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Chemokine receptor utilization and macrophage signaling by human immunodeficiency virus type 1 gp120: Implications for neuropathogenesis

Yanjie Yi,[1] ChuHee Lee,[1] Qing-Hua Liu,[2] Bruce D Freedman,[2] and Ronald G Collman[1]

[1]*Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA;*
[2]*Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, Pennsylvania, USA*

Human immunodeficiency virus type 1 (HIV-1) uses the chemokine receptors CCR5 and CXCR4 for entry. Macrophages and microglia (M/M) are the principal productively infected brain cells in HIV encephalopathy (HIVE), and neuronal injury is believed to result both from direct effects of viral proteins and indirect effects mediated by macrophage activation and secretion of neurotoxic products. *In vitro*, direct injury by the viral envelope glycoprotein gp120 can be mediated by neuronal CXCR4, but most HIV-1 isolates from the central nervous system (CNS) studied to date use CCR5 (R5 strains) rather than CXCR4 (X4 or R5X4 strains). Additionally, it remains unknown how HIV induces M/M activation and neurotoxin secretion. To address these issues, the authors analyzed a CNS-derived primary isolate, TYBE, and showed that it uses CXCR4 only and replicates efficiently in macrophages through CXCR4-mediated entry. The authors also showed that both R5 and X4 gp120 activate intracellular signals in macrophages through CCR5 and CXCR4, including calcium elevations; K^+, Cl^- and nonselective cation channel activation; phosphorylation of the nonreceptor tyrosine kinase Pyk2; and activation of p38 and SAPK/JNK mitogen-activated protein kinases (MAPKs). Finally, the authors showed that macrophages stimulated with gp120 produce soluble factors through MAPK-dependent pathways, including β-chemokines implicated in HIVE pathogenesis. The findings emphasize that both X4 and R5 HIV-1 isolates may contribute to HIVE pathogenesis, and that gp120/chemokine receptor interactions in M/M trigger specific signal transduction pathways that may affect M/M function and provide a mechanism underlying CNS injury. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 91–96.

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Introduction

Human immunodeficiency virus (HIV) encephalopathy (HIVE) generally occurs late in HIV-1 infection and is associated with direct viral infection of the central nervous system (CNS). Macrophages and microglia (M/M) are the principal target cells for productive infection in the CNS. Both infection and activation of M/M appear to be critical for the development of dementia (reviewed in Kaul *et al*, 2001), but how HIV elicits cellular M/M activation in the CNS is not known.

HIV-1 enters target cells through sequential interactions of its envelope glycoprotein gp120 with CD4 and one of two seven-transmembrane G protein–coupled chemokine receptors, either CCR5 or CXCR4. Although HIV-1 has subverted the

Address correspondence to Ronald G. Collman, MD, University of Pennsylvania School of Medicine, 522 Johnson Pavilion, 36th and Hamilton Walk, Philadelphia, PA 19104, USA. E-mail: collmanr@mail.med.upenn.edu

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chemokine receptors for its own use, their normal function is to elicit downstream signaling and activation in response to extracellular ligands. Prototype macrophage (M)-tropic HIV-1 that replicate *in vitro* in M/M and lymphocytes but not cell lines use CCR5 as a coreceptor (R5 strains) and are non–syncytia-inducing (NSI) in culture, while T-cell line (T)-tropic viruses replicate in T-cell lines and lymphocytes but not M/M, are syncytia-inducing (SI), and use CXCR4 (X4 strains). Dual-tropic prototypes use both CCR5 and CXCR4 (R5X4 strains), replicate in all three cell types, and induce syncytia *in vitro* (reviewed in Berger *et al*, 1999).

Prototype HIV-1 isolates from the CNS are typically R5 NSI and M-tropic, consistent with M/M as the main productively infected cells. Recently, however, we and others found that M/M express CXCR4 as well as CCR5, and that macrophage CXCR4 supports infection by some primary X4 HIV-1 isolates (Yi *et al*, 1998; Simmons *et al*, 1998; Albright *et al*, 1999) Furthermore, several groups have shown that CXCR4 is expressed on a number of cell types in the CNS (Vallat *et al*, 1998; Lavi *et al*, 1997), and can mediate toxic effects of gp120 (Zheng *et al*, 1999; Hesselgesser *et al*, 1998). Our studies described here have focused on two aspects of how gp120–chemokine receptor interactions may participate in neurological damage: (a) might HIV-1 lead to M/M activation directly by triggering intracellular signals through gp120 interactions with these chemokine receptors, and (b) does the CNS harbor X4 variants, in addition to R5 variants, which might interact directly with neuronal targets and induce injury?

CXCR4-using HIV-1 isolates in the CNS

CXCR4 is widely expressed in M/M, neurons, and other cells in the CNS (Albright *et al*, 1999; Lavi *et al*, 1997; Vallat *et al*, 1998). Importantly, both X4 gp120 and intact virions induce neuronal injury in a variety of *in vitro* cell models (Hesselgesser *et al*, 1998; Ohagen *et al*, 1999; Zheng *et al*, 1999). These observations emphasize the potential importance of X4 strains for infection and neuronal injury in CNS. However, the CNS-derived HIV-1 isolates studied to date all display an R5 NSI phenotype, and so it is unclear whether X4 strains may be present in the CNS. Thus, mechanisms by which X4 gp120 elicits neuronal injury through CXCR4 are relevant only if X4 HIV-1 may be present within the CNS.

To address coreceptor diversity among CNS variants, we analyzed a panel of HIV-1 primary isolates derived from cerebrospinal fluid (CSF) of infected individuals with neurological symptoms and focused on one, designated as TYBE, that differed from prototype CNS isolates in that it replicated in cell lines and was SI in MT2 cells (Yi *et al*, 2003). This isolate was subjected to biological cloning by limiting dilution to ensure that a single viral species rather than a mixture of viruses was being analyzed. Like the prototype CNS-derived R5 NSI M-tropic strain JRFL, strain TYBE replicated efficiently in primary monocyte-derived macrophages (MDMs) (Figure 1A). The R5X4 prototype 89.6 also replicated in macrophages, whereas the X4 prototype 3B did not. We then tested the specific CXCR4 antagonist AMD3100 and found that blocking CXCR4 completely prevented TYBE infection of macrophages, suggesting that entry was mediated by CXCR4. In contrast, neither JRFL nor 89.6 were inhibited by blocking CXCR4 (Figure 1A). We also examined macrophages lacking CCR5 (from donors homozygous for the ccr5Δ32 deletion allele) and found that, unlike JRFL, TYBE replicated efficiently despite the absence of CCR5, and infection was also blocked by CXCR4 inhibition (data not shown). Thus, macrophage infection by TYBE is mediated exclusively by CXCR4.

To confirm the coreceptor utilization pattern of TYBE, we generated a full-length *env* clone by polymerase chain reaction (PCR) and analyzed its ability to mediate cell-cell fusion with cells expressing CD4 in conjunction with either CCR5 or CXCR4. As shown in Figure 1B, TYBE exclusively used CXCR4 for fusion and did not fuse with cells expressing CD4 and CCR5. *env* sequence analysis revealed a pattern typical of T-tropic X4 or dual-tropic R5X4 variants, with high positive charge of +7 in the V3 region (data not shown). Thus, TYBE represents a novel type of HIV-1 from the CNS that is exclusively CXCR4-dependent yet highly macrophage-tropic through the use of macrophage CXCR4 for entry.

gp120 activation of ionic signals through CCR5 and CXCR4 in macrophages

Although infection of cells in the CNS is necessary for the development of HIVE, M/M activation appears to be a critical element of pathogenesis (Glass *et al*, 1995). How HIV-1 leads to M/M activation is not known, however. The chemokine receptors used by HIV-1 for entry into M/M normally transduce activation signals elicited by chemokines, and several groups have demonstrated signaling responses in lymphocytes to HIV-1 gp120 (Weissman *et al*, 1997; Davis *et al*, 1997). Therefore, we asked whether HIV-1 gp120 might act as a ligand for macrophage CCR5 and CXCR4 and, if so, what pathways and functional responses would be elicited that might contribute to the pathogenesis of HIVE.

Both ion currents and elevation of intracellular calcium regulate activation of many cell types, and previous data showed that R5 gp120 elevated intracellular Ca^{2+} in T cells (Weissman *et al*,1997). We therefore examined $[Ca^{2+}]_i$ and ion currents in MDMs exposed to gp120. Both R5 (JRFL) and X4 (3B) gp120 elevated $[Ca^{2+}]_i$ (data not shown). Of note, JRFL-induced Ca^{2+} elevations typically reached ~2-fold higher peak and decayed to somewhat higher steady-state level than those induced by 3B. Ionic

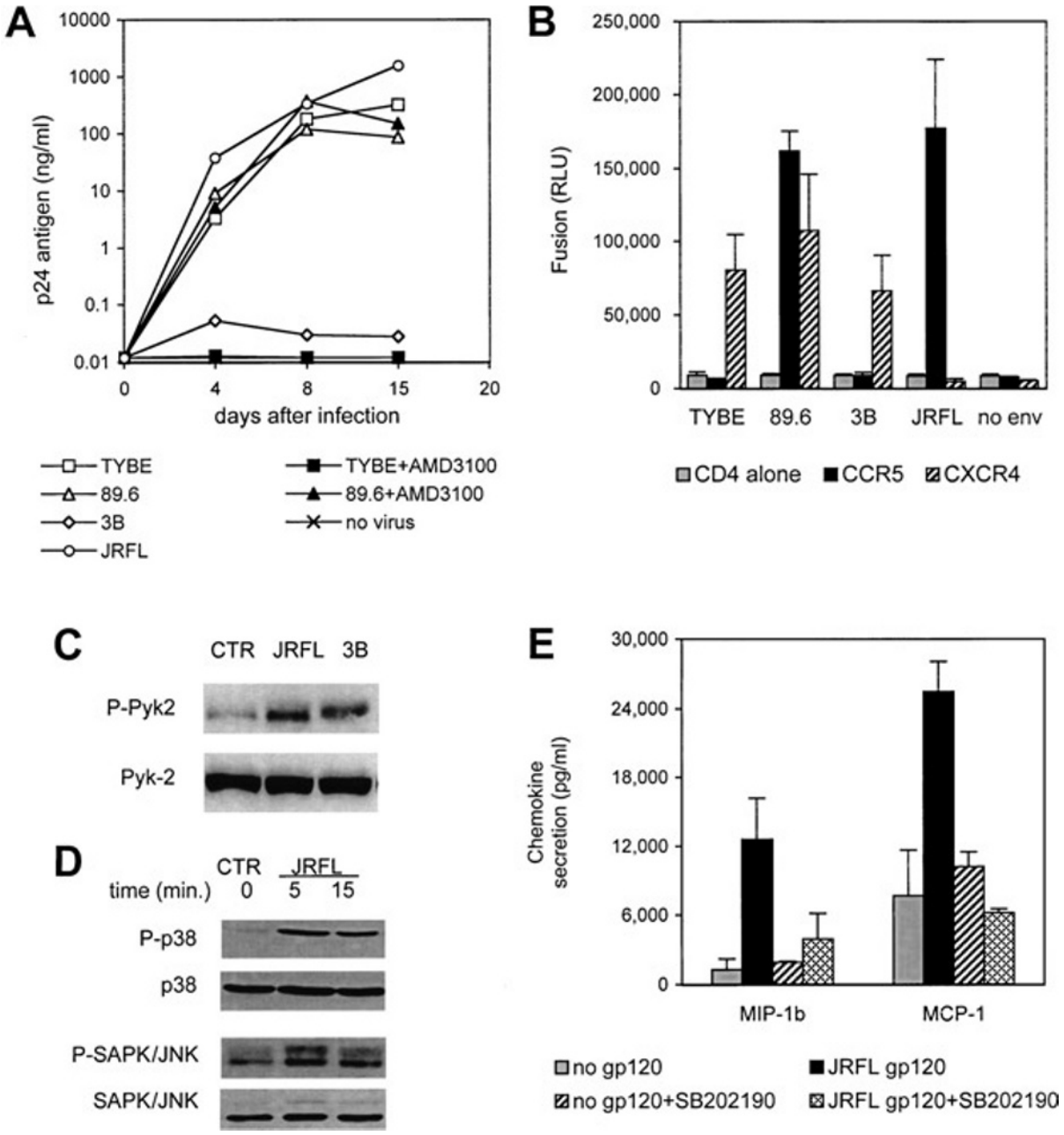

Figure 1 Identification of an X4 HIV-1 primary isolate from the CNS and macrophage signaling and secretory responses to gp120. (**A**) Infection of macrophages by isolate TYBE is CXCR4 dependent. Monocyte-derived macrophages (MDMs) were infected overnight with TYBE, the CNS-derived R5 prototype JRFL, the R5X4 strain 89.6, and the X4 strain 3B. Infections were carried out in the present or absence of the CXCR4 antagonist AMD3100 (1 μg/ml) and supernatant was sampled for p24 antigen by ELISA. TYBE infection of MDMs was blocked by AMD3100, indicating that CXCR4 mediates entry. (**B**) TYBE Env-mediated cell-cell fusion is CXCR4 restricted. The TYBE *env* gene under control of the T7 promoter was transfected into 293T cells, which were also infected with a recombinant vaccinia virus expressing the T7 RNA polymerase. Cells were mixed with QT6 cells that had been cotransfected with plasmids encoding CD4 alone, or CD4 plus, CCR5 or CXCR4, and or CXCR4, and a T7-driven luciferase reporter gene. Luciferase expression was measured 6 h later as an indication of cell-cell fusion, which allows cytoplasmic mixing and T7-driven reporter gene transactivation. TYBE uses CXCR4 but not CCR5 for fusion. (**C**) R5 and X4 gp120 activate Pyk2 in macrophages. MDMs were treated with gp120 (2.5 μg/ml) for 5 min. Whole-cell lysates were then analyzed by Western blot with an antibody specific for the phosphorylated form of Pyk2 or an antibody specific for total Pyk2. Both JRFL and 3B gp120 induce Pyk2 phosphorylation. (**D**) gp120 induces MAPK activation in macrophages. MDM were stimulated with JRFL gp120 (2.5 μg/ml) and cell lysates were analyzed by Western blot with antibodies specific for the phosphorylated or total forms of p38 MAPK and JNK/SAPK. Both p38 MAPK and JNK/SAPK are phosphorylated in response to gp120. (**E**) gp120 induces chemokine secretion through a MAPK-dependent pathway. MDMs were incubated for 1 h with or without the specific p38 MAPK blocker SB-202190 (1 μg/ml) and then exposed to JRFL gp120 (1 μg/ml). Supernatants were harvested 24 h later and MIP-1β and MCP-1 levels were determined by ELISA. MIP-1β and MCP-1 secretion occurs in response to gp120 and is inhibited by blocking MAPK activation.

currents were then examined by patch-clamp analysis of voltage-clamped MDMs (data not shown). We found that gp120 activated a brief outward current followed by a slowly developing, longer duration inward current. Although JRFL usually evoked both outward and inward currents, 3B typically evoked only the inward current. Based on sensitivity to the pharmacological inhibitor charybdotoxin and a current reversal potential (Er) of −70 mV, we identified the outward current as a K^+ current. The slower inward current was identified as a Cl^- current based on sensitivity to Cl^- channel antagonists such as NPPB [5-nitro-2-(3-phenyl propylamino) benzoic acid], and an Er of 0 mV that was shifted in low-chloride bath solution. Both macrophage inhibitory protein (MIP)-1β and stromal cell-derived factor (SDF)-1α, natural ligands of CCR5 and CXCR4, respectively, elicited currents that were similar to their corresponding R5 and X4 gp120 molecules. AMD3100 completely blocked 3B-induced activation but not that elicited by JRFL, whereas JRFL failed to elicit currents in CCR5-negative MDMs but 3B did (data not shown), confirming that these currents were specifically activated through the chemokine receptors. These results also indicate that CD4 is not responsible for gp120-induced signals, because neither AMD3100 blocking of CXCR4 nor the absence of CCR5 would interfere with gp120 binding to CD4.

Nonselective cation (NSC) currents may also reverse at 0 mV (Liu *et al*, 2000), and so we tested whether a NSC current might also be activated within the inward ionic current seen. Conditions were established that would block any K^+ or Cl^- currents by inclusion of pharmacological inhibitors and replacement of K^+ and Cl^- by impermeant ions in bath solutions. Under these conditions, JRFL and 3B gp120 still evoked an inward current, whereas MIP-1β and SDF-1α did not. We confirmed that this was a NSC current by a shift when Na^+ was partially replaced by an impermeant cation. Surprisingly, neither MIP-1β and SDF-1α elicited inward current under identical conditions, indicting that the NSC current was only induced by gp120 and not by chemokine ligands (data not shown). This unexpected result suggests that signals triggered by gp120 are similar to but not absolutely identical to activated by the receptors' natural chemokine ligands. We are currently exploring the role of CD4 co-activation in modifying the chemokine receptor response to gp120 stimulation.

Protein phosphorylation responses to R5 and X4 gp120 in macrophages

Pyk2 is a nonreceptor tyrosine kinase that, in many cell types, links chemokine receptor activation and downstream signaling pathways, may be triggered by $[Ca^{2+}]_i$ increases, and may even modulate ion channel function (Lev *et al*, 1995). As shown in Figure 1C, we found that MDMs express abundant Pyk2 protein, and that both R5 and X4 HIV-1 gp120 as well as chemokines induced rapid Pyk2 phosphorylation (Del Corno *et al*, 2001). Virion-associated gp120 also activated Pyk2, with ~100-fold greater potency than soluble gp120 based on molar content (data not shown). Like ion channel activation, Pyk2 phosphorylation was mediated specifically through CCR5 and CXCR4, and was not triggered by CD4 binding alone (data not shown).

Because Pyk2 activation is regulated by $[Ca^{2+}]_i$ in some cells, we addressed the linkage between gp120-induced $[Ca^{2+}]_i$ elevation and Pyk2 phosphorylation in macrophages. JRFL gp120-induced Pyk2 activation was blocked both by the Ca^{2+} chelator EGTA, and by lanthanum, an inhibitor of calcium release–activated Ca^{2+} (CRAC) channels (data not shown). This result indicates that gp120-induced Pyk2 activation is linked to a CRAC channel–mediated Ca^{2+} influx triggered through the chemokine receptors. In contrast, Pyk2 activation was insensitive to pertussis toxin, indicating that chemokine receptor signaling in primary macrophages is not mediated by Gαi.

We next focused on the mitogen-activated protein kinase (MAPK) signaling molecules, an important family of protein kinases that are activated by extracellular stimuli through dual phosphorylation. In lymphocytes, R5 gp120 were previously shown to induce MAPK activation (Popik and Pitha, 1998). We found that JRFL gp120 induced rapid phosphorylation of p38 and JNK/SAPK (Figure 1D). In contrast, p44/42 MAPK had a high basal level of phosphorylation and no further activation was seen (data not shown). However, MAPK activation by 3B gp120 was inconsistent, suggesting that X4 gp120 had a lower propensity for MAPK activation (data not shown). Pyk2 is an upstream regulator of MAPK in some cell types (Lev *et al*, 1995), so we are currently trying to determine the relationship between Pyk2 and MAPK in macrophages.

Mechanisms underlying macrophage secretion of soluble mediators in response to gp120

Many reports over the past decade have shown that macrophage exposure to gp120 or virions results in secretion of a variety of mediators, including tumor necrosis factor (TNF)-α, β-chemokines, interleukin (IL)-1, IL-10 and other products (Merrill *et al*, 1989; Herbein *et al*, 1994; Fantuzzi *et al*, 2001, and others), but the mechanisms responsible are unknown. We hypothesized that the pathways triggered by gp120/chemokine receptor interactions identified here might be involved. To test this notion, we focused on the β-chemokines MIP-1β and monocyte chemoattractant protein (MCP)-1 because both are powerful chemoattractants and macrophage activators. In addition, MCP-1 has been implicated in the pathogenesis of HIVE (Kelder *et al*, 1998; Cinque *et al*, 1998). JRFL gp120 induced macrophage secretion of both MIP-1β (~10-fold) and MCP-1 (~4-fold), and inhibiting p38 MAPK activation with the

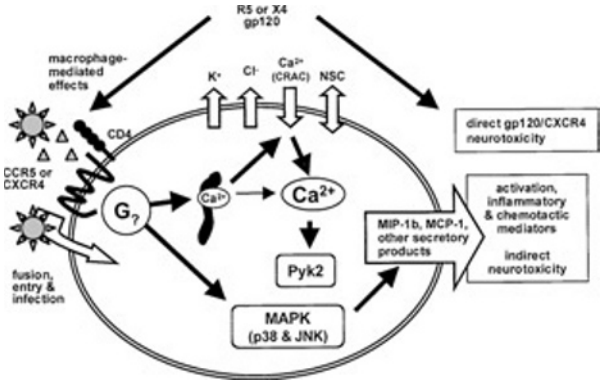

Figure 2 Overview of macrophage signaling responses identified and a model for how these gp120-chemokine receptor interactions may contribute to HIVE pathogenesis. Brain M/M may support both R5 and X4 infection and direct neuronal toxicity may result from gp120 interactions with CXCR4 on neurons. Indirect injury may result from g120-induced activation of both infected and uninfected M/M, with release of inflammatory, chemotactic, and neurotoxic products. Proposed relationships between intracellular Ca^{2+}, ionic currents, and protein kinase activation pathways defined in this work are indicated.

specific blocker SB-202190 inhibited secretion of both (Figure 1E). Consistent with the other signaling responses described above, gp120-induced MIP-1β and MCP-1 production was insensitive to pertussis toxin (data not shown) and thus independent of $G\alpha_i$. Therefore, gp120 stimulates macrophage β-chemokine secretion through MAPK-mediated pathways, and studies are currently under way examining other macrophage secretory and functional responses to gp120.

Significance of X4 CNS isolates and gp120/chemokine receptor–mediated macrophage activation for HIVE pathogenesis

Macrophages may serve a tripartite role in HIVE pathogenesis. One critical function is as a reservoir for HIV-1 production in the CNS, with the consequence that viral gene products may directly injure neurons. Tat, Nef, Env, and other viral proteins have been implicated in neuronal injury, but Env has received the greatest attention and direct effects of Env on neurons are well-described. However, most studies of Env-mediated neurotoxicity have utilized X4 virus variants and implicate gp120-CXCR4 interactions and, in fact, X4 Env appear to be considerably more neurotoxic than R5 variants (Hesselgesser *et al*, 1998; Ohagen *et al*, 1999; Zheng *et al*, 1999). Our studies address the issue of CXCR4-dependent strains in the CNS by demonstrating that such variants do exist and can establish robust infection in primary M/M cells, and provide a prototype for such a CNS-derived X4 variant (Figure 2). Similar observation have recently been reported by others (Gorry *et al*, 2001), but how widely such variants exist and whether CXCR4-dependent mechanisms of neurotoxicity identified *in vitro* actually operate *in vivo*, remain to be determined.

Another role served by macrophages in AIDS neuropathogenesis is as an amplifier of CNS inflammation. Although the presence of virus within the brain is necessary for neurological injury, there is an even closer correlation between neurological dysfunction and the degree of macrophage activation and infiltration (Glass *et al*, 1995). Our studies address this issue by showing that gp120 activation through the pathways defined here can lead to both cellular activation and secretion of inflammatory products, including chemoattractants that have been implicated in pathogenesis *in vivo*, such as MCP-1. Macrophages may be exposed to gp120 on infectious or defective virions, or as free glycoprotein (Jones *et al*, 2000), leading to secretion of these inflammatory and chemotactic molecules with recruitment of additional cells and further M/M activation (Figure 2). Such a cascade could serve to extend the effect beyond the relatively restricted number of directly infected cells.

Finally, it is believed that indirect mechanisms of neuronal damage play a central role in HIVE pathogenesis, and that brain M/M are the principal source of the cellular products that injure neurons. Many substances have been implicated (reviewed in Kaul *et al*, 2001), including metabolic amines such as quinolinic acid; cytokines such as TNF-α; nitric oxide; chemokines; and small molecules such as arachadonic acid metabolites and platelet-activating factor. Although the soluble products of HIV-macrophage interactions that injure neurons has been the subject of extensive study, how HIV may activate macrophages to produce such factors has not been elucidated. Our results suggest a mechanism by which virus may trigger macrophages to secrete such neurotoxic products. Indeed, in preliminary studies using an *in vitro* neuronal cell line model (Chen *et al*, 2002), we have found that supernatant of macrophages exposed to gp120 injures neurons, and that blockade of macrophage MAPK pathways prevents the neurotoxic effect (data not shown). Thus, these results (Figure 2) may both enhance our understanding of the mechanisms underlying HIV-macrophage-neuronal interactions, and provide a basis for the ultimate development of therapeutic approaches to interfere with this cascade.

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