibited a polygonal shape typical of astrocytes after 3 days in culture, and an adherent cell monolayer was formed in less than 7 days (Figure 2b). After 7 days in culture, CD44$^+$ and CD44$^-$ cells continued to show the presence or absence of CD44 immunocytochemical staining, respectively (Figure 2c). Moreover, the neuron-specific cell surface protein NCAM-L1 was detected on neurites and cell bodies of CD44$^-$ but not CD44$^+$ cells (Figure 2c). In addition to expressing the neuron-specific antigen MAP2 (Figure 2a), CD44$^-$ cells also expressed other neuronal markers such as neuron-specific nuclear protein (NeuN) and β-tubulin isotype III (data not shown). Together, these results suggest that primary human neurons isolated by negative selection maintain their cell type specificity in culture and undergo neuronal differentiation.

Isolated CD44$^-$ cells were evaluated for intracellular synthesis and/or storage of two neurotransmitters, glutamate and γ-aminobutyric acid (GABA). More than 90% of isolated CD44$^-$ cells stained strongly with mAb against glutamate in the cytoplasm after 7 days in culture (Figure 2d). Around 5% stained strongly with mAb against GABA in the cytoplasm (Figure 2d), indicating the presence of a minor population of GABAergic neurons.

To examine whether isolated CD44$^-$ cells were capable of calcium signaling in response to depolarization and other stimuli, potassium chloride

Table 2 Phenotypic analysis of primary human fetal brain cell cultures before and after purification

	CD44$^-$ (%)	*CD44$^+$ (%)*	*MAP-2$^+$ (%)*	*GFAP$^+$ (%)*	*CD45$^+$ (%)*
Total HBCs*	50.7 ± 12.8	49.3 ± 12.8	43.1 ± 10.2	54.1 ± 7.3	0.7 ± 0.5
	[26.1, 63.6]	[36.4, 73.9]	[28.2, 51.1]	[49.4, 64.9]	[0.22, 1.41]
Purified CD44$^-$ cells	98.2 ± 1.1	1.8 ± 1.1	95.5 ± 0.3	1.2 ± 0.3	0.0 ± 0.0
	[96.4, 99.6]	[0.4, 3.6]	[95.2, 95.8]	[0.8, 1.5]	[0.0, 0.0]
Purified CD44$^+$ cells	2.5 ± 1.5	97.5 ± 1.5	1.8 ± 1.3	96.8 ± 2.4	0.1 ± 0.2
	[0.5, 4.4]	[95.6, 99.5]	[0.8, 3.3]	[94, 98.4]	[0.0, 0.3]

Note. Single-cell suspensions were prepared from primary human fetal brain cell cultures after 1 to 2 weeks in culture. Cells before and after magnetic selection were stained with the indicated antibodies (surface staining for CD44 and CD45 and intracellular staining for MAP-2 and GFAP) as described in Materials and Methods and then subjected to flow cytometric analysis. Data are summarized from 3 to 7 experiments using cells from different donors. Upper values indicate averages ± SD. Lower values (in brackets) indicate ranges.
* = Floating microglia (1 to 2% of total cells) were removed before dissociation of adherent cells and were not included in the analysis.

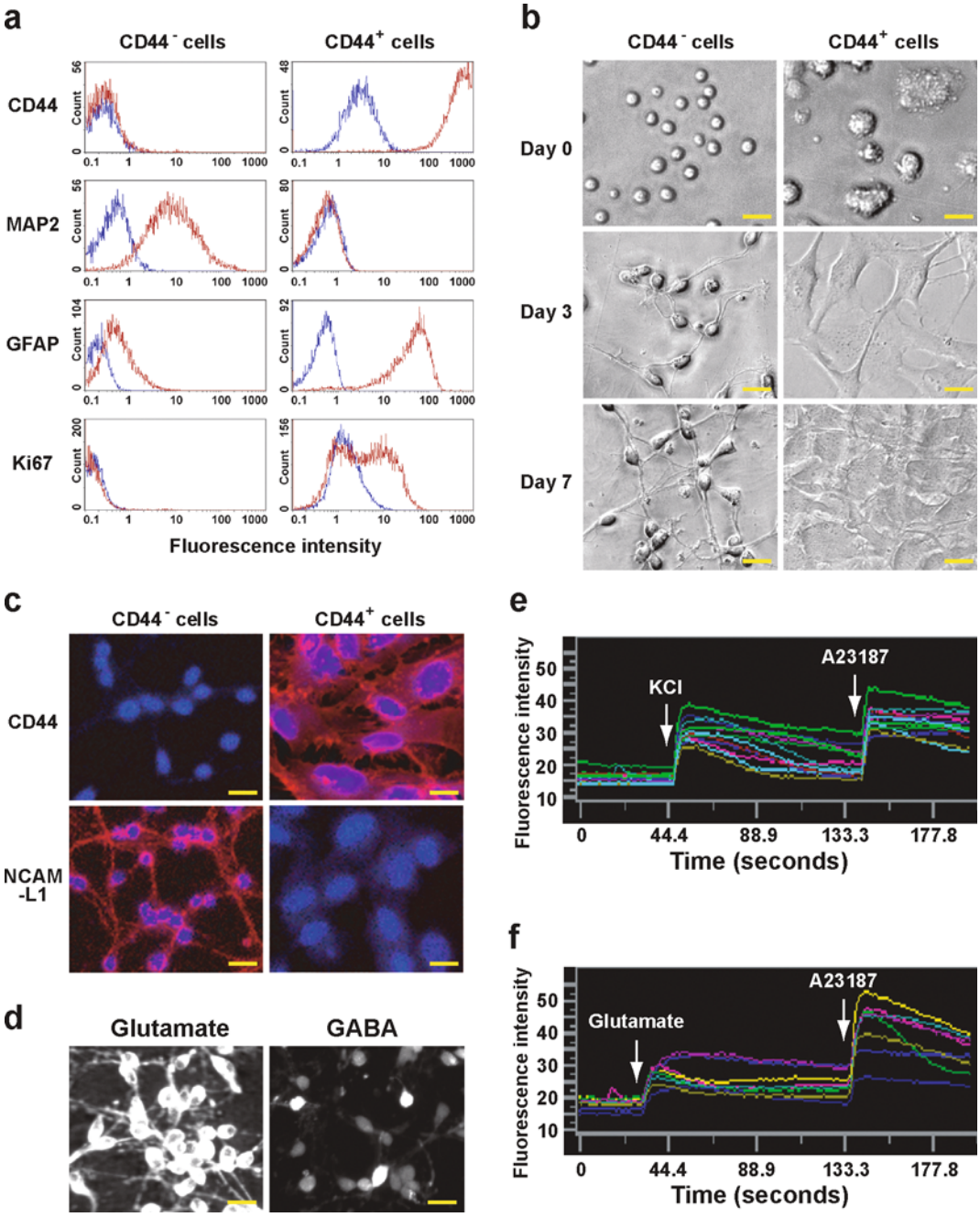

Figure 2 Characterization of freshly isolated and cultured CD44⁻ and CD44⁺ cells. (**a**) Immunophenotyping of freshly isolated CD44⁻ and CD44⁺ cells. Isolated CD44⁻ and CD44⁺ cells were stained with PE-conjugated anti-CD44 mAb, or were fixed, permeabilized, and stained with PE-conjugated anti-MAP2, or FITC-conjugated anti-GFAP or -Ki67 mAbs as indicated in red lines, and then subjected to flow cytometric analysis. Cells stained with isotype-matched control mAb are indicated by blue lines. (**b**) Phase-contrast images of isolated CD44⁻ and CD44⁺ cells cultured on poly-lysine—coated slides or plates for 2 h, 3 days, or 7 days. (**c**) Immunocytochemistry of isolated cells. Isolated CD44⁻ and CD44⁺ cells cultured for 7 days were stained with PE-conjugated anti-CD44 or -NCAM-L1 (red). Cell nuclei were then counterstained with Hoechst 33342 (*blue*). (**d**) Expression of neurotransmitters in isolated CD44⁻ cells. Isolated CD44⁻ cells cultured for 7 days were fixed, permeabilized, and then stained with anti-glutamate or anti-GABA mAbs. (**e**, **f**) Induction of calcium signaling in isolated CD44⁻ cells. Cells were loaded with Fluo-3/AM and then stimulated with the indicated stimuli. Calcium influx was recorded with a CCD camera and data were analyzed with MetaMorph software. Scale bar represents 20 μm (**b** to **d**). Results shown in **a** to **f** are representative of three to seven experiments.

(KCl)-induced depolarization and responses to extracellular glutamate stimulation were tested in calcium mobilization assays. Ca^{2+} ionophore A23187 was used as a positive control. As expected, CD44⁻ cells (Figure 2e and 2f), but not CD44⁺ cells (data not shown), exhibited calcium influx in response to stimulation with 50 mM KCl or 100 μM glutamate. These results suggest that isolated CD44⁻

cells are capable of neuronal calcium signaling responses.

Expression of HIV receptors on isolated CD44$^-$ and CD44$^+$ cells

Isolated populations of primary human neurons and astrocytes were used to investigate mechanisms involved in HIV-induced neurodegeneration. We first investigated expression of the HIV receptors CD4, CXCR4, and CCR5 on neurons and astrocytes by flow cytometry analysis. CXCR4 but not CD4 nor CCR5 was detected on isolated CD44$^-$ neurons (10% to 30%) and CD44$^+$ astrocytes (>90%) (Figure 3a). The specificity of CXCR4 staining with anti-CXCR4 mAb was demonstrated by preincubation with AMD3100, a small molecule CXCR4 antagonist (Donzella *et al*, 1998; Schols *et al*, 1997) (Figure 3b). We also used SDF-Ig to test the ability of CXCR4 expressed on neurons and astrocytes to bind its natural ligand, SDF-1α. SDF-Ig bound to CD44$^-$ neurons and CD44$^+$ astrocytes in a pattern similar to that detected by staining with a CXCR4 mAb, and binding was blocked by pre-incubating of cells with AMD3100 (Figure 3b). CXCR4 expression on CD44$^-$ neurons was confirmed by immunocytochemistry. By staining cells before fixation, CXCR4 was detected on the surface of ~10% of neurons mainly on neuronal processes and cell bodies (Figure 3c, *left*). In contrast, neurons stained strongly in the cytoplasm with anti-CXCR4 after fixation and permeabilization (Figure 3c, *right*). These data suggest that CXCR4 but not CCR5 or CD4 is expressed on the surface of primary human fetal neurons and astrocytes.

Binding of HIV gp120 to CD44$^-$ neurons and CD44$^+$ astrocytes

Apoptosis of neurons and astrocytes is induced by HIV infection *in vitro* and has been demonstrated in autopsy brain tissues from children and adult with AIDS (Petito and Roberts, 1995; Vallat *et al*,

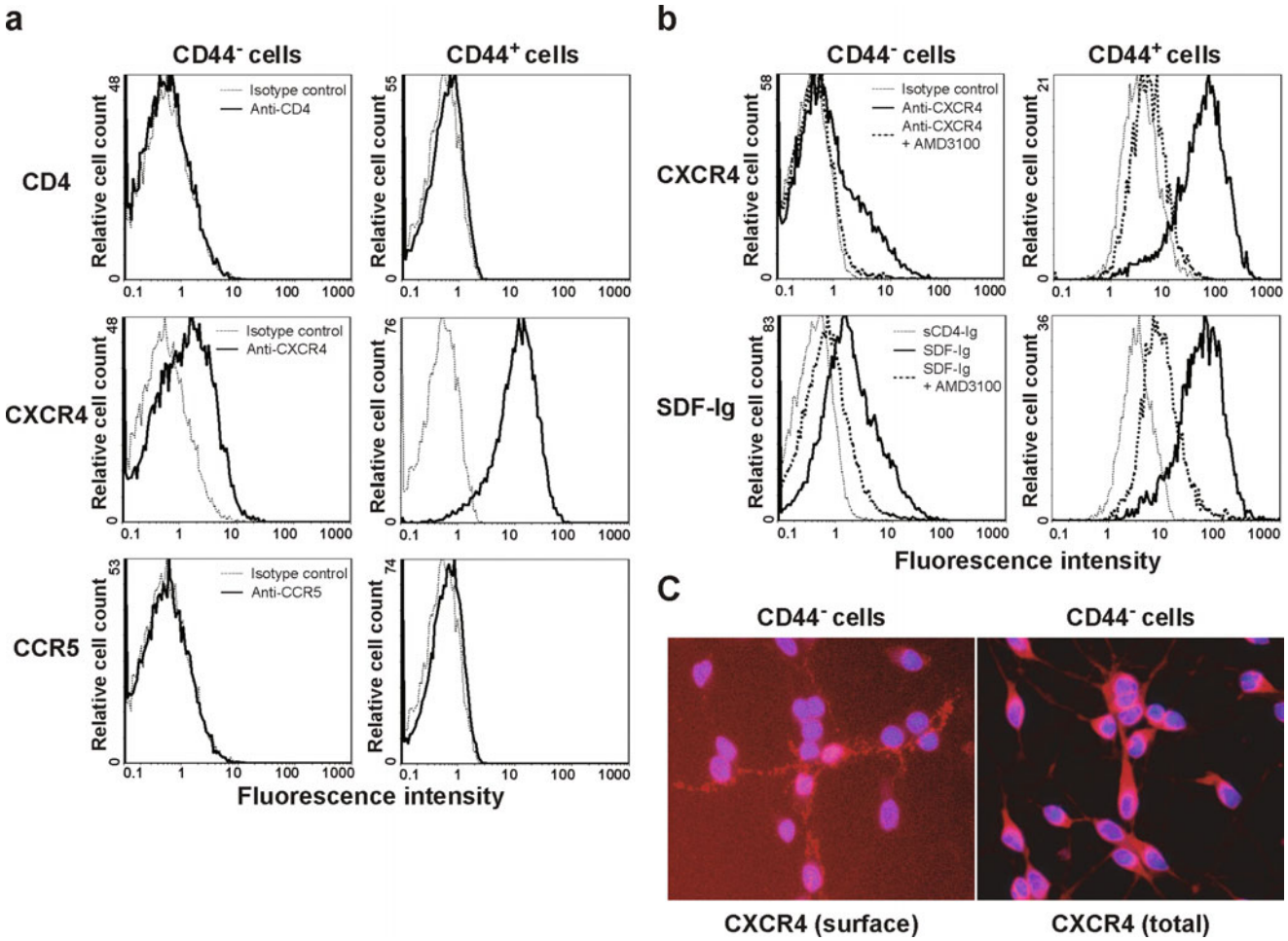

Figure 3 Cell surface expression of CD4, CXCR4, and CCR5 on neurons and astrocytes. (**a**) Isolated CD44$^-$ and CD44$^+$ cells were stained with PE-conjugated mAbs as indicated and analyzed by flow cytometry. (**b**) HBCs were preincubated with AMD3100 or culture medium at 4°C for 30 min and then subjected to double staining with anti-CD44 and the indicated reagents. Stained cells were analyzed by flow cytometry. (**c**) Isolated CD44$^-$ cells were cultured for a week and then stained with anti-CXCR4 before (surface) or after (total) fixation. Results shown in **a** to **c** are representative of two to four experiments.

1998; Zheng *et al*, 1999). The HIV envelope glycoprotein gp120 induces neuronal apoptosis *in vitro* and *in vivo*, and has been proposed to contribute to neuronal injury in HIV-associated dementia. However, it remains controversial whether gp120-induced neuronal apoptosis occurs via direct interaction of gp120 with neurons or indirectly via stimulation of glia to release neurotoxic factors (Bezzi *et al*, 2001; Hesselgesser *et al*, 1998; Kaul and Lipton, 1999; Meucci *et al*, 1998). To address this issue, we investigated the ability of R5 and X4 gp120s to bind to $CD44^-$ neurons and $CD44^+$ astrocytes using a previously established method (Wang *et al*, 2004). In the absence of soluble CD4 (sCD4), X4 gp120 exhibited detectable binding to $CD44^-$ neurons and $CD44^+$ astrocytes only when used at >0.83 μM and R5 gp120 binding was undetectable (Figure 4). In the presence of sCD4, significant binding of X4 gp120 to both $CD44^-$ neurons and $CD44^+$ astrocytes was detected (Figure 4). In contrast, binding of R5 gp120 to $CD44^-$ neurons was not detected, and binding to $CD44^+$ astrocytes was detected only when gp120 was used at >0.83 μM. These results suggest that gp120 binding to neurons and astrocytes requires the presence of sCD4, and support the idea that gp120-induced neuronal apoptosis occurs indirectly via stimulation of brain macrophages/microglia to release neurotoxic factors.

Distinct roles of microglia and astrocytes in HIV-induced neuronal loss

To investigate the roles of astrocytes and macrophages/microglia in HIV-induced neuronal loss, we reconstituted mixed brain cell cultures using purified neurons (N) cocultured with astrocytes (A) and/or macrophages/microglia (hereafter referred to as microglia, M). Reconstituted cultures were then either mock-infected or infected with R5 HIV, and virus replication was monitored by p24 enzyme-linked immunosorbent assay (ELISA). Significant virus replication and neuronal loss were observed only in cultures containing microglia (Figure 5a and b). Infection with R5 HIV induced 12.6% ± 2.1% (N + M) and 25.2% ± 5.0% (N + A + M) ($n = 5$) neuronal loss in cultures containing microglia, with two-fold increased neuronal loss in cultures containing astrocytes (N + A + M) compared to cultures without astrocytes (N + M). Preincubation with the small molecule CCR5 antagonist TAK779 (Baba *et al*, 1999), but not AMD3100, inhibited infection and also abolished the neurotoxic effects of R5 HIV (Figure 5c and d). Thus, infection of microglia is required for neurotoxic effects of HIV in this assay system. This conclusion was further supported by data from experiments using AT-2–inactivated HIV (Rossio *et al*, 1998), which failed to induce significant neuronal loss (Figure 5e). Neurotoxic effects of AT-2– inactivated virions were observed only when cultures were treated with very high concentrations of virions that are nonphysiological (>500 ng p24 per ml) (data not shown). Thus, microglia infection was required for HIV-induced neuronal loss, and astrocytes promoted this neurotoxic effect.

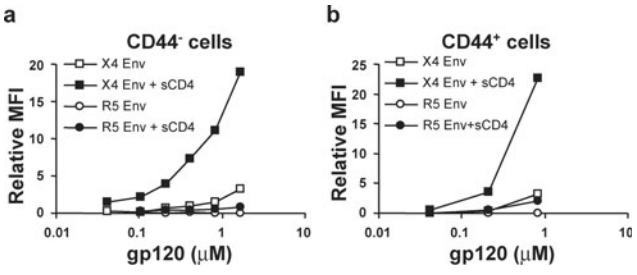

Figure 4 Binding of HIV gp120 to neurons and astrocytes is CD4-dependent. Isolated $CD44^-$ (**a**) or $CD44^+$ cells (**b**) were incubated with X4 or R5 HIV Env glycoprotein gp120 in the absence or presence of soluble CD4 (sCD4), followed by staining with anti-gp120 mAb (C11) and PE-conjugated anti-hIgG pAb as described in Material and Methods. Stained cells were analyzed by flow cytometry. Results shown are representative of three experiments.

Production of soluble neurotoxic factors by activated microglia in HIV-infected brain cell cultures

To investigate whether HIV-infected microglia induce neuronal loss via direct interaction with neurons and/or astrocytes or indirectly via release of soluble neurotoxic factors, a transwell system was employed. Neurons and astrocytes were cultured in 24-well plates, and microglia were cultured either in direct contact with neurons and astrocytes (mixed cultures) or were separated from neurons and astrocytes by transwells with a pore size of 0.4 μm (transwell cultures). R5 HIV infection caused 30.5% ± 3.5% and 29.0% ± 7.1% neuronal loss ($P = .81$, Student's t test) in mixed and transwell cultures, respectively (Figure 6a and b). Levels of p24 in supernatants from mixed and transwell cultures were 32.1 ± 0.3 and 25.4 ± 5.0 ng/ml, respectively, at 2 weeks post infection. These results suggest that HIV-induced neuronal loss is mediated by soluble factors produced by infected microglia and does not require direct cell-cell contact between infected microglia and neurons or astrocytes.

Previous studies demonstrated increased expression of MHC class II molecules and other activation markers on brain macrophages/microglia of HIV-infected patients (Achim *et al*, 1991). To investigate whether microglia become activated in HIV-infected cultures, we examined expression of several activation markers including major histocompatibility complex (MHC) class II and the adhesion molecule CD44 on microglia after mock or R5 HIV infection. MHC class II and CD44 expression was up-regulated three- and fourfold compared to control levels, respectively, on microglia in cultures infected with R5 HIV compared to mock infection (Figure 6c). No significant difference in MHC class II and CD44 expression was observed between $p24^+$ and $p24^-$ microglia in HIV-infected cultures (data not shown). Surface

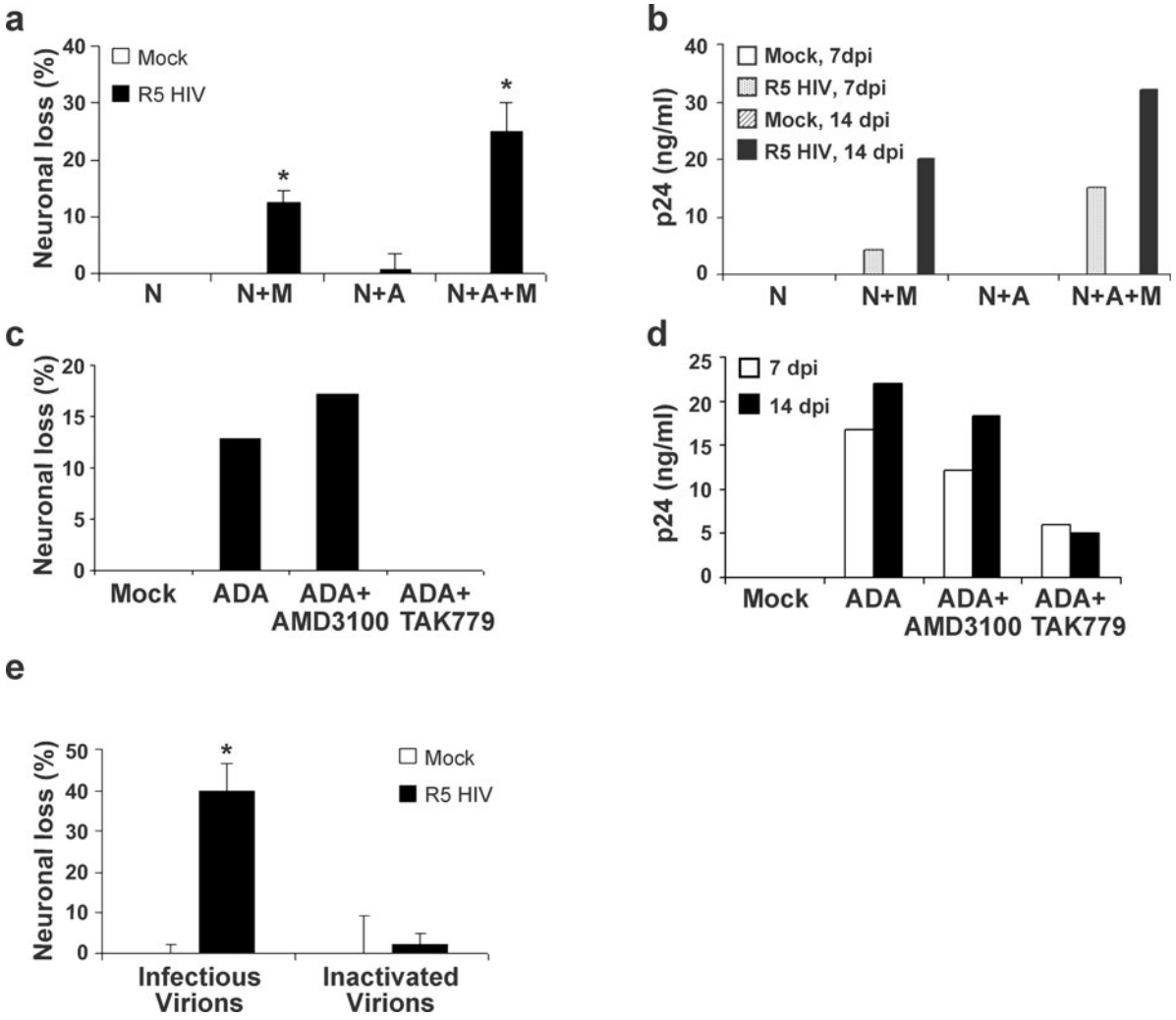

Figure 5 Neuronal loss in reconstituted brain cell cultures infected with HIV. (**a**) Isolated neurons (N) cultured in the absence or presence of astrocytes (A) and/or microglia (M) were mock infected or infected with R5 HIV. Cells were collected 2 weeks post infection and neuronal loss was examined by analysis of cells stained with FITC-conjugated Annexin V, PE-conjugated anti-CD44, cychrome-conjugated anti-CD45, and 7-AAD as described in Materials and Methods. Data are summarized from five experiments. Error bars correspond to SEM of 5 samples. * $P < .05$ compared with mock-infected control. (**b**) Virus titer in culture supernatants from (**a**) was quantified by p24 ELISA. One representative experiment of five is shown. (**c**) Reconstituted brain cell cultures containing neurons, astrocytes, and microglia were mock infected or infected with R5 HIV ADA in the absence or presence of AMD3100 or TAK779. Cells were collected 2 weeks post infection and neuronal loss was examined. One representative experiment of two is shown. (**d**) Virus titer in culture supernatants from (**a**) was quantified by p24 ELISA. (**e**) Reconstituted brain cell cultures containing neurons, astrocytes, and microglia were infected with R5 HIV or AT-2 inactivated R5 HIV. Cells were collected 2 weeks post infection and neuronal loss was examined. Data are presented as means ± SD for duplicate samples. One representative experiment of three is shown.

expression of the activation markers CD40, CD80, and CD86 was not detected on microglia (data not shown), probably due to the high autofluorescence of these cells. These results indicate that both infected and uninfected microglia become activated in HIV-infected cultures.

Activated astrocytes promote HIV replication in microglia

The preceding experiments suggested that astrocytes amplify the neurotoxic effects of HIV-infected microglia (Figure 5). To better understand the underlying mechanisms, we examined whether astrocytes promote HIV replication in microglia (Figure 6d). Microglia cultured in the absence or presence of neurons and/or astrocytes were infected with R5 HIV and viral replication was monitored. Cultures containing astrocytes (M + A or M + N + A) supported 5 to 25-fold higher levels of HIV replication compared to cultures without astrocytes (M or M + N). In contrast, the presence of neurons had no significant effect on HIV replication (M + A *versus* M + N + A) (Figure 6d). These results suggest that astrocytes promote HIV replication in microglia.

Reactive astrogliosis is a common neuropathological finding in the brains of HIV infected individuals

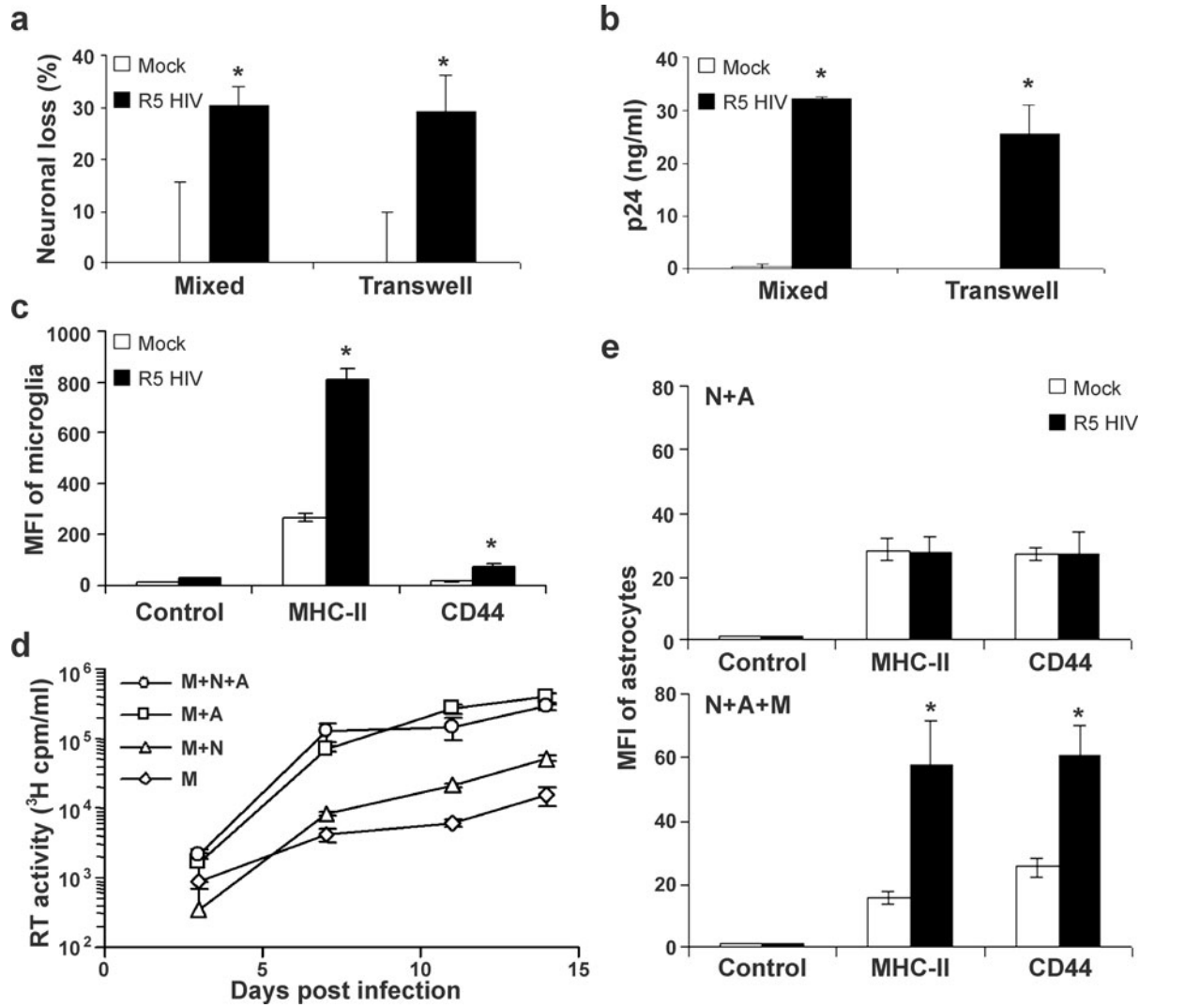

Figure 6 Activated astrocytes promote HIV replication in reconstituted brain cell cultures. (**a**) Microglia were either directly added into cultures containing neurons and astrocytes (mixed cultures) or were separated from neurons and astrocytes by a transwell with a pore size of 0.4 μm (transwell cultures). Cultures were then either mock infected or infected with R5 HIV and neuronal loss was examined 2 weeks post infection. (**b**) Virus titer in culture supernatants from **a** was quantified by p24 ELISA. (**c**) Reconstituted brain cell cultures containing neurons, astrocytes, and microglia were collected 2 weeks post infection and stained with FITC-conjugated anti-CD45 and the indicated antibodies conjugated with PE. Mean fluorescence intensity (MFI) of the staining of gated CD45$^+$ cells is shown. (**d**) Microglia (M) were cultured in the absence or presence of neurons (N) and/or astrocytes (A) and infected with R5 HIV. Half of the culture medium was replaced with fresh medium every 3 to 4 days. Cell culture supernatants were subjected to reverse transcriptase (RT) assay to measure virus titer. (**e**) Reconstituted brain cell cultures containing neurons, astrocytes, and microglia were collected 2 weeks post infection for flow cytometry analysis. Mean florescence intensity (MFI) of the staining of gated CD44$^+$ cells were shown. Data in **a** to **e** are presented as means $\pm$ SD for duplicate determinants. Results shown are representative of two to four experiments. * $P < .05$ compared with mock-infected control.

(Brack-Werner, 1999; Price, 1996; Sabri *et al*, 2003). To test whether astrocytes become activated in HIV-infected brain cell cultures, we examined the surface expression of MHC class II and CD44 on astrocytes. In the absence of microglia (N + A), R5 HIV had no effect on the expression of either MHC class II or CD44 on astrocytes (Figure 6e). However, in the presence of microglia (N + A + M), surface expression of MHC class II and CD44 was enhanced to 3.7- and 2.4-fold of control levels, respectively, on astrocytes in R5 HIV-infected cultures compared to mock-infected cultures (Figure 6e). These findings suggest that astrocytes become activated in the presence of HIV-infected microglia. Thus, HIV infection of microglia induces activation of astrocytes, which in turn promote virus replication in microglia.

Cytokine profiling demonstrates upregulation of IGFBP-2, IL-6, and CCL8/MCP-2 in HIV-infected brain cell cultures

To identify soluble neurotoxic factors produced by R5 HIV-infected microglia and/or activated

microglia/astrocytes, we used a cytokine antibody array to screen for 120 soluble factors in supernatants of R5 HIV-infected brain cell cultures. More than 30 cytokines/proteins were detected in culture supernatants of mock or R5 HIV-infected brain cells at 1 week post infection (Figure 7a–c). Compared to mock-infected cultures, R5 HIV-infected brain cell cultures produced significantly higher levels of CCL8/MCP-2, CXCL6/GCP-2, IL-6, and IGFBP-2 (Figure 7a–c). Expression of a few cytokines/proteins was downregulated in R5 HIV-infected brain cell cultures. However, down-regulation of these cytokines/proteins and up-regulation of CXCL6 were not observed in repeated experiments using cell cultures from different donors, possibly reflecting donor variation or because expression levels of most of these cytokines/proteins were close to the limit of detection. The up-regulation of IL-6 in HIV-infected brain cell cultures is consistent with previous studies demonstrating induction of this cytokine in activated or HIV-infected macrophages and microglia (Albright and Gonzalez-Scarano, 2004; Nakajima *et al*, 1989; Trentin *et al*, 1992; Xu *et al*, 2004). CCL8/MCP-2 and IGFBP-2 levels in mock and R5 HIV-infected brain cell cultures were quantified by ELISA (Figure. 7d and e). CCL8/MCP-2 was detected in supernatants of cultures infected with R5 HIV (63.7 $\pm$ 6 pg/ml), but was undetectable in uninfected control supernatants at 7 days post infection. IGFBP-2 was induced by four-fold in R5 HIV-infected compared to mock-infected cultures at 7 days post infection (69.1 $\pm$ 5.7 versus 273.3 $\pm$ 47.9 ng/ml, $P = .027$). These results suggest that IL-6, CCL8/MCP-2, and IGFBP-2 are selectively induced in HIV-infected brain cell cultures.

IGFs are neuroprotective in HIV-infected brain cell cultures, and IGFBP-2 decreases neuronal survival

CCL8/MCP-2 is a monocyte chemotactic protein that shares 62% amino acid sequence identity with CCL2/MCP-1 and 58% with CCL7/MCP-3 (Gong *et al*, 1997; Proost *et al, 1996*). Previous reports suggest that CCL8/MCP-2 activates CCR5, and blocks CCR5-mediated HIV entry and replication (Gong *et al*, 1998; Yang *et al*, 2002). Thus, up-regulation of CCL8 could potentially reduce levels of HIV infection. Alternatively, increased CCL8/MCP-2 levels might contribute to neuropathogenesis by enhancing neuronal injury and/or recruiting monocytes or other leukocyte populations to the brain. To investigate effects of CCL8/MCP-2 on neuronal survival in HIV-infected brain cell cultures, we first tested the effects of an anti-CCL8/MCP-2 neutralizing antibody. However, the antibody as well as an isotype control antibody alone induced neuronal loss in uninfected brain cultures (data not shown), probably due to nonspecific binding and/or activation of macrophages/microglia mediated through cell surface Fc receptors. We also tested whether CCL8/MCP-2 enhances neuronal survival by adding CCL8/MCP-2 to mock- or R5 HIV-infected neuronal cultures. However, addition of CCL8/MCP-2 had no significant effect on neuronal loss either in the absence (data not shown) or presence of astrocytes (Figure 7f), suggesting that CCL8/MCP-2 has no direct effect on neuronal survival *ex vivo*.

The insulin-like growth factor-binding proteins (IGFBPs) comprise a family of six related peptides that interact with high affinity with IGFs. IGFBPs compete with IGF receptors for IGF binding, and consequently can adversely affect cell survival, differentiation, and mitogenesis (Bach *et al*, 2005; Denley *et al*, 2005; Firth and Baxter, 2002). IGFBP-2, the second most abundant IGFBP in serum, binds both IGF-1 and IGF-2 and therefore has the potential to inhibit the bioactivity of these growth factors (Carrick *et al*, 2001; Firth and Baxter, 2002; Rajaram *et al*, 1997). IGFs (particularly IGF-1) promote cell proliferation and differentiation during normal brain development and maturation, and may play a neuroprotective (antiapoptotic) role during certain neuropathological conditions (Beck *et al*, 1995; Stewart and Rotwein, 1996; Ying Wang *et al*, 2003). In HIV-infected patients, decreased concentrations of IGF-1, IGF-2, and IGFBP-3, and increased levels of IGFBP-1 and -2 have been demonstrated in serum (Congote, 2005; Rondanelli *et al*, 2002; Ying Wang *et al*, 2003), but IGF and IGFBP levels have not been characterized in brain.

To test effects of IGFBP-2 on neuronal survival, we added IGFBP-2 alone or in combination with IGF-1 and IGF-2 to neuronal cultures in the presence of astrocytes and microglia. In the absence of HIV infection, IGFBP-2 alone induced neuronal loss by 11%. IGF-1 and IGF-2 enhanced neuronal survival by 26%, whereas the combination of IGFBP-2 with IGF-1 and IGF-2 significantly reduced the neuroprotective effect of IGF-1 and IGF-2 (Figure 7g). In the presence of HIV infection, which induced neuronal loss by 18%, addition of IGFBP-2 had no effect on neuronal loss, whereas addition of IGF-1 and IGF-2 protected neurons from HIV-induced neuronal loss and enhanced neuronal survival to levels similar to those in mock-infected control cultures. Addition of IGFBP-2 to HIV-infected brain cell cultures in the presence of IGF-1 and IGF-2 had no significant effect compared to cultures treated with these IGFs alone, probably because the concentration of exogenous IGFBP-2 was not sufficient to completely neutralize the effects of these growth factors. These data suggested that up-regulation of IGFBP-2 can result in decreased neuroprotection mediated by IGFs. These findings reveal a novel pathway that may contribute to HIV-induced neurodegeneration, and suggest that IGF-1 and IGF-2 are potential neuroprotective factors in the pathogenesis of HAD.

Discussion

In this study, we developed a novel method to isolate primary human neurons and astrocytes and used

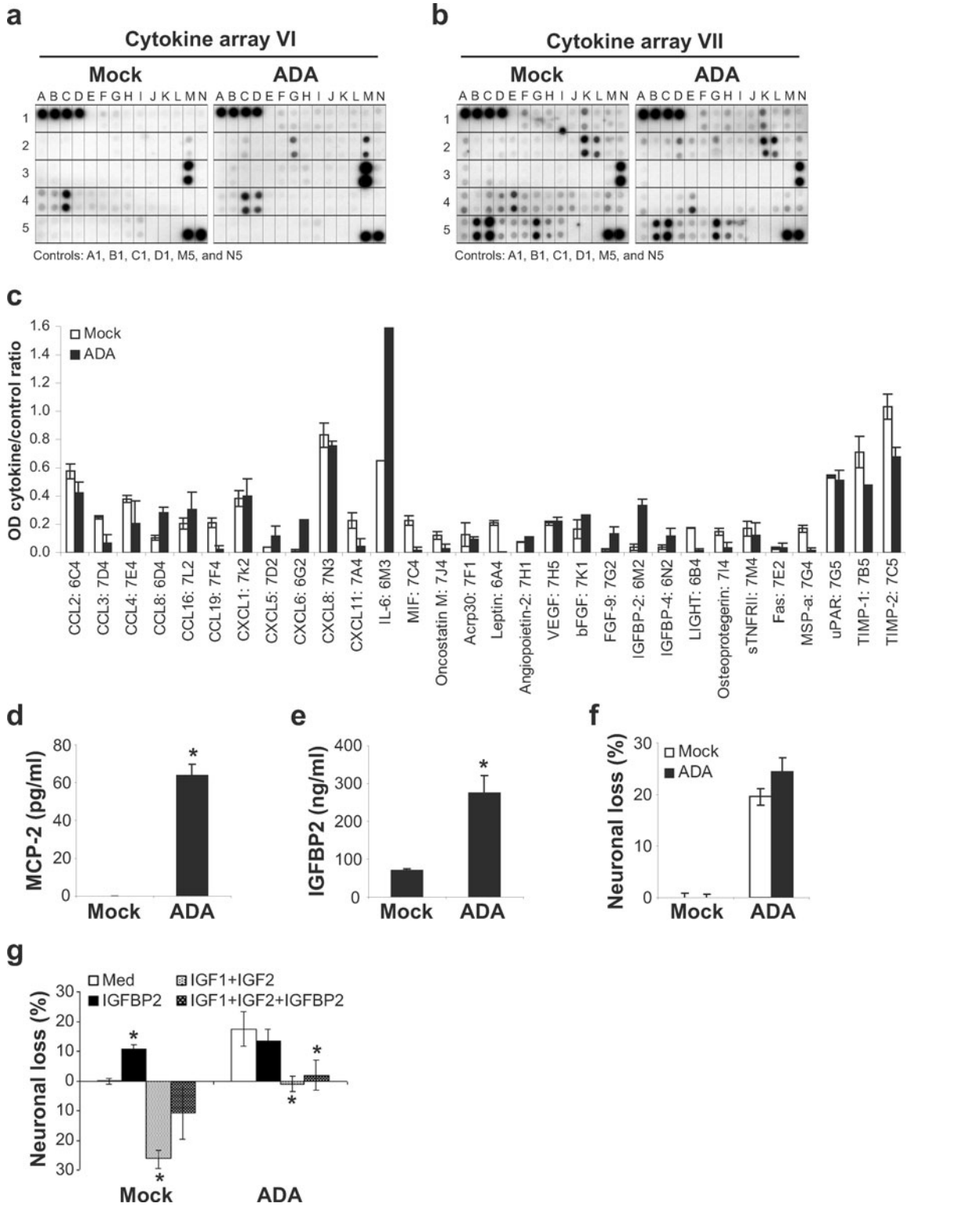

Figure 7 Cytokine profiling demonstrates upregulation of IGFBP-2, IL-6, and CCL8/MCP-2 in HIV-infected brain cell cultures. (**a** and **b**) Supernatants from mock- or R5 HIV ADA–infected brain cell cultures were harvested 7 days post infection and then blotted against a membrane spotted with anti-cytokine Abs (cytokine antibody array C1000 VI and VII, Raybiotech). (**c**) The optical density (OD) of each spot was determined and the ratio between the OD of cytokine/protein and positive-control spots was calculated. (**d** and **e**) CCL8/MCP-2 and IGFBP-2 levels in supernatants from mock- or ADA-infected brain cell cultures were quantified by ELISA. (**f** and **g**) Reconstituted brain cell cultures containing neurons, astrocytes, and microglia were mock-infected or infected with ADA. CCL8/MCP-2, IGF-1, IGF-2, or IGFBP-2 were added to cultures at 16 h post-infection. Cells were collected 2 weeks post infection and neuronal loss was quantified as described in Materials and Methods. Data in **c** to **g** are presented as means ± SD for duplicate determinants. Results shown in **a** to **g** are representative of two to four experiments. $^{*}P < .05$ compared with mock-infected (**c** to **e**) or medium-treated (**f** and **g**) controls.

isolated populations of neurons, astrocytes, and microglia to reconstitute HIV-induced neurodegeneration. We screened more than 80 cell surface antigens to identify a marker that readily distinguishes between neurons and astrocytes, and developed a novel method to isolate primary neurons and astrocytes from cultured human brain based on differential expression of CD44. Using reconstituted brain cell cultures, we demonstrated that HIV infection of microglia induces cellular activation and production of soluble neurotoxic factors that act directly on neurons or indirectly through stimulation of uninfected microglia and astrocytes. Activated astrocytes promoted HIV replication in microglia, thereby amplifying HIV-induced neurotoxicity and implying the importance of cooperative microglia-astrocyte interactions in neurodegenerative mechanisms associated with HIV infection. Cytokine profiling demonstrated up-regulation of IGFBP-2, IL-6, and CCL8/MCP-2 in supernatants of HIV-infected cultures. Furthermore, we provide evidence that IGFBP-2 decreases neuronal survival by blocking neurotrophic effects of IGF-1 and IGF-2 in HIV-infected brain cell cultures. These findings reveal a novel pathway that may contribute to HIV-induced neurodegeneration, and suggest that IGF-1 and IGF-2 are potential neuroprotective factors in the pathogenesis of HAD.

The method of neuronal isolation we describe yields highly pure (>98%) populations of neurons. In comparison with existing methodologies, advantages of the method include high yield (more than 2–5 $\times$ 10^7 neurons can easily be isolated), high purity, no requirement for costly equipment, and simultaneous isolation of astrocytes and macrophages/microglia derived from the same donor. Another important advantage is that the isolated neurons are neither genetically modified nor antibody bound. In contrast to neurons isolated from adult tissues, which have limited viability and rarely grow neurites in culture (Konishi *et al*, 2002), more than 95% of isolated $CD44^-$ neurons derived from HBCs and cultured for 2 weeks *in vitro* are viable based on analysis of cell cultures stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (data not shown). Furthermore, isolated $CD44^-$ neurons are capable of neuronal differentiation and calcium signaling. Thus, this experimental system will be useful for basic research on neuronal functions and pathogenic mechanisms in neurological disorders, as well as neuroactive drug screening. Postmitotic neurons are able to engraft rodent brains *in vivo* (Sawamoto *et al*, 2001; White *et al*, 1999), raising the possibility that it might be feasible to use isolated primary human neurons for neuronal engraftment in the treatment of neurological diseases.

CD44 was considered to be the best marker for isolation of neurons by negative selection because of its high level of surface expression on astrocytes and other non-neuronal cells, and its resistance to trypsin treatment. However, other surface antigens such as CD9 and CD95 were also selectively expressed on astrocytes but not neurons (Table 1) and might therefore be useful as markers alone or in combination with CD44 for negative selection of neurons. Further studies are needed to investigate such alternative approaches. Among the antigens tested, NCAM-L1 was the only cell surface marker that appeared to be neuron specific. However, NCAM-L1 was not suitable for isolation of neurons because of its sensitivity to trypsin treatment. Moreover, a significant fraction of neurons did not have a sufficient level of NCAM-L1 surface expression. Thus, we were not able to isolate neurons by positive selection.

Isolated populations of primary human neurons, astrocytes, and macrophages/microglia were used to investigate mechanisms involved in HIV-induced neurodegeneration. We investigated expression of HIV receptors on primary human fetal neurons and astrocytes and demonstrated that CXCR4 but not CCR5 or CD4 is expressed on the surface of neurons and astrocytes. Previous studies reported contradictory results regarding expression of CCR5 and CXCR4 on primary fetal neurons and astrocytes (Coughlan *et al*, 2000; Klein *et al*, 1999; Sabri *et al*, 1999). Klein *et al*. detected CCR5 and CXCR4 expression on both neurons and astrocytes (Klein *et al*, 1999), whereas Coughlan *et al*. detected weak CCR5 staining (Coughlan *et al*, 2000), and Sabri *et al*. failed to detect CCR5 and CXCR4 on astrocytes (Sabri *et al*, 1999). These discrepancies are probably due to differences in experimental methods. In particular, CXCR4 expression on astrocytes declines after prolonged culture *in vitro* (Klein *et al*, 1999). Furthermore, a significant proportion of CCR5 and CXCR4 was detected in the cytoplasm (Coughlan *et al*, 2000; Klein *et al*, 1999). In the present study, only cell surface expression of CCR5 and CXCR4 was detected by flow cytometry analysis, because live cells were used for staining and nonviable cells were excluded by 7-AAD staining. Consistent with the lack of surface CD4 and CCR5 expression on primary neurons and astrocytes, R5 gp120 failed to bind to neurons and astrocytes, and X4 gp120 binding required the presence of sCD4 (Figure 4). These findings may explain why microglia, which express CD4, CXCR4, and CCR5, are required for gp120-induced neuronal apoptosis (Kaul and Lipton, 1999). Low levels of X4 gp120 binding to neurons and astrocytes were detected in the absence of sCD4 when gp120 was used at high concentrations. However, these high concentrations of gp120 may not present *in vivo* (Klasse and Moore, 2004).

To investigate the role of astrocytes and microglia in HIV-induced neuronal loss, we reconstituted mixed brain cell cultures using isolated populations of neurons cultured in the presence or absence of astrocytes and/or microglia. Infection of reconstituted cultures with R5 HIV demonstrated that microglia are required for HIV-induced neuronal loss. Preincubation of cultures with TAK779 inhibited infection and abolished the neurotoxic effects of R5 HIV, and AT-2–inactivated HIV failed to induce significant neuronal

loss. These findings suggest that HIV infection rather than virion-associated gp120 induced neuronal loss in this assay system. Both infected and uninfected microglia in HIV-infected cultures became activated as indicated by upregulation of MHC class II, consistent with studies of brain tissues from HIV-infected patients (Achim *et al*, 1991). Utilizing a transwell system, we demonstrated that neuronal loss is initiated by soluble factors produced by HIV-infected microglia, which then act directly on neurons or exert neurotoxic effects indirectly through stimulation of uninfected microglia and astrocytes.

Reactive astrogliosis is a common neuropathological finding in the brains of HIV infected individuals, but whether astrocyte activation promotes HIV-induced neuronal apoptosis is unclear (Brack-Werner, 1999; Price, 1996; Sabri *et al*, 2003). Reactive astrocytes are functionally distinct from quiescent astrocytes, and may play key roles in some CNS disorders (Miller, 2005). Activated astrocytes produce neurotrophic factors as well as pro inflammatory cytokines, and also exhibit reduced glutamate uptake or may even release glutamate themselves (Bezzi *et al*, 2001; Gonzalez-Scarano and Martin-Garcia, 2005; Kaul *et al*, 2001). We found that expression of MHC class II was up-regulated on astrocytes upon HIV infection of microglia, an effect that may be mediated through the release of proinflammatory cytokines such as tumor necrosis factor(TNF)-α from HIV-infected microglia (Dong and Benveniste, 2001). Previous studies suggested that astrocytes can either amplify or attenuate the production of neurotoxic factors from monocyte-derived macrophages (MDMs) (Genis *et al*, 1992; Hori *et al*, 1999; Nottet *et al*, 1995). However, these studies used astrocytes and MDM from different donors. Using reconstituted cultures containing astrocytes and microglia from the same donor, we demonstrated that activated astrocytes promote HIV replication in microglia, thereby amplifying HIV-induced neurotoxicity. Activated astrocytes produce cytokines such as macrophage colony-stimulating factor (M-CSF), IL-1β, and TNF-α which are known to promote HIV replication in microglia (Genis *et al*, 1992; Hori *et al*, 1999; Nottet *et al*, 1995), and may also stimulate microglia to produce factors that enhance HIV replication. We did not detect TNF-α in supernatants from HIV-infected brain cultures using cytokine antibody arrays. However, in other studies we detected TNF-α levels of 30 to 60 pg/ml in HIV-infected brain cultures compared to $<$10 pg/ml in uninfected cultures by ELISA (D. Gabuzda, unpublished data). These low levels may be below the limit of detection of the cytokine antibody arrays used in the present study. Reduced glutamate uptake or the release of other neurotoxic factors by activated astrocytes (i.e., glutamate, arachidonic acid and its metabolites, IL-1β, and TNF-α) may also contribute to neurotoxicity (Bezzi *et al*, 2001; Gonzalez-Scarano and Martin-Garcia, 2005; Kaul *et al*, 2001; Genis *et al*, 1992; Hori *et al*, 1999; Nottet *et al*, 1995). Thus, activated astrocytes are likely to contribute to neuropathogenic effects during HIV infection through mechanisms similar to those implicated in other neurodegenerative disorders (D'Ambrosio, 2004; Miller, 2005; Mrak and Griffin, 2005).

Using reconstituted human brain cell cultures, we showed that IGFBP-2 is up-regulated by infection with R5 HIV. Our studies show that IGF-1 and IGF-2 enhanced neuronal survival in uninfected human brain cell cultures and protected neurons from HIV-induced neurodegeneration in R5 HIV-infected cultures, whereas addition of IGFBP-2 inhibited the pro-survival effects of IGF-1 and IGF-2. Furthermore, IGFBP-2 alone induced neuronal loss in uninfected cultures, most likely by antagonizing neuroprotective effects of endogenous IGF-1 and IGF-2. Previous studies demonstrated elevated expression of IGFBP-2 in activated macrophages/microglia and/or astrocytes in brain tissues with active multiple sclerosis lesions, glioma tumors, and hypoxia-ischemia (Chesik *et al*, 2004; Elmlinger *et al*, 2001; Gveric *et al*, 1999; O'Donnell *et al*, 2002; Zhang *et al*, 2002), so these cells are the likely source of up-regulated IGFBP-2 in our cell culture system. HIV infection has been associated with reduced IGF-1, IGF-2, and IGFBP-3, and increased IGFBP-1 and-2 levels in serum (Congote, 2005; Jain *et al*, 1998; Laue *et al*, 1990; Rondanelli *et al*, 2002). Furthermore, abnormalities in circulating levels of IGFs/IGFBPs has been linked to weight loss and fat redistribution (wasting syndrome) in HIV-infected patients (Congote, 2005; Rondanelli *et al*, 2002). Previous studies demonstrated the perturbation of lipid metabolism in brain from patients with HAD (Haughey *et al*, 2004; Power and Patel, 2004). However, little is known about the role of the IGF system in HIV neuropathogenesis. One study reported that IGF-1 mRNA levels were not decreased in brain from patients with HAD, but the number of subjects was small, IGF-1 mRNA levels were variable, and control subjects were older (Boven *et al*, 1999). IGFs are well-known survival factors for many different cell types, including neurons. IGF-1 was shown to be neuroprotective against neuronal death induced by TNF-α, or by supernatants from HIV-infected cells, in a rodent neuronal cell line (differentiated PC12 cells) *in vitro* (Mackay *et al*, 2003; Ying Wang *et al*, 2003). These studies together with our findings suggested that up-regulation of IGFBP-2 can reduce neuronal survival through interference with neuroprotective effects of IGFs. Thus, our studies implicate dysregulation of the IGF system in contributing to HIV-induced neurodegeneration.

Our finding that CCL8/MCP-2 is up-regulated in supernatants from HIV-infected brain cultures is consistent with previous studies that demonstrated up-regulation of this chemokine in activated or HIV-infected monocytes/macrophages and microglia (Albright and Gonzalez-Scarano, 2004; Jiang *et al*, 2004; Suzuki *et al*, 2000; Woelk *et al*, 2004; Xu *et al*, 2004) . Furthermore, CCL8/MCP-2

mRNA levels in peripheral blood mononuclear cells (PBMCs) from HIV-infected subjects were significantly decreased following anti-retroviral therapy (Wasmuth *et al*, 2004). CCL8/MCP-2, a ligand for CCR1, CCR2, and CCR5, is chemotactic for monocytes, lymphocytes, basophils, and eosinophils (Gong *et al*, 1997; Proost *et al*, 1996). Previous reports suggested that CCL8/MCP-2 activates CCR5 and blocks CCR5-mediated HIV-1 entry and replication in cell lines and primary CD4 T cells (Gong *et al*, 1998; Yang *et al*, 2002). However, we found that addition of CCL8/MCP-2 to HIV-infected brain cell cultures did not enhance neuronal survival, suggesting that up-regulated production of CCL8/MCP-2 is not neuroprotective in this cell culture system. Furthermore, the levels of CCL8/MCP-2 in HIV-infected brain cell cultures were probably not sufficient to block HIV entry. Addition of CCL8/MCP-2 to mock-infected brain cell cultures did not induce neuronal loss, suggesting that CCL8/MCP-2 has no direct neurotoxic effect *ex vivo*. However, CCL8/MCP-2 may contribute to neuropathogenesis *in vivo* by mechanisms not detectable in our experimental system, such as recruiting circulating monocytes or other leukocytes to sites of active HIV replication in brain and contributing to chronic inflammation. In addition to up-regulation of CCL8/MCP-2, we also found up-regulation of IL-6 in supernatants of HIV-infected brain cell cultures. This finding is consistent with previous studies that demonstrated up-regulation of IL-6 in HIV-infected macrophages/microglia (Albright and Gonzalez-Scarano, 2004; Nakajima *et al*, 1989; Trentin *et al*, 1992; Xu *et al*, 2004), and in brain or CSF of acquried immunodeficiency syndrone (AIDS) patients with HAD (Gallo *et al*, 1989; Merrill and Chen, 1991; Perrella *et al*, 1992; Persidsky *et al*, 1997). Thus, CCL-8 and IL-6 may contribute to HIV neuropathogenesis by promoting chronic inflammation.

Over the past decade, a growing body of evidence suggests reciprocal communication between neurons, astrocytes, and microglia (Araque *et al*, 2001; Parpura *et al*, 1994). Astrocytes not only respond to neuronal signaling but also modulate functions of neighboring neurons through release of glutamate and other soluble factors. The simultaneous isolation of neurons, astrocytes, and macrophages/microglia from a single donor will facilitate studies on neuron-glia and glia-glia interactions that influence normal neuronal functions as well as the pathogenesis of neurological diseases. By culturing neurons in the absence or presence of astrocytes and/or microglia from the same donor, we demonstrated that astrocytes enhance HIV replication in microglia and thereby amplify HIV-induced neuronal loss, implying the importance of cooperative microglia-astrocyte interactions in neurodegenerative mechanisms associated with HIV infection. Thus, the ability to reconstitute brain cell cultures using isolated populations of neurons, astrocytes, and microglia will be valuable for future studies on pathogenic mechanisms involved in HAD and other neurological disorders, and will also facilitate neuroactive drug screening.

Materials and methods

Antibodies

Monoclonal antibodies (mAbs) used for immunophenotyping of neurons and astrocytes are summarized in Table 1. Anti-glial fibrillary acidic protein (GFAP), microtubule associated protein 2 (MAP2), glutamate, and γ-aminobutyric acid (GABA) mAbs were from Sigma (Saint Louis, MO). FluoroTagTM fluorescein isothiocyanate (FITC) conjugation kit (Sigma) and Phycolink R-phycoerythrin (RPE) conjugation kit (Prozyme, San Leandro, CA) were used to prepare FITC- and PE-conjugated antibodies, respectively.

Preparation and culture of brain cells

Human fetal brain tissues (16 to 22 weeks) were procured in accordance with institutional and federal regulations. Tissues were cut into small pieces, treated with 0.25% trypsin at 37°C for 20 min unless otherwise indicated, and dissociated by gentle pipetting. Dead cells were removed by gradient centrifugation on 42.5% Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden). Cells were then cultured on poly-L-lysine-coated multi-well plates or dishes in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, and 10% (*v*/*v*) heat-inactivated bovine calf serum (BCS) (HyClone, Logan, UT).

Flow cytometric analysis

Single cell suspensions were prepared from primary human fetal brain cell cultures (HBCs) by 0.25% trypsin digestion at 37°C for 5 to 10 min. Alternatively, single -cell suspensions were prepared by mechanical dissociation (i.e., without trypsin) to test expression of selected cell surface antigens sensitive to trypsin treatment. For direct staining, HBCs were incubated with FITC- or PE-conjugated primary Abs against cell surface antigens at 4°C for 30 min. For indirect staining, cells were first incubated at 4°C for 30 min with unconjugated primary antibodies. Cells were then incubated with FITC- or PE-conjugated goat anti-mouse immunoglobulin (Ig) (or anti-rat Ig) for 30 min at 4°C. In some experiments, cells were double stained with PE- or FITC-conjugated anti-CD44. 7-AAD (BD Pharmingen, San Diego, CA) was included in the staining buffer at the last step to exclude nonviable cells from the analysis. Cells were then fixed with phosphate buffered saline (PBS) containing 1% (*w*/*v*) paraformaldehyde. Stained cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL). For surface staining of purified cell populations, the same procedure was adapted with modifications. To test the specificity of the binding of anti-CXCR4 Ab, cells

were preincubated with 1 μM AMD3100 at 4°C for 20 min before addition of anti-CXCR4 Ab. For intracellular staining of GFAP and MAP2, cells were permeabilized with Cytofix/Cytoperm solution (BD Pharmingen).

Immunocytochemistry
Brain cells cultured on multiwell plates or slides were chilled on ice for 10 min and incubated with primary antibodies (either unconjugated or directly conjugated with FITC or PE) against cell surface antigens in cold RPMI 1640 medium containing 2.5% (*v*/*v*) heat-inactivated fetal bovine serum (FBS) and 0.02% NaN_3, at 4°C for 30 min. If unconjugated primary antibodies were used, cells were then incubated with FITC- or PE-conjugated secondary antibodies at 4°C for 30 min. Hoechst 33342 (Molecular Probes, Eugene, Oregon) was added for 10 min to counterstain cell nuclei. Cells were then fixed with 4% paraformaldehyde in PBS. For intracellular staining, cells were permeabilized with Cytofix/Cytoperm solution. Data were acquired with a CCD camera connected to a Nikon TE300 microscope.

Isolation of neurons
Primary HBCs cultured for 1 to 2 weeks, unless otherwise indicated, were incubated with 0.25% trypsin at 37°C for 5 to 10 min, and dissociated into a single cell suspension by gentle pipetting. Brain macrophages/microglia were depleted by automated magnetic cell sorting (autoMACS) after incubation of cells with anti-CD45–conjugated magnetic microbeads according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). $CD45^-$ cells were then incubated with anti-CD44 mAb, which was preincubated with anti-mIgG linked to Dynabeads through a DNA linker (Dynal Biotech, Brown Deer, WI), at 4°C for 30 min. $CD44^+$ cells were then isolated and treated with DNase to obtain bead-free cells (astrocytes). $CD44^-$ cells (neurons) were further purified by depletion of $CD44^+$ cells after staining with PE-conjugated anti-CD44 mAb and microbead-conjugated anti-PE mAb using an autoMACS system (Miltenyi Biotec). Isolated cells were cultured in DMEM containing B-27 and 10% (*v*/*v*) FBS unless otherwise indicated. For some $CD44^-$ preparations, to improve purity of neurons, the CD44-based depletion procedure was repeated 1 to 2 days later. Alternatively, isolated $CD44^-$ cells were maintained in serum-free media.

Calcium mobilization assay
Cells grown in 48-well plates were incubated with 2 to 5 μM Fluo-3/AM (Molecular Probes) in 0.2 ml of CI buffer (Hanks' buffered saline solution, 10 mM HEPES [pH 7.2], 0.1% [*w*/*v*] bovine serum albumin) for 30 min at 37°C and washed. Calcium influx in response to the indicated stimuli in CI buffer containing 1 mM $CaCl_2$ and 0.5mM $MgCl_2$ was then recorded with a CCD camera connected to a Nikon TE300 microscope. More than 100 cells were analyzed using MetaMorph software (Universal Imaging, Downingtown, PA).

HIV gp120 and SDF-1α binding assays
X4 (HXBc2) and R5 (YU2, ADA, or JR-FL) gp120s were purified from the culture supernatant of 293T cells transiently transfected with the pSVIIIexE7 plasmid containing the corresponding gp120 sequences as described previously using a F105 (anti-gp120 mAb) affinity column (Babcock et al, 2001). To examine gp120 binding, cells were incubated with gp120 that had been pre-incubated in the presence or absence of 5 to 25 μg/ml sCD4 at 37°C for 30 to 60 min at the indicated concentrations in binding buffer (2.5% FBS, 0.05% NaN_3 in PBS, pH 7.4) at 37°C for 30 min and washed. Cells were then incubated with a human anti-gp120 mAb (C11) at 4°C for 30 min and washed. At the third step, cells were stained with PE-conjugated anti-human immunoglobulin G(IgG) pAb at 4°C for 30 min. For staining experiments using cells, 7-AAD (BD Pharmingen) was included in the staining buffer at the last step to exclude nonviable cells from the analysis. If HBCs were used, FITC-conjugated anti-CD44 mAb was also included in the staining buffer at the last staining step. Cells were washed and fixed with PBS containing 1% (*w*/*v*) paraformaldehyde and then analyzed using an EPICS XL flow cytometer (Beckman Coulter). The amount of ligand bound to cells was determined by the relative mean fluorescence intensity (MFI), which is calculated as (MFIspe - MFIcon)/MFIcon. MFIspe is the MFI of cells incubated with the indicated concentrations of gp120. MFIcon is the MFI of control cells incubated in the absence of gp120. Immunoglobulin (Ig) tagged SDF-1α (SDF-1α-Ig) was produced as a secreted protein as described previously (Babcock et al, 2001). For SDF-1α binding assays, cells were preincubated with or without 1.2 μM AMD3100 at 4°C for 30 min, and incubated with SDF = 1α1g at 4°C for 30 min and washed. Cells were then stained with PE-conjugated anti-human IgG pAb at 4°C for 30 min.

Preparation of HIV stocks
R5 HIV (ADA or JR-FL) virus stocks were prepared by transfection of 293T cells or infection of PM1 cells. Briefly, 293T cells were transfected with 20 μg of proviral DNA plasmids by the calcium phosphate method. Supernatants containing virus were collected 48 h after transfection, filtered (0.45 μm pore-size filter), and quantified by reverse transcriptase (RT) assay using [^{3}H]dTTP incorporation, as described (Ohagen et al, 1999). PM1 cells were infected with R5 HIV and supernatants were collected every 3 to 4 days for up to 3 weeks. Virus preparations were then concentrated and purified by ultracentrifugation through a 20% sucrose cushion at 27,000 rpm for 1 h at 4°C, followed by depletion of microvesicles from PM1-derived virus preparations with anti-CD45 coated microbeads or Dynalbeads (Trubey et al, 2003). To prepare 2,2'-dithiodipyridine (aldrithiol-2

or AT-2, Sigma)-inactivated virus, concentrated virus stocks were incubated with 1 mM AT-2 at 37°C for 1 h (Rossio et al, 1998). AT-2 was then removed by ultrafiltration with a centrifugal filtration device (Vivascience, Lincoln, UK). Concentrated purified virions (either AT-2 inactivated or not) were then aliquoted and stored at −80°C. Control supernatants from 293T and PM1 cells were similarly prepared, concentrated, purified, and used for mock infections. Virus titer was quantitated by RT assay or p24 ELISA (PerkinElmer Life Sciences, Boston, MA).

HIV replication assays

Microglia were purified from mixed brain cell cultures by gentle shaking and washing as described (Wang *et al*, 2002). The purity of microglia obtained by this method was ∼95%, as determined by staining with anti-CD45. Microglia cultured in the presence or absence of neurons and/or astrocytes, were infected by incubation with R5 HIV (25,000 ^{3}H cpm RT units per ml). After 16 h of incubation at 37°C, the medium was removed and washed twice before addition of fresh medium. A 50% medium exchange was performed every 7 days. HIV replication was examined by monitoring RT activity or p24 levels in the culture supernatants.

Neurotoxicity assay

Isolated neurons (N) were cultured in the absence or presence of astrocytes (A) and/or microglia (M) for 4 to 7 days on poly-lysine–coated 48-well or 24-well plates in high-glucose DMEM containing 10% (*v*/*v*) heat-inactivated calf serum. Cultures were then infected by incubation with R5 HIV (10 to 35 ng p24) or mock infected with the same volume of control supernatants. In some experiments, cultures were preincubated with or without 1 μM AMD3100 or 100 nM TAK779 for 2 h at 37°C before addition of HIV. After 16 h incubation at 37°C, the medium was removed and cultures were washed twice before addition of fresh medium containing 1% (*v*/*v*) heat-inactivated calf serum and 5 ng/ml M-CSF. In some experiments, 10 ng/ml of CCL8/MCP-2, 10 ng/ml of IGF-1, 10 ng/ml IGF-2, or 100 ng/ml IGFBP-2 was also included in the culture medium. A 50% medium exchange was performed every 7 days. Productive HIV infection was confirmed by monitoring RT activity or p24 levels in culture supernatants. After 2 weeks in culture, cells were dissociated from plates with 0.25% trypsin, washed, and incubated with FITC-conjugated annexin-V, PE-conjugated anti-CD44, cychrome-conjugated anti-CD45, and 7-AAD at 4°C for 20 min. Cells were then analyzed immediately after the addition of 2×10^4 flow-count beads using an EPICS XL (Beckman Coulter) flow cytometer. Percentages of neuronal loss were calculated as $(N_{mock}\text{-}N_{hiv})/N_{mock} \times 100$. N_{mock} is the number of viable neurons (annexin-V-CD44-CD45-7-AAD-) in mock-infected cultures. N_{hiv} is the number of viable neurons in HIV-infected cultures.

Cytokine antibody array and ELISA

Two cytokine Ab arrays (VI and VII) were used to detect expression of 120 cytokines/proteins in brain cell culture supernatants (RayBiotech, Norcross, GA). The expression of each cytokine/protein was evaluated by measuring the optical density (OD) of duplicate spot using Eagle Sight Software. The ratio between each cytokine spot and the positive control was calculated. Levels of CCL8/MCP-2 and IGFBP-2 in cell culture supernatants were quantified by ELISA (RayBiotech).

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