Journal of NeuroVirology, 7: 196–207, 2001
 ISSN 1355–0284/01 $12.00+.00

Interactions between HIV-1 gp120, chemokines, and cultured adult microglial cells

Andrew V Albright,[1] Julio Mart´n,[1] Michael O'Connor,[2] and Francisco González-Scarano[1]

[1]*Departments of Neurology and Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA and* [2]*Department of Neurosurgery, Thomas Jefferson University School of Medicine, Philadelphia, Pennsylvania, USA*

HIV dementia (HIVD), a disease that is apparently mediated by neurotoxins and viral proteins secreted by HIV infected microglia, is characterized neuropathologically by an increased number of activated microglia in the brains of affected individuals. Consequently, the rational design of potential therapeutic strategies should take into account the mechanisms that lead to microglial activation and to their increased prominence in the adult brain. In this regard, one leading hypothesis proposes that microglia are recruited to specific sites in the central nervous system (CNS) as a result of interactions between microglial chemokine receptors and chemokines, or even the viral glycoprotein gp120, which binds chemokine receptors in the process of cellular entry. Adult microglia express the functional chemokine receptors CCR5 and CXCR4 molecules that mediate chemotaxis in these and other cell types. We determined that purified adult microglial cultures contain a heterogeneous population with respect to their ability to respond to the α- and β-chemokines, SDF1α, and MIP-1β. A mean of 14.6% of the microglia assayed responded to both α- and β-chemokines ($CCR5^+CXCR4^+$ phenotype); 45.4% of microglia were phenotyped as $CCR5^+CXCR4^-$; 12.9% of the microglia were $CXCR4^+CCR5^-$; and 27.0% of microglia did not respond to either chemokine. No increase in intracellular calcium levels was seen in the vast majority of microglia exposed to the soluble HIV envelope protein, gp120, or to HIV envelope (gp120/gp41) expressed on MLV virus pseudotypes. However, exposure of microglia to soluble fractalkine or to other chemokines resulted in an intracellular calcium flux. Our results raise the possibility of microglial heterogeneity with respect to their response to chemokines, and indicate that any effects due to gp120 are likely to be considerably less robust than the response of microglia to the natural ligands of their chemokine receptors, for example SDF1α and MIP-1β.*Journal of NeuroVirology*** (2001) **7,** 196–207.

Introduction

Microglia play an essential role in human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system (CNS), and are key to its principal neuropathogenetic manifestation, HIV dementia (HIVD). First, microglia/brain macrophages are the primary HIV-1 cellular target in the CNS, since microglia have been consistently identified as expressing the vast majority of HIV-1 proteins and RNA detected in the brain (Gabuzda *et al*, 1986; Gartner *et al*, 1986; Koenig *et al*, 1986; Budka, 1990). Second, HIVD brains have increased numbers of microglia/brain macrophage, microglial nodules, and multinucleated giant cells (MGCs) (Glass *et al*, 1993); these histopathological changes demonstrate the fusogenic and pathogenic potential of HIV-1 glycoproteins within the CNS (Porwit *et al*, 1989). Third, microglia in autopsy specimens from patients with HIVD are "activated," as defined by their expression of MHC class II and CD4 molecules (Soontornniyomij *et al*, 1998; Kaul and Lipton, 1999) and their secretion of putative neurotoxins (Tyor *et al*, 1993; Kolson *et al*, 1998). Microglial toxicity due to "activated" microglial cells has also been implicated in a number of inflammatory and degenerative diseases with disparate etiologies such as multiple sclerosis (Bo *et al*,

Address correspondence to Andrew V Albright, Dept. of Neurology, University of Pennsylvania School of Medicine, 255 Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104-6146, USA. E-mail: albrigh@mail.med.upenn.edu

Received 17 August 2000; revised 10 November 2000; accepted 7 February 2001.

1994), Alzheimer's disease (Kalaria, 1993), cerebral malaria, and tuberculosis (Medana and Hunt, 1997; Sciorati *et al*, 1999; Stoll and Jander, 1999). Nevertheless, detectable HIV-1 infection within the CNS is apparently not an absolute requirement for the microglial activation associated with HIVD (Glass *et al*, 1995), even though HIV-1 infection and the signature alterations associated with activation coincide frequently (Weis *et al*, 1994; Dick *et al*, 1997).

A potential alternative scenario of microglial activation and subsequent neurotoxicity that does *not* involve productive infection is that the HIV-1 surface glycoprotein gp120 is released or "shed" from virions or infected cells, binds the appropriate receptors, and triggers an intracellular signal in microglia. Recombinant gp120 and gp120/gp41 reportedly induce alterations in intracellular Ca^{2+} levels in a number of cell types, which renders this theory plausible. A decade ago, Dreyer and colleagues induced an intracellular calcium signal in mixed rat neuronal cells using recombinant gp120 (Dreyer *et al*, 1990). The mechanism was not known. Then, after the discovery that gp120 binds to CD4 and chemokine receptors in 1996 (Feng *et al*, 1996), similar experiments in lymphocytes and macrophages, which express an array of chemokine receptors, demonstrated both positive and negative results using calcium mobilization assays (Davis *et al*, 1997; Herbein *et al*, 1998; Liu *et al*, 2000), Weissman *et al*, 1997). Neurons and cells transfected with chemokine receptors were also examined for the ability of gp120 to induce intracellular signaling (Meucci and Miller, 1996; Madani *et al*, 1998; Arthos *et al*, 2000).

An important consideration is the question of whether soluble, free gp120 is or could be present *in vivo*. Prior to recognition that T-cell line adapted (TCLA) HIV-1 isolates constituted a unique subgroup of HIV-1 strains, investigators determined that gp120 from those isolates could be shed as a soluble protein into culture supernatant (Schneider *et al*, 1986; Pauli *et al*, 1987; Earl *et al*, 1991), possibly after its interaction with CD4 (Thali *et al*, 1992). This "shed" gp120 could theoretically be soluble in blood or tissue and bind to the cell surface. However, macrophage-tropic and other primary isolates do not shed their gp120 as readily as do TCLA isolates, even when treated with soluble CD4 (sCD4) (Gu *et al*, 1993; Moore *et al*, 1993), which makes any theories regarding soluble gp120 less attractive. Lastly, shed HIV-1 gp120 has been found in the serum of some AIDS patients (Capobianchi *et al*, 1992); however, there is no evidence that soluble gp120 plays a direct role in human pathogenesis.

Theoretically, chemokines themselves could bind to chemokine receptors on microglia, which alone might be sufficient to activate microglia and trigger their toxic potential. Chemokines are a family of small (8–14 kDa), chemoattractant pro-inflammatory cytokines. They play a major role in the recruitment of immune cells, such as microglia, to sites of inflammatory and immune responses (reviewed in Campbell and Butcher, 2000; Murdoch and Finn, 2000). Chemokine receptors, particularly the alpha (α) chemokine receptor CXCR4 and the beta (β) chemokine receptor CCR5, work in conjunction with CD4 to mediate entry of HIV into susceptible cells (Alkhatib *et al*, 1996; Choe *et al*, 1996; Deng *et al*, 1996; Doranz *et al*, 1996; Feng *et al*, 1996; Wu *et al*, 1996). Since microglia express a number of chemokines and chemokine receptors, including functional CCR5 and CXCR4, this third hypothesis of microglial activation is viable (Albright *et al*, 1999). Additional support for this hypothesis includes evidence that other CNS cells types also secrete chemokines (Hesselgesser and Horuk, 1999; Bacon and Harrison, 2000), and that other neurological diseases could be linked to chemokines and their chemokine receptors (Glabinski and Ransohoff, 1999; Zhang *et al*, 2000).

To determine whether treatment of microglia with gp120 would alter their intracellular calcium concentration, a common manifestation of chemokine-mediated intracellular signal transduction, we used microscopic techniques that detect such changes in single cells (Itoh *et al*, 1998). We found no evidence to support a *prominent* role for gp120 in microglial signaling, at least in comparison to the consistent responses detected with α- and β-chemokines. Moreover, we found an unexpected level of heterogeneity among individual cells. Our results raise the possibility of chemokine receptor heterogeneity among microglia from the same donor, and indicate that any response to gp120 is likely to be considerably less robust than a chemokine-induced calcium response.

Materials and methods

Isolation and culture of microglia

Microglia were isolated from fresh human brain tissue obtained during temporal lobe surgery for intractable epilepsy, and cultured for 4–7 days in microglia media, which consists of Dulbecco's Minimal Essential Media (DMEM) supplemented with 5% fetal bovine serum (FCS) (Atlanta Biologicals), 5% giant cell tumor supernatant (GCTS) (Fisher), 4.5 g/l glucose, 50 μg/ml gentamicin, and 1 mM sodium pyruvate (Strizki *et al*, 1996). These microglial cultures were washed three times with PBS and incubated with 0.25% trypsin. Ninety to 95% of the adherent microglia were detached and centrifuged for 10 min at 1750 $\times$ g on a Beckman tabletop centrifuge. Microglia were plated at 3–4 $\times 10^4$ microglia per well in 8-well Lab-Tek Permanox chamber slides (Nalge Nunc International, Naperville, IL), and cultured in microglia media. The purity of the microglia in culture was determined to be greater than 95% using diI-Ac-LDL (Biomedical Technologies Inc, Stoughton, MA) as previously described (Strizki *et al*, 1996).

Intracellular calcium assay

Unless otherwise specified, all intracellular calcium $[Ca^{2+}]_i$ measurements were performed at room temperature (23–25°C). Coverslips (22 × 22 mm), containing $2–4 \times 10^4$ microglia were inverted, and attached to the upper side of a perfusion chamber (RC-21B, Warner Instrument Corp., CT), which was then mounted on the stage of an upright microscope (Optiphot, Nikon, Japan) equipped for epifluorescence detection. The microglia were loaded with 5 μM fura-2/AM in 0.02% Pluronic F-127 (Molecular Probes) for 30 min (Itoh *et al*, 1998; Albright *et al*, 1999). Excess fura-2/AM was washed out of the chamber and the cells maintained in mock recording media for an additional 15 min to completely hydrolyze the internalized fura-2/AM (Itoh *et al*, 1998). The microglia were exposed to 60–66 nM macrophage inflammatory protein 1β (MIP-1β) or stromal cell-derived factor 1α (SDF-1α) (Peprotech, Rocky Hill, NJ), while being illuminated by a 75-watt Xe arc lamp through a 340 and then 380 nm excitation filter, and controlled by a computer-assisted filter changing device (LAMBDA-10, Sutter Ins. Co., CA). Emission fluorescence images were collected every 15 s through a 20× objective lens (CF Fluor DL, numerical aperture: 0.75, Nikon, Japan) and a 510 nm barrier filter by a SIT camera (C2400-08, Hamamatsu Photonics KK, Japan), and converted to digital data by an image-processing system (ARGUS-50, Hamamatsu Photonics KK, Japan). Each frame of digital data was stored in a PC-based computer as both numerical and color images (512 × 382 pixels) and converted to single-cell temporal 510-nm emission plots. These data were plotted at 15-s intervals either as single cell tracings or as the average of all cell recordings from a single experiment.

For soluble HIV gp120 assays, 8–25 nM gp120 was added to microglia in the manner previously described for chemokines (Albright *et al*, 1999). The intracellular calcium recordings were scored as being either above or below baseline levels. For all experiments, the viability of the microglia was confirmed by their response to the β-chemokines, MIP-1β, and RANTES. The chemokine receptor tropism of each of these HIV-1 gp120s is listed in Table 1.

Heterogeneity/homogeneity analysis of functional chemokine receptors

The different microglial populations were scored according to their ability to respond to α- and/or β-chemokines, as judged by a noticeable change in intracellular calcium levels over background. Microglia were separated into four groups ($CCR5^{+/-}$ $CXCR4^{+/-}$) based on the induction of intracellular calcium concentration changes when exposed to α- and β-chemokines. The means and standard deviations of the intracellular calcium concentration plots for each group of microglia were calculated from six separate experiments in cells from separate brain preparations. The Wilcoxon Sum of Ranks statistical test was performed using SPSS for MS Windows, to determine if there was any statistical difference between the four phenotypic groups of microglia. MDM, isolated and cultured as previously described (Strizki *et al*, 1996), were assayed in the same manner.

Table 1

gp120 strain	*Tropism*[3]	*Requirement for CD4*	*Protein quality assay*[4]
8×V3BaL[1]	R5	No	Western, ELISA
JrFL	R5	Yes	Western, ELISA
8×V3IIIB[1]	X4	No	Western
CM235	R5	Unknown	Western, ELISA
Clade E[2]	NSI	Unknown	Western
SF2	X4	Unknown	nd[5]
IIIB	X4	Yes	Western, ELISA

[1]Hoffman *et al*, 1999.
[2]Clade E 93TH975.
[3]X4 = (CXCR4-using) and R5 = (CCR5-using).
[4]Western Blot and ELISA as described in Materials and methods, and Results sections.
[5]nd = not done.

Analysis of calcium flux data

Each experiment was scored according to the following scheme: "0" when there was no discernible increase in the emission ratio of fura-2 at 340/380 nm ratio of intracellular calcium concentration; "1" when the ratio increased 0–0.5-fold over the background ratio; "2" if the ratio increased 0.5–1-fold; "3" if the ratio increased 1–2-fold; and "4" if the increase was >2-fold. The average of this score was calculated for 5–16 experiments performed for each chemokine.

Source of gp120s

Purified, soluble gp120 proteins from HIV-$1_{Jr-Fl,\ 8\times V3IIIB,\ and\ 8\times V3BaL}$ strains were generous gifts from Trevor Hoffman and Carrie McManus of the laboratory of Dr Robert Doms (Dept. of Pathology, University of Pennsylvania School of Medicine) (Doranz *et al*, 1999; Hoffman *et al*, 1999). Briefly, these soluble gp120 proteins were constructed by inserting a stop codon into the *env* gene at the 3′ end of gp120; the gp120 protein was synthesized with a vaccinia-virus T7 expression system in 293T cells. The gp120 protein was incubated with Galanthis Novalis (Snow Drop) Lectin I beads and eluted (Doranz *et al*, 1999; Hoffman *et al*, 1999). The purified gp120s have been shown to bind to CD4 and chemokine receptors and were tested for purity [see next two sections]. The other soluble HIV-1 gp120s used in our experiments were obtained from the AIDS Reagent Program as purified, soluble gp120s synthesized from envelope clones from HIV-$1_{CM235,93TH975,IIIB}$ strains in baculovirus.

Western blot and ECL detection

CM235, clade E, IIIB, 8×V3IIIB, 8×V3BaL, MN, and JrFL gp120s were individually diluted in fresh 2×SDS loading buffer (pH 6.5–6.8), boiled for 4 min, placed

immediately on ice, centrifuged at 13 000 rpm for 1 min, and electrophoresed on a precast, 8 cm × 10 cm × 1 mm, 10% Tris-Glycine pAGER FMC gel (Bio Whittaker, Rockland, MA). Rainbow (RPN 756). Prestained Protein Range Marker (New England BioLabs) was loaded in lanes next to the gp120. All proteins were transferred using a semi-dry transfer to an Immobilon-P PVDF membrane (Millipore). This membrane was probed with 1:2000 dilution of rabbit anti-HIV envelope (#R1104) for 1 h at 4°C, washed in Blotto, and then probed for 15 min with a 1:5000 dilution of the secondary antibody, goat anti-rabbit IgG conjugated to HRP (Boehringer). Positive binding was detected with the ECL + Western Blotting Detection kit (Amersham #2132) as per the manufacturer's instructions. Once the substrate was added, Action-Scientific X-ray film was exposed to the blot for 15–30 s and promptly developed.

Anti-gp120 ELISA and soluble CD4 (sCD4) binding
A 96-well microtiter plate was coated with 10 μg/ml of the sheep anti-gp120 antibody D7324, which binds to the 15 carboxy-terminal amino acids of gp120, diluted in bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plate was washed twice with Wash buffer (PBS with 0.05% Tween 20) and incubated with blocking solution (10% heat-inactivated FCS in Wash buffer) for 1 h at 37°C. After washing with Wash buffer, 8–25 nM of soluble gp120 (see previously) was added to the plate and incubated for 2 h at 37°C. After five washes with Wash buffer, 0.1 μg/ml of HIV-1 gp120 monoclonal antibody 17b (Thali *et al*, 1993) was added to all wells, either in the presence or absence of 5 μg/ml sCD4, for 1 h at 37°C. Antibody 17b binds to a conformation-dependent epitope on gp120, in the bridging peptide between the V3 and V1/V2 loops, with increased affinity after gp120 is bound by soluble or membrane-bound CD4. After washing five times with Wash buffer, peroxidase conjugated anti-human IgG, with specificity for heavy and light chains (Pierce), was diluted 1:5000 in wash buffer, added to the microtiter plate, and incubated for 1 h at 37°C. After washing, 1-Step ABTS substrate (Pierce) was added, and the absorbance read at 405 nm after 30–60 min incubation at room temperature.

Preparation of MLV pseudotype virus
The plasmids pCgp, pCnβg, and DS-env were cotransfected into 293T cells to produce a pseudotype virus containing β-galactosidase gene with functional HIV-$1_{DS\text{-}br}$ envelope protein (Albright *et al*, 1999; Ma *et al*, 1999). Briefly, supernatant containing this pseudotyped virus was centrifuged to remove cellular debris and ultracentrifuged to increase the viral titer 25–40-fold. The viral pellet was resuspended to ensure no residual calcium phosphate remained from the original transfectant. The $HIV_{DS\text{-}br}$ envelope/MLV pseudotyped virus was tested for its ability to infect cells transfected with CD4 and CCR5. Viral stocks had a titer of 1–5 × 10^4 IU/ml as judged by their ability to infect U373-MAGI $CD4^+CCR5^+$ cells, but not $CD4^+CCR5^-$ or $CD4^+CXCR4^+$ U373-MAGI cells (Vodicka *et al*, 1997).

Results

Categorization of microglia based on their expression of functional α- and β-chemokine receptors reveals heterogeneity
Cultured microglia and monocyte-derived macrophages (MDM), exposed to α- and β-chemokines, transduce a signal via mobilization of calcium (Herbein *et al*, 1998; Albright *et al*, 1999). To further our understanding of the expression of functional chemokine receptors on microglia, we analyzed the calcium responses of single microglia when exposed to α- and β-chemokines, the natural ligands for CXCR4 and CCR5. These experiments tested the hypothesis that in a population of microglia, chemokine receptor responses fall into different phenotypic groups, which would reflect variability in the expression of functional receptors.

Free intracellular calcium levels were measured in individual microglia after exposure to 60–66 nM of α- and β-chemokines (Albright *et al*, 1999). The CXCR4 ligand SDF-1α induced a mild change in intracellular calcium levels in a subset of the cultured microglia (Figure 1A). Treatment with the CCR5 ligand MIP-1β resulted in a more robust signal in a higher proportion of the microglia in culture (Figure 1A). These data are representative of six separate microglial experiments, each from a separate donor. The mean emission ratios were plotted versus time (Figure 1B) and are consistent with the greater mean expression of CCR5 antibody binding sites (45 000) versus the mean number of CXCR4 antibody binding sites (20 000) on microglia (Albright *et al*, 1999).

Of 289 microglial cells assayed during the six separate experiments, 47 microglia responded to both MIP-1β and SDF-1α, and were categorized as physiologically double positive ($CCR5^+CXCR4^+$); therefore, a mean of 14.6% of the microglia assayed responded to both α- and β-chemokines (Figure 2). The largest percentage (45.4%) of microglia (145 cells) responded to MIP-1β only (Figure 2) and were classified as $CCR5^+CXCR4^-$. Conversely 30 cells (12.9%) responded only to SDF-1α, and a significant proportion (67 cells or 27.0% of the total) did not respond to either chemokine (Figure 2). The differences in the size of the $CCR5^+CXCR4^-$ and the $CCR5^-CXCR4^+$ groups reached statistical significance ($P = 0.0435$) using the Wilcoxon Sum-of-Ranks [Mann–Whitney]. None of the other differences were confirmed statistically. In summary, our experiments confirmed the hypothesis that, in a population of cultured microglia, single microglial cells have heterogeneous calcium signal transduction phenotypes when exposed to

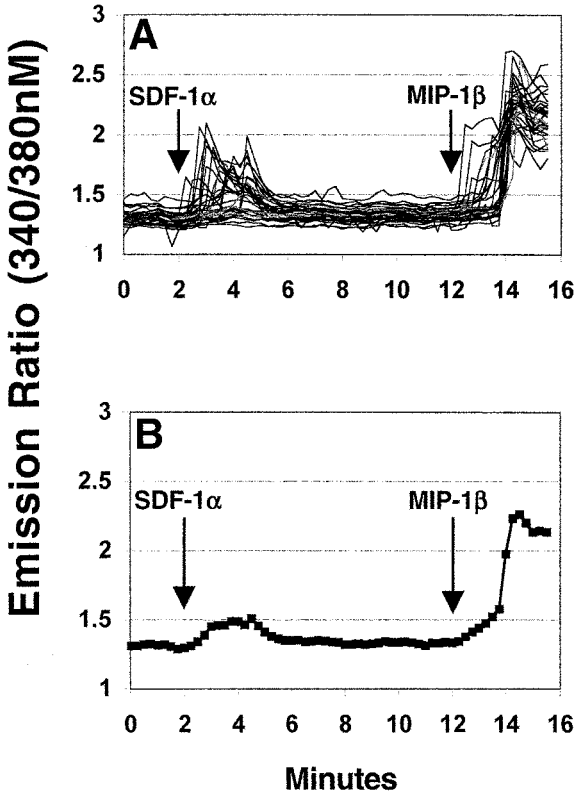

Figure 1 Microglial cultures consist of a heterogeneous population with respect to their response to α- and β-chemokines. (**A**) Individual cells in a microglial culture exposed to SDF-1α and MIP-1β have a specific response pattern. Each line represents the calcium measurements of a single microglial cell, loaded with fura-2, taken at 15-s intervals after chemokine exposure (Albright *et al*, 1999 and Materials and methods). The emission ratio at 510 nm was plotted on the y-axis and the time in minutes on the x-axis. This experiment is representative of six separate experiments with microglia from six individual brain preparations. (**B**) The average of all of the individual microglial calcium measurement of the experiment in panel (**A**) was plotted in the same manner.

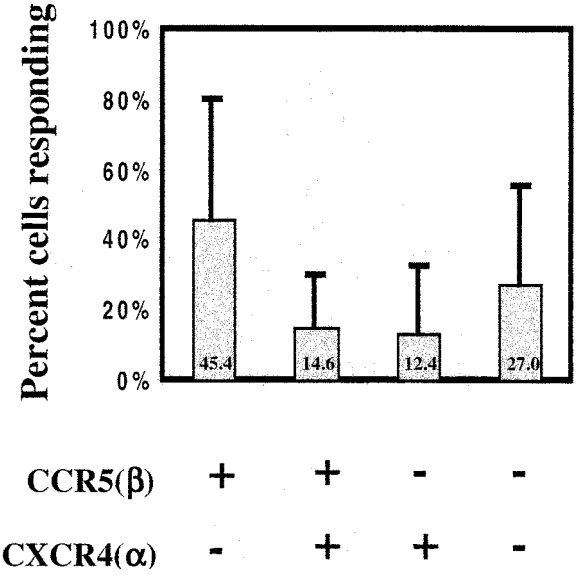

Figure 2 Summary of the heterogeneous calcium flux response in microglia. (**A**) Data from six calcium flux experiments were plotted with functional chemokine receptor phenotype on x-axis, and percentage of cells responding in calcium flux assay in the y-axis. Microglia responding to both MIP-1β and SDF-1α made up an average of 14.6% of the cells in the cultures, 45.4% responded to MIP-1β only, 12.9% to SDF-1α only, and 27.0% responded to neither chemokine. A "+" for CCR5 indicates a response to MIP-1β and a "+" for CXCR4 response to SDF-1α.

α- and β-chemokines. These results may reflect variability in chemokine receptor expression.

The same analysis was performed on monocyte-derived macrophages (MDM): 21.1% of cultured MDM responded to both MIP-1β and SDF-1α; 21.2% to only MIP-1β, 10.0% to SDF-1α only, and 47.8% of MDM responded to neither chemokine (data not shown). Therefore, taking into account experimental variability, MDM and microglia had similar, but not identical, functional α- and β-chemokine receptors expression patterns. Other researchers, using a variety of experimental systems, have reported intracellular calcium signaling in MDM after treatment with chemokines (Weissman *et al*, 1997; Meucci *et al*, 1998; Arthos *et al*, 2000).

gp120 induction of signal transduction in microglia? We then tested whether gp120 molecules with different coreceptor utilization specificity could mimic α- and β-chemokines by inducing the mobilization of the intracellular calcium stores in microglia. We used soluble gp120, presumably monomeric in form, and gp120 expressed on the surface of murine leukemia virus (MLV) pseudotyped virus, which is most likely in an oligomeric state (Kwong *et al*, 2000; Weiss *et al*, 1990).

The purity of these gp120 preparations was analyzed by Western blotting (Figure 3, and compared to total protein detected with a Ponceau stain (data not shown). This Western blot, in conjunction with the Ponceau protein staining, demonstrated no detectable impurities in these protein preparations. The band with faster mobility, particularly noted in the JrFL preparation, is probably due to partial glycosylation, but it did not affect binding to CD4 (Doranz *et al*, 1999). Furthermore, among the gp120 proteins used, 8×V3BaL, 8×V3IIIB, and Jr-FL gp120s have been shown to bind to chemokine receptors in an assay using cells expressing CCR5 or CXCR4 (Doranz *et al*., 1999; Hoffman *et al*, 1999).

We determined that the soluble gp120 preparations were pure and able to bind to CD4 and an antibody directed against the 17b epitope on gp120, which is close to the chemokine receptor binding site (Figure 4) (Wyatt *et al*, 1995). The anti-gp120 antibody, D7324, which binds to a conserved peptide in the carboxy terminus of gp120, captured the gp120 on ELISA microtiter plates. Jr-FL, IIIB, CM-235, and 8×V3BaL gp120 proteins were then added at concentrations ranging from 10–810 ng/well after preincubation with 5 μg/ml of sCD4. It is hypothesized

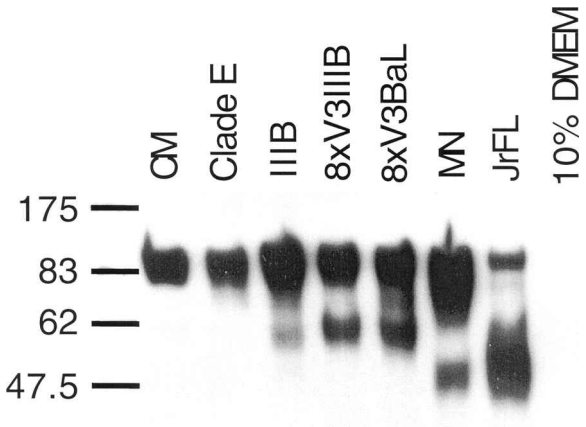

Figure 3 Western blot of gp120 proteins used in signaling assays. Purified, soluble gp120s: CM, clade E, IIIB, 8×V3IIIB, 8×V3BaL, MN, JrFL were reduced, electrophoresed on a 10% SDS-PAGE gel, transferred to a PVDF membrane, bound by an anti-gp120 antibody, #R1104, and developed using the ECL-Western Detection System. Rainbow markers were resolved in the left-most lane, and are indicated by a black band drawn over them. These markers indicate the presence of a band of approximately 120-kD size in all of the gp120 preparations. The membrane was also stained for total protein using the Ponceau stain with no additional bands being detected (not shown).

that when gp120 binds to CD4, a conformational change occurs in gp120, exposing the 17b epitope. As shown in Figure 4, soluble JrFL and CM235 gp120s demonstrated very little, if any, binding to 17b (0.1 μg/ml) in the absence of sCD4 pre-incubation. However, there was clearly antibody binding after sCD4 treatment. The IIIB gp120 bound 17b with or without sCD4 pretreatment; however, binding was increased after sCD4 treatment. The CD4-independent 8×V3BaL gp120 bound 17b equally well before and after sCD4 treatment, as previously described (Hoffman *et al*, 1999).

Microglia loaded with fura-2 demonstrated no evidence of an intracellular calcium flux induction when treated with 8×V3BaL or 8×V3IIIB gp120s (Figure 5 and Table 2). Similar analyses were performed with other gp120 proteins (Figure 6 and Table 2). Exposure of microglia to CM235, Clade E, SF2 gp120s resulted in no change in intracellular calcium concentrations in two to five experiments for each gp120 (Table 2). However, in one experiment, all three of these gp120s induced a calcium flux (Figure 6A). A different order of gp120 addition was used in other experiments, so that each strain of gp120 was added first at least once (not shown). These data are summarized in Table 2. Although there was a hint of intracellular calcium flux in microglia following treatment with some gp120 preparation in our experiments, in general, this calcium response was much less prominent than the calcium response previously seen after chemokine treatment (Albright *et al*, 1999 and Figure 1). Although it has been previously shown that gp120 is present in the brain of patients with HIVD, the concentration was not estimated (Nath *et al*, 2000).

Next, we examined whether HIV-$1_{DS\text{-}br}$ envelope (gp12/gp41), functionally incorporated into MLV pseudotypes, could induce a change in intracellular calcium levels in microglia. The HIV-$1_{DS\text{-}br}$ envelope/MLV pseudotyped viruses were able to specifically infect CCR5+ cells (data not shown), confirming that DS-br gp120 was functionally incorporated onto the virions. This observation is in contrast to envelopes from other HIV-1 strains tested, whose envelopes did not pseudotype well with MLV (data not shown). Microglia exposed to DS-br/MLV pseudotypes demonstrated no significant change in free intracellular calcium concentrations (data not shown). This experiment was repeated 13 times with uniformly negative results.

Calcium signaling experiments with non-α-/β-chemokines

The response to non-α-/β-chemokines in human adult microglia and their potential importance in CNS function and pathogenesis has not been described to any great extent. Therefore, we examined whether fractalkine, a CX_3C chemokine, could induce a response in these cells. Fractalkine exists in both membrane-attached and soluble forms (Bazan *et al*, 1997). In the CNS, fractalkine is expressed by neurons, while the fractalkine receptor (CX_3CR1) is expressed on microglia (Nishiyori *et al*, 1998). Since fractalkine induces microglial cell migration (Harrison *et al*, 1998; Nishiyori *et al*, 1998) and activation, this chemokine might play a role in microglia–neuron communication. We determined that soluble fractalkine induced intracellular calcium signal in cultured, human microglia (Table 2A), which is similar to findings in rat microglia (Boddeke *et al*, 1999; Maciejewski-Lenoir *et al*, 1999).

Conversely, vMIP-II, which is a homologue of human MIP-1α encoded by human herpesvirus type-8 (HHV-8), did not induce any consistent change in intracellular calcium levels. In one of six experiments performed, there were only small changes in calcium levels (data not shown). Our results are in agreement with vMIP-II calcium flux experiments performed on CHO cells stably transfected with CCR5 (Kledal *et al*, 1997). We were unable to block HIV-1 infection of microglia with vMIP-II (data not shown), which others have suggested may be possible (Boshoff *et al*, 1997; Kledal *et al*, 1997). Lastly, the two chemokines interferon (IFN)-γ-inducible protein-10 (IP-10) and monokine induced by IFN-γ (Mig), two ligands of the chemokine receptor CXCR3, did not induce a change in intracellular calcium levels in these cells (Table 2A). This was an expected result since CXCR3 has not been detected on microglia.

Therefore, based on the calcium flux scoring system described in the Materials and methods, we

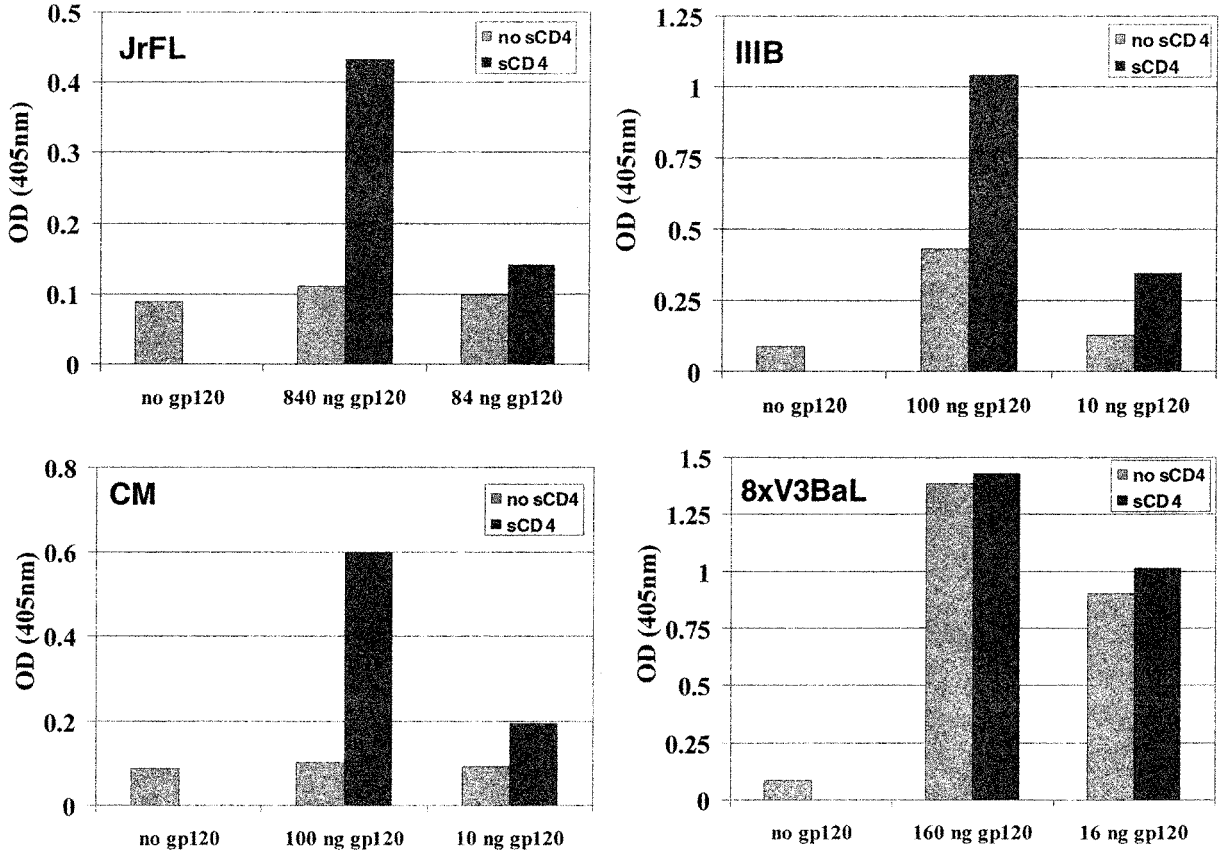

Figure 4 ELISA to detect exposure of the conformation-dependent 17b epitope. Ninety-six-well microtiter plates were coated with D7324 (a sheep antibody against the C-terminus of gp120) then incubated with 10–810 ng of gp120. The conformation-dependent anti-gp120 antibody (17b) that recognizes the putative chemokine receptor-binding region of gp120 was added either in the presence or absence of 5 μg/mL of soluble CD4 (sCD4). The anti-gp120 antibodies used recognized Jr-FL, IIIB, CM, and 8×V3BaL gp120s. After treatment with sCD4, gp120s from Jr-FL, IIIB, and CM bound more 17b antibody, consistent with a predicted conformational change exposing this epitope. The gp120 from 8×V3BaL has a highly exposed 17b epitope prior to sCD4 treatment, consistent with its CD4-independence (Hoffman *et al*, 1999).

determined that fractalkine, SDF-1α, RANTES, and MIP-1β formed a distinct group of chemokines, which induced reproducible changes in intracellular calcium in microglia (Table 2, top panel). Conversely, vMIP-II, Mig, and IP-10 resulted in very little or no change in intracellular calcium levels in a number of experiments.

Discussion

By analyzing the free intracellular calcium concentration in cultured microglia, we determined that human adult microglia have a heterogeneous response to the chemokines SDF-1α and MIP-1β (Figure 2), with the majority of the cell population responding best to MIP-1β. This is consistent with previously published data indicating that in bulk, quantitative flow cytometry (QFC) analyses with monoclonal antibodies, the β-chemokine receptor CCR5 is more abundant than the α-chemokine receptor CXCR4 (Albright *et al*, 1999). However, there were subpopulations of cells that demonstrated a more robust response to SDF-1α (Figure 1A/2), a result which would not have been predicted from the antibody data. Additionally, there were some "null" cells that did not respond to either chemokine. Furthermore, relatively large variations among experiments hint that cells obtained from different subjects are likely to display additional heterogeneity, although this was not studied directly. These data are congruent with previous observations that the replication of HIV in microglia, which requires the presence of certain chemokine receptors and particularly CCR5, can vary among cells from different donors (Collman *et al*, 1989; Strizki *et al*, 1996), and could relate to variations in the number of activated microglia seen in the brains of HIV infected individuals (Janssen *et al*, 1992; McArthur *et al*, 1994).

There is a smaller percentage of microglia expressing functional chemokine receptors, CXCR4 (39%) and CCR5 (60.0%) in Figures 1 and 2, than the percentage of microglia expressing detectable levels of anti-CXCR4 (78%) and anti-CCR5 (91%) binding sites as determined by QFC (Albright *et al*, 1999). One

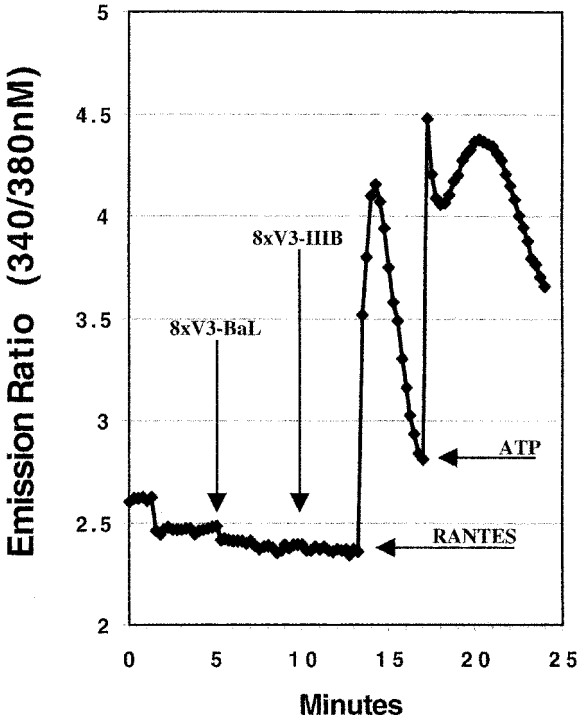

Figure 5 Ca^{2+} mobilization in microglia treated with CD4-independent gp120s. Two CD4-independent monomeric gp120s, 8×V3IIIB, and 8×V3BaL with different HIV coreceptor tropisms, CXCR4 and CCCR5 respectively, were synthesized with a vaccinia expression system, and microglia were exposed to these preparations as indicated in Materials and methods. Neither protein induced a change in the free intracellular calcium concentration in the cultured microglia. This specific graph is representative of four separate experiments. Positive controls, namely RANTES and ATP, were used in each experiment, but the order of addition was altered such that each of the gp120s was added "first" in at least one experiment. Table 2 is a summary of these data.

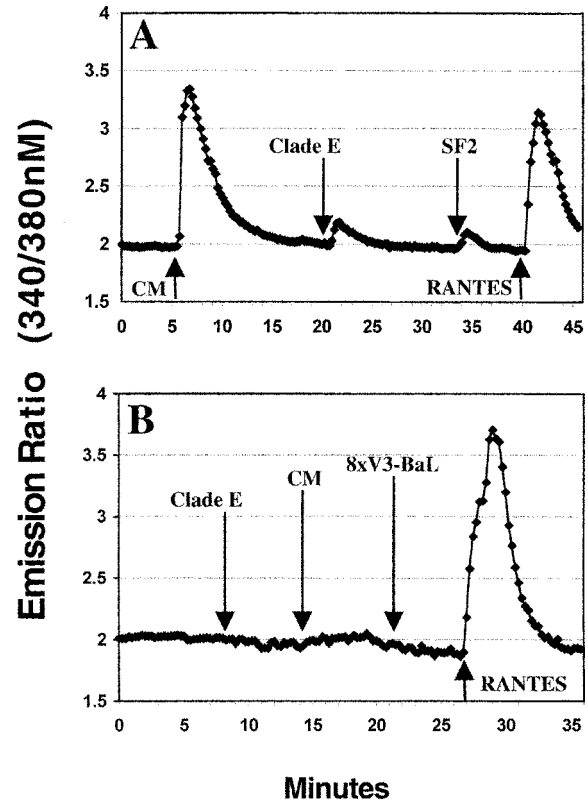

Figure 6 Variable intracellular calcium responses in human, adult microglia exposed to various monomeric gp120 proteins. (**A**) Purified, soluble gp120s (CM, clade E, SF2) induced an increase in the intracellular calcium levels in one of three to six experiments. RANTES was used as a positive control. (**B**) The majority of microglial cultures did not have a calcium flux in response to treatment by gp120's (clade E, CM, 8×V3BaL). This negative result is representative of three to five experiments for each gp120.

might conclude that some microglia, which express detectable levels of CCR5 and CXCR4 on their surfaces, do not respond to their cognate α- and β-chemokines. However, there is an important caveat to this type of analysis. First, in both the calcium flux and QFC assays a subjective determination was made as to what constitutes a "positive cell," that is a cell that responds or expresses chemokine receptors above a subjective background level. Since the background level chosen might differ between both types of assays, one of these assays may be inherently more sensitive.

Ideally, the level of expression of each of these chemokine receptors should be determined in the microglia of individuals with and without HIV dementia. It is unlikely that such a study could be performed, since microglia can only be obtained through brain biopsies, and immunohistochemical analyses of fixed brain are not as quantitative. However, monocyte or MDM populations may serve as surrogates for this type of analyses, since preliminary quantification indicates that levels of functional receptor expression on MDM and microglia, while not identical, are similar (Albright *et al*, 1999; Lee *et al*, 1999). Indeed,

Table 2

Chemokine	*% of positive experiments*	*# of positives / Total # of experiments*	*Signaling score*
RANTES	82%	9/11	1.4
MIP-1β	63%	10/16	1.3
SDF-1α	60%	3/5	1.2
Fractalkine[1]	50%	1/2	1.0
vMIP-II	14%	1/7	0.1
Mig	0%	0/5	0.0
IP-10	0%	0/6	0.0

gp120 protein	*% of positive experiments*	*# of positives / Total # of experiments*	*Signaling score*
CM235	17%	1/6	0.2
Clade E	25%	1/4	0.3
SF2	33%	1/3	0.3
8×V3BaL	0%	0/5	0.0
JrFL	0%	0/5	0.0
8×V3IIIB	0%	0/4	0.0
IIIB	0%	0/1	0.0

[1]Fractalkine studies were limited as this has been studied in mouse microglia with full-length fractalkine.

other investigators have proposed that differences in monocyte populations might be potential predictors of the development of HIV dementia (Pulliam *et al*, 1997).

Because a number of factors could affect our results, which differ from those described for MDM, we took measures to insure there was not a trivial explanation for our negative results. First, we used seven different envelope strains from different gp120 preparations. Several of these proteins were tested in an ELISA that detected their ability to undergo a conformational change in response to CD4 binding, a reasonably rigorous test of structural integrity. Moreover, we were able to functionally express one HIV envelope on a murine leukemia virus pseudotype, where presumably it would be close to its native state.

A potential alternative explanation for the absence of a cellular response to gp120 was that microglia express a relatively low level of CD4. This could in turn affect gp120's ability to interact with chemokine receptors, since the chemokine receptor core-binding site is exposed only after a gp120-CD4 interaction. However, several experiments indicate that this explanation is unlikely. First, gp120s from 8×V3BaL and 8×V3IIIB did not trigger a calcium flux; these proteins can respectively bind the chemokine receptors CCR5 and CXCR4 absent an initial interaction with CD4. Second, pre-exposure of gp120s to soluble CD4 had no effect on their ability to trigger a cellular response.

Using purified gp120 proteins from several different X4 (CXCR4-using) and R5 (CCR5-using) HIV strains, we found no consistent evidence for gp120-induced calcium signaling in microglia. Therefore, we conclude that the triggering of an intracellular signal by binding of gp120 to the surface of microglial cells is at best a rare event, and certainly harder to detect than their response to chemokines themselves, which stimulate robust intracellular calcium mobilization in microglia. Overall, our studies provide little evidence that HIV virions or their shed gp120, were they present in the CNS in sufficient concentrations, could interact with microglial cells to mediate downstream effects.

However, our results do not rule out the possibility that gp120 transduces an intracellular signal that is below the detection threshold of this assay. Experiments analyzing signal transduction events downstream of calcium mobilization may reveal a response not noted here. One such pathway involves the phosphorylation of the cellular kinase py2k, which could be used as a noncalcium intracellular signaling marker for gp120-induced intracellular signaling (Davis *et al*, 1997).

Recent reports indicate that MDM and fetal microglia exposed to recombinant gp120 exhibited a transient increase in intracellular Ca^{2+} levels (Herbein *et al*, 1998; Liu *et al*, 2000). Liu and colleagues (2000) reported that both HIV-1 gp120 and chemokines activate calcium-activated potassium (K_{ca}), chloride, and calcium-permeant nonselective cation channels in primary MDM via cell surface CCR5 and CXCR4. The specificity of these interactions was proven using $CCR5^-$ macrophages and a CXCR4 inhibitor. The differences between our results are likely due to differences in the cell types assayed; and possibly to the participation of unknown molecules, perhaps even chemokine receptors, that are not shared among these cell types. In this vein, it appears that cultured adult and fetal microglia isolated from human brain have differential CCR3 expression levels. For example, He *et al.* (1997) reported that HIV infection of cultured fetal microglia could be blocked by anti-CCR3 antibodies; a result not seen in adult microglial cultures (Albright *et al*, 1999). A recent publication indicated that fetal microglia could be induced to signal by treatment with eotaxin, a CCR3-binding chemokine (Hegg *et al*, 2000), while we have reported the opposite (Albright *et al*, 1999). Therefore, it is reasonable to expect careful comparisons between microglia and MDM may also reveal similar differences in receptor expression.

Neurons have also been reported to undergo intracellular changes in response to HIV proteins. It will be interesting to determine how CD4-dependent gp120s can mediate a calcium flux in these $CD4^-$ cells. New data on the *in vivo* conformation states of gp120/41 and the synthesis of physiological relevant forms and concentrations of gp120 may provide insight into this mechanism.

In conclusion, we determined that microglial cultures consist of a heterogeneous population of cells with respect to their response to α- and β-chemokines. Overall, we found no substantial evidence that gp120 interacts with microglial chemokine receptors to generate downstream events transduced via intracellular calcium fluxes. Despite using seven different soluble gp120s from several sources, our results did not support the hypothesis that gp120 could interact with otherwise functional chemokine receptors on the surface of microglia and transmit a repeatable intracellular signal as judged by a flux in intracellular calcium levels. However, our results leave open the possibility that more sensitive assays could detect changes not seen with these microscopic techniques.

Acknowledgements

Supported by NIH grants MH-58958, NS-35743, and NS-27405. We thank Robert Doms, Trevor Hoffman, and Carrie McManus for the gift of the Jr-FL, 8×V3BaL, and 8×V3IIIB gp120 preparations, and for many helpful comments and suggestions, and Wei Cao and Linda Shawver for their excellent technical help. The other gp120 protein reagents were obtained from the AIDS Reagent Program.

References

Albright AV, Shieh JTC, Itoh T, Lee B, Pleasure D, O'Connor MJ, Doms RW, Gonzalez-Scarano F (1999). Microglia express CCR5, CXCR4, and CCR3, but of these, CCR5 is the principal coreceptor for HIV-1 dementia isolates. *J Virol* **73**: 205–213.

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA (1996). CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**: 1955–1958.

Arthos J, Rubbert A, Rabin RL, Cicala C, Machado E, Wildt K, Hanbach M, Steenbeke TD, Swofford R, Farber JM, Fauci AS (2000). CCR5 signal transduction in macrophages by human immunodeficiency virus and simian immunodeficiency virus envelopes. *J Virol* **74**: 6418–6424.

Bacon KB, Harrison JK (2000). Chemokines and their receptors in neurobiology: perspectives in physiology and homeostasis. *J Neuroimmunol* **104**: 2–7.

Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik ASTJ (1997). A new class of membrane-bound chemokine with a CX3C motif. *Nature* **385**: 640–644.

Bo L, Dawson TM, Wesselingh S, Mork S, Choi S, Kong PA, Hanley D, Trapp BD (1994). Induction of nitric oxide synthase in demylinating regions of multiple sclerosis brains. *Ann Neurol* **36**: 778–786.

Boddeke EW, Meigel I, Frentzel S, Biber K, Renn LQ, Gebicke-Harter P (1999). Functional expression of the fractalkine (CX3C) receptor and its regulation by lipopolysaccharide in rat microglia. *Eur J Pharmacol* **372**: 309–313.

Boshoff C, Endo Y, Collins PD, Takeuchi Y, Reeves JD, Schweickart VL, Siani MA, Sasaki T, Williams TJ, Gray PW, Moore PS, Chang Y, Weiss RA (1997). Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. *Science* **278**: 290–294.

Budka H (1990). Human immunodeficiency virus (HIV) envelope and core proteins in CNS tissues of patients with the aquired immune deficiency syndrome (AIDS). *Acta Neuropathol (Berl)* **79**: 611–619.

Campbell JJ, Butcher EC (2000). Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Curr Opin Immunol* **12**: 336–341.

Capobianchi MR, Ankel H, Ameglio F, Paganelli R, Pizzoli PM, Dianzani F (1992). Recombinant glycoprotein 120 of human immunodeficiency virus is a potent interferon inducer. *AIDS Res Hum Retroviruses* **8**: 575–579.

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu J, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J (1996). The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**: 1135–1148.

Collman R, Hassan NF, Walker R, Godfrey B, Cutilli J, Hastings J, Freidman H, Douglas SD, Nathanson N (1989). Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). *J Exp Med* **170**: 1149–1163.

Davis CB, Dikic I, Unutmaz D, Hill CM, Arthos J, Siani MA, Thompson DA, Schlessinger J, Littman DR (1997). Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J Exp Med* **186**: 1793–1798.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**: 661–666.

Dick AD, Pell M, Brew BJ, Foulcher E, Sedgwick JD (1997). Direct ex vivo flow cytometric analysis of human microglial cell CD4 expression: examination of central nervous system biopsy specimens from HIV-seropositive patients and patients with other neurological disease. *AIDS* **11**: 1699–1708.

Doranz BJ, Baik SS, Doms RW (1999). Use of a gp120 binding assay to dissect the requirements and kinetics of human immunodeficiency virus fusion events. *J Virol* **73**: 10346–10358.

Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, Doms RW (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**: 1149–1158.

Dreyer EB, Kaiser PK, Offermann JT, Lipton SA (1990). HIV-1 coat protein neurotoxicity prevented by calcium channel antagonists. *Science* **248**: 364–367.

Earl PL, Moss B, Doms RW (1991). Folding, interaction with GRP78–BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. *J Virol* **65**: 2047–2055.

Feng Y, Broder CC, Kennedy PE, Berger EA (1996). HIV-1 entry cofactor:functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**: 872–877.

Gabuzda DH, Ho DD, de la Monte SM, Hirsch MS, Rota TR, Sobel, RA (1986). Immunohistochemical identification of HTLV-III antigen in brains of patients with AIDS. *Ann Neurol* **20**: 289–295.

Gartner S, Markovits P, Markovitz DM, Betts RF, Popovic M (1986). Virus isolation from and identification of HTLV-III/LAV-producing cells in brain tissue from a patient with AIDS. *JAMA* **256**: 2365–2371.

Glabinski AR, Ransohoff RM (1999). Chemokines and chemokine receptors in CNS pathology. *J Neurovirol* **5**: 3–12.

Glass JD, Fedor H, Wesselingh SL, McArthur JC (1995). Immunocytochemical quantitation of human immunodeficiency virus in the brain: correlations with dementia. *Ann Neurol* **38**: 755–762.

Glass JD, Wesselingh SL, Selnes OA, McArthur JC (1993). Clinical-neuropathological correlation in HIV-associated dementia. *Neurol* **43**: 2230–2237.

Gu R, Westervelt P, Ratner L (1993). Role of HIV-1 envelope V3 loop cleavage in cell tropism. *AIDS Res Hum Retroviruses* **9**: 1007–1015.

Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB, Feng L (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc Natl Acad Sci USA* **95**: 10896–10901.

Hegg CC, Hu S, Peterson PK, Thayer SA (2000). Beta-chemokines and human immunodeficiency virus type-1 proteins evoke intracellular calcium increases in human microglia. *Neurosci* **98**: 191–199.

Herbein G, Mahlknecht U, Batliwalla F, Gregersen P, Pappas T, Butler J, O'Brien WA, Verdin E (1998). Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. *Nature* **395**: 189–194.

Hesselgesser J, Horuk R (1999). Chemokine and chemokine receptor expression in the central nervous system. *J Neurovirol* **5**: 13–26.

Hoffman TL, LaBranche CC, Zhang W, Canziani G, Robinson J, Chaiken I, Hoxie JA, Doms RW (1999). Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein. *Proc Natl Acad Sci USA* **96**: 6359–6364.

Itoh T, Itoh A, Horiuchi K, Pleasure D (1998). AMPA receptor-mediated excitotoxicity in human NT2-N neurons results from loss of intracellular Ca2+ homeostasis following marked elevation of intracellular Na+. *J Neurochem* **71**: 112–124.

Janssen RS, Nwanyanwu OC, Selik RM, Stehr-Green J (1992). Epidemiology of HIV encephalopathy in the United States. *Neurol* **42**: 1472–1476.

Kalaria RN (1993). The immunopathology of Alzheimer's disease and some related disorders. *Brain Pathol* 333–347.

Kaul M, Lipton SA (1999). Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. *Proc Natl Acad Sci USA* **96**: 8212–8216.

Kledal TN, Rosenkilde MM, Coulin F, Simmons G, Johnsen AH, Alouani S, Power CA, Luttichau HR, Gerstoft J, Clapham PR, Clark-Lewis I, Wells TNC, Schwartz TWA (1997). Broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science* **277**: 1656–1659.

Koenig S, Gendelman HE, Orenstein JM, Dal Canto MC, Pezeshkpour GH, Yungbluth M, Janotta F, Aksamit A, Martin MA, Fauci AS (1986). Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* **233**: 1089–1093.

Kolson DL, Lavi E, Gonzalez-Scarano F (1998). The effects of Human Immunodeficiency Virus in the central nervous system. *Adv Virus Res* **50**: 1–47.

Kwong PD, Wyatt R, Sattentau QJ, Sodroski J, Hendrickson WA (2000). Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. *J Virol* **74**: 1961–1972.

Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW (1999). Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proc Natl Acad Sci USA* **96**: 5215–5220.

Liu QH, Williams DA, McManus C, Baribaud F, Doms RW, Schols D, De Clercq E, Kotlikoff MI, Collman RG, Freedman BD (2000). HIV-1 gp120 and chemokines activate ion channels in primary macrophages through CCR5 and CXCR4 stimulation. *Proc Natl Acad Sci USA* **97**: 4832–4837.

Ma M, Kersten DB, Kamrud KI, Wool-Lewis RJ, Schmaljohn C, Gonzalez-Scarano F (1999). Murine leukemia virus pseudotypes of La Crosse and Hantaan Bunyaviruses: a system for analysis of cell tropism. *Virus Res* **64**: 23–32.

Maciejewski-Lenoir D, Chen S, Feng L, Maki R, Bacon KB (1999). Characterization of fractalkine in rat brain cells: migratory and activation signals for CX3CR-1-expressing microglia. *J Immunol* **163**: 1628–1635.

Madani N, Kozak SL, Kavanaugh MP, Kabat D (1998). gp120 envelope glycoproteins of human immunodeficiency viruses competitively antagonize signaling by coreceptors CXCR4 and CCR5. *Proc Natl Acad Sci USA* **95**: 8005–8010.

McArthur JC, Selnes OA, Glass JD, Hoover DR, Bacellar H (1994). HIV dementia: incidence and risk factors. In *HIV, AIDS and the brain*. Price RW, Perry SW (eds). New York: Raven Press, Ltd, pp 251–272.

Medana IM, Hunt NH, Chan-Ling T (1997). Early activation of microglia in the pathogenesis of fatal murine cerebral malaria. *Glia* **19**: 91–103.

Meucci O, Fatatis A, Simen AA, Bushell TJ, Gray PW, Miller RJ (1998). Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc Natl Acad Sci USA* **95**: 14500–14505.

Meucci O, Miller RJ (1996). gp120-induced neurotoxicity in hippocampal pyramidal neuron cultures: protective action of TGF-beta1. *J Neurosci* **16**: 4080–4088.

Moore JP, Thali M, Vignaux F, Poon S-W, Charles M, Sun B, Durda PJ, Akerblom L, Alkhatib G, Ho DD, Sattentau QJ, Sodroski J (1993). Immunochemical analysis of the gp120 surface glycoprotein of human immunodeficiency virus type 1: probing the structure of the C4 and V4 domains and the interaction of the C4 domain with V3 loop. *J Virol* **67**: 4785–4796.

Murdoch C, Finn A (2000). Chemokine receptors and their role in inflammation and infectious diseases. *Blood* **95**: 3032–3043.

Nath A, Haughey NJ, Jones M, Anderson C, Bell JE, Geiger JD (2000). Synergistic neurotoxicity by human immunodeficiency virus proteins tat and gp120: protection by memantine. *Ann Neurol* **47**: 186–194.

Nishiyori A, Minami M, Ohtani Y, Takami S, Yamamoto J, Kawaguchi N, Kume T, Akaike A, Satoh M (1998). Localization of fractalkine and CX3CR1 mRNAs in rat brain: does fractalkine play a role in signaling from neuron to microglia? *FEBS Lett* **429**: 167–172.

Pauli G, Hausmann E, Weiss R, Clapham P, Schneider J, Hunsmann, Gallo RC, Daniel MD, Koch MA, Gelderblom H (1987). LAV/HTLV-III: fine-structure analysis, localization of structural proteins, and detection of envelope antigens by patient sera. *Hamatol Bluttransfusion* **31**: 407–409.

Porwit A, Parravicini C, Petren AL, Barkhem T, Costanzi G, Josephs S, Biberfeld P (1989). Cell association of HIV in AIDS-related encephalopathy and dementia. *APMIS* **97**: 79–90.

Pulliam L, Gascon R, Stubblebine M, McGuire D, McGrath MS (1997). Unique monocyte subset in patients with AIDS dementia. *Lancet* **349**: 692–695.

Schneider J, Kaaden O, Copeland TD, Oroszlan S, Hunsmann G (1986). Shedding and interspecies type sero-reactivity of the envelope glycopolypeptide gp120 of the human immunodeficiency virus. *J Gen Virol* **67**: 2533–2538.

Sciorati C, Rovere P, Ferrarini M, Paolucci C, Heltai S, Vaiani R, Clementi E, Manfredi AA (1999). Generation of nitrix oxide by the inducible nitric oxide synthease protects gamma delta T cells from Mycobacterium tuberculosis-induced apoptosis. *J Immunol* **163**: 1570–1576.

Soontornniyomij V, Wang G, Pittman CA, Wiley CA, Achim CL (1998). Expression of brain-derived neurotrophic factor protein in activated microglia of human

immunodeficiency virus type 1 encephalitis. *Neuropathol Appl Neurobiol* **24**: 453–460.

Stoll G, Jander S (1999). The role of microglia and macrophages in the pathophysiology of the CNS. *Prog Neurobiol* **58**: 233–247.

Strizki JM, Albright AV, Sheng H, O'Connor M, Perrin L, Gonzalez-Scarano F (1996). Infection of primary human microglia and monocyte-derived macrophages with human immunodeficiency virus type 1 isolates: evidence of differential tropism. *J Virol* **70**: 7654–7662.

Thali M, Furman C, Helseth E, Repke H, Sodroski J (1992). Lack of correlation between soluble CD4-induced shedding of the human immunodeficiency virus type 1 exterior envelope glycoprotein and subsequent membrane fusion events. *J Virol* **66**: 5516–5524.

Thali M, Moore JP, Furman C, Charles M, Ho DD, Robinson J, Sodroski J (1993). Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J Virol* **67**: 3978–3988.

Tyor WR, Glass JD, Baumrind N, McArthur JC, Driffin JW, Becker PS, Griffin DE (1993). Cytokine expression of macrophages in HIV-1-associated vacuolar myelopathy. *Neurol* **43**: 1002–1009.

Vodicka MA, Goh WC, Wu LI, Rogel ME, Bartz SR, Schweickart VL, Raport CJ, Emerman M (1997). Indicator cell lines for detection of primary strains of human and simian immunodeficiency viruses. Virology **233**: 193–198.

Weis S, Neuhaus B, Mehraein P (1994). Activation of microglia in HIV-1 infected brains is not dependent on the presence of HIV-1 antigens. *Neuroreport* **5**: 1516–6.

Weiss CD, Levy JA, White JM (1990). Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. *J Virol* **64**: 5674–5677.

Weissman D, Rabin RL, Arthos J, Rubbert A, Dybul M, Swofford R, Venkatesan S, Farber JM, Fauci AS (1997). Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* **389**: 981–985.

Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MBA (1986). Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proc Natl Acad Sci USA* **83**: 7089–7093.

Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, Borsetti A, Cardoso AA, Desjardin E, Newman W, Gerard C, Sodroski J (1996). CD4-induced interaction of primary HIV-1gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384**: 179–187.

Wyatt R, Moore J, Accola M, Desjardin E, Robinson J, Sodroski J (1995). Involvement of the V1/V2 variable loop structure in the exposure of Human Immunodeficiency Virus type 1 gp120 epitopes induced by receptor binding. *J Virol* **69**: 5723–5733.

Zhang GX, Baker CM, Kolson DL, Rostami AM (2000). Chemokines and chemokine receptors in the pathogenesis of multiple sclerosis. *Mult Scler* **6**: 3–13.