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Gp120-mediated cytotoxicity of human brain microvascular endothelial cells is dependent on p38 mitogen-activated protein kinase activation

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Breakdown of the blood-brain barrier has been shown to contribute to neurological disorders that are prevalent in human immunodeficiency virus type 1 (HIV-1)–infected individuals, but the mechanisms involved in HIV-1–associated blood-brain barrier dysfunction remain incompletely understood. Using human brain microvascular endothelial cells (HBMECs) that constitute the blood-brain barrier, the authors determined the cytotoxic effects of gp120 on HBMECs. The authors showed that gp120 induced cytotoxicity of HBMECs derived from children, which required cotreatment with interferon (IFN)-γ. IFN-γ treatment exhibited up-regulation of the chemokine receptors CCR3 and CCR5 in children's HBMECs. In contrast, HBMECs isolated from adults were not responsive to gp120-mediated cytotoxicity. Peptides of gp120 representing binding regions for CD4 and chemokine receptors as well as CD4 antibody inhibited gp120-mediated cytotoxicity of HBMECs. RANTES, as expected, inhibited M-tropic gp120-mediated HBMEC cytotoxicity, whereas stromal cell–derived factor (SDF)-1α failed to inhibit T-tropic gp120-mediated cytotoxicity. Of interest, gp120 peptides representing non-CD4/non–chemokine receptor binding regions inhibited gp120-mediated HBMEC cytotoxicity. In addition, the authors showed that gp120-mediated HBMEC cytotoxicity involved p38 mitogen-activated protein kinase pathway. Taken together, these findings showed that gp120, in the presence of IFN-γ, can cause dysfunction of the blood-brain barrier endothelium via MAPK pathways involving several gp120-HBMEC interactions. *Journal of NeuroVirology* (2007) **13,** 242–251.

Keywords: blood-brain barrier; cytotoxicity; Gp120; human brain microvascular endothelial cells; MAPK

Introduction

Several investigators have shown that structural and functional perturbations of the blood-brain barrier occur commonly during human immunodeficiency virus type 1 (HIV-1) infection (Dallasta *et al,* 1999; Toborek *et al,* 2005). Breakdown of the blood-brain barrier has been shown to contribute to neurological disorders that are prevalent in HIV-1–infected individuals, but the mechanisms involved in HIV-1–associated blood-brain barrier dysfunction remain incompletely understood.

HIV-1–associated blood-brain barrier dysfunction is likely to involve many viral and host factors. For example, HIV-1 proteins, such as HIV-1 envelope glycoprotein or gp120, along with inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interferon (IFN)-γ, released by HIV-1–infected cells have been shown to contribute to HIV-1–associated blood-brain barrier dysfunction (Annunziata *et al,* 2002; Kanmogne *et al,* 2005).

The viral and host factors that contribute to the pathogenesis of HIV-1 encephalopathy may include

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(i) direct viral infection of the brain tissue, (ii) neurotoxic effects of viral proteins, and/or (iii) virally induced cytokines (Epstein *et al,* 1984; Meyenhofer *et al,* 1987; Mirra and Del Rio, 1989; Sharer *et al,* 1986; Weiser *et al,* 1990). The brain tissue is protected by the presence of a highly selective and tight blood-brain barrier, i.e., cerebral capillary endothelium. In addition to its function as a selective permeable transport barrier, the blood-brain barrier plays an important role in the modulation of immune and inflammatory responses by restricting pathogen/toxin entry into the central nervous system (CNS). Several lines of evidence suggest that HIV-1 enters into the CNS by directly crossing the blood-brain barrier and/or crossing via infected immune cells using Trojan horse mechanisms (Hurwitz *et al,* 1994; Moses *et al,* 1993; Moses and Nelson, 1994). In the former, HIV-1 interactions with the brain microvascular endothelial cells, which constitute the blood-brain barrier, are mediated by viral coat glycoprotein gp120, resulting in viral uptake and traversal of the blood-brain barrier (Banks *et al,* 1997). An alternative mechanism may involve alterations in the function and/or integrity of the blood-brain barrier, which may facilitate HIV-1 encephalopathy by allowing virus or viral/cellular proteins access to the brain tissue (Stins *et al,* 2003, 2004). Using human brain microvascular endothelial cells (HBMECs) that constitute the blood-brain barrier, here we determined the cytotoxic effects of gp120 in the presence or absence of a proinflammatory cytokine IFN-γ on HBMECs isolated from children and adults and studied the associated mechanisms.

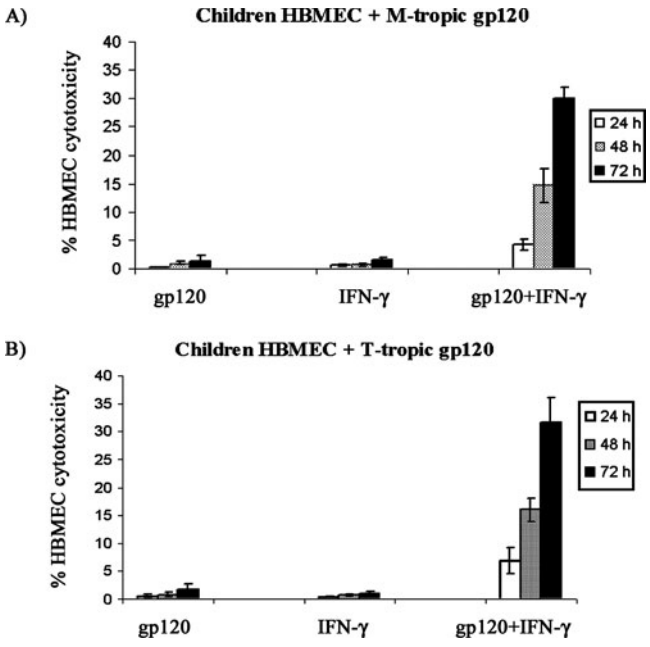

Figure 1 HIV-1 gp120-induced cytotoxicity of brain microvascular endothelial cells (HBMECs) derived from children. Confluent cultures of primary HBMECs derived from children were incubated with IFN-γ (2 ng/ml) and Gp120 (0.5 μg/ml) derived from M-tropic strains of HIV-1 (**A**) and T-tropic strains of HIV-1 (**B**). Cultures were incubated at 37°C in a 5% CO_2 incubator for 24, 48, and 72 h. At the end of the incubation period, supernatants were collected and analyzed for LDH release and converted into percentage cytotoxicity as described in Materials and Methods. Note that both M- and T-tropic gp120-induced cytotoxicity of HBMECs, but only in the presence of IFN-γ (**A**). Results represent means of three independent experiments. Error bars represent standard deviation.

Results

HIV-1 gp120 in the presence of IFN-γ induces cytotoxicity of HBMECs derived from children but not from adults

To determine the effects of gp120 on HBMEC cytotoxicity *in vitro*, assays were performed with HBMECs derived from both children and adults. We determined that gp120 (0.5 μg/ml) from both T-tropic and M-tropic strains of HIV-1 were able to produce cytotoxicity of HBMECs derived from children in a time-dependent manner, but the HBMEC cytotoxicity required IFN-γ. As shown in Figure 1A and B, more than 30% cytotoxicity was demonstrated as compared to HBMECs treated with gp120 or IFN-γ alone. These gp120-mediated effects were demonstrated with gp120 derived from T-tropic and M-tropic HIV-1 strains (Figure 1A and B). In contrast, no significant effects of cytotoxicity were demonstrated when HBMECs derived from adults were treated with gp120 of T-tropic and M-tropic HIV-1 and IFN-γ, alone and in combination (Figure 2A and B). Given that HBMECs derived from adults were not responsive to gp120/IFN-γ–mediated cytotoxicity, all subsequent experiments were performed using HBMECs derived from children.

IFN-γ treatment leads to up-regulation of CCR3 and CCR5 but not CCR2 and CXCR4 expression in HBMECs

As noted above, gp120-mediated HBMEC cytotoxicity was demonstrated only in combination with IFN-γ. Gp120 has been shown to interact with CD4 and chemokine receptors. To determine whether the expression of chemokine receptors is affected by IFN-γ treatment, reverse transcriptase–polymerase chain reaction (RT-PCR) analyses were performed using primers specific for chemokine receptors (Table 1). As shown in Figure 3, HBMECs were found to exhibit transcriptional expression of CCR1, CCR2, CCR5 and CXCR4, but failed to exhibit CCR3 at the baseline. However, IFN-γ treatment increased expression of CCR3 and CCR5 mRNA levels, whereas mRNA levels of CCR1 and CXCR4 were unchanged (Fig. 3). Of interest, the expression of CCR2 was down-regulated by IFN-γ treatment. Amplification of a 294-bp β-actin mRNA fragment gave comparable bands from both IFN-γ–treated and untreated samples, confirming that the same amounts of cDNA were used (Fig. 3).

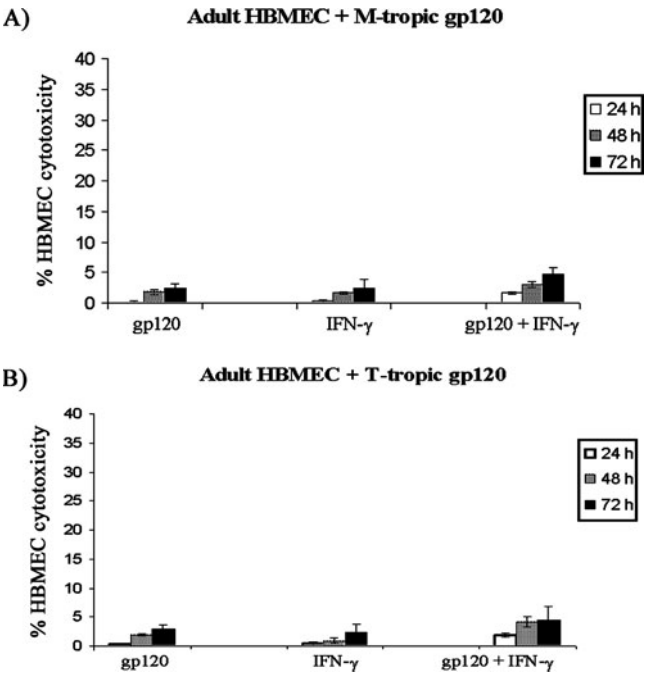

Figure 2 HIV-1 gp120 did not induce cytotoxicity of HBMECs derived from adults. Confluent cultures of HBMECs derived from adults were incubated with IFN-γ (2 ng/ml) and Gp120 (0.5 μg/ml) derived from M-tropic strains of HIV-1 (**A**) and T-tropic strains of HIV-1 (**B**). Cultures were incubated at 37°C in a 5% CO_2 incubator for 24, 48, and 72 h as described in Figure 1. Neither M- nor T-tropic gp120 had any significant cytotoxic effect on HBMECs. Results represent means of three independent experiments. Error bars represent standard deviation.

Overall, these findings indicate that the expressions of CCR3 and CCR5 were increased by IFN-γ treatment, suggesting that IFN-γ–mediated up-regulation of CCR3 and CCR5 may play a role in gp120-induced cytotoxicity.

HIV-1 gp120–mediated cytotoxicity of HBMECs requires tyrosine kinase signaling pathways

To determine the host cell signaling pathways that are involved in gp120- and IFN-γ–induced cytotoxicity, HBMEC monolayers were pretreated with genistein (25 μM) (a protein tyrosine kinase inhibitor) before stimulation with gp120 and IFN-γ. HIV-1 gp120–induced cytotoxicity was inhibited by more than 50% with genistein, suggesting the involvement of tyrosine kinase signaling pathways in gp120- and IFN-γ-dependent cytotoxicity (Figure 4). Genistein treatment inhibited cytotoxicity of gp120 from both T-tropic and M-tropic strains of HIV-1. These data led us to investigate signaling molecules involved in gp120- and IFN-γ–induced cytotoxicity.

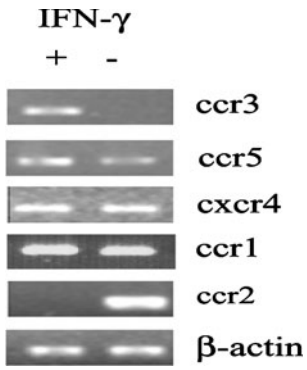

Figure 3 IFN-γ treatment of HBMECs up-regulates CCR3 and CCR5 expression. Confluent cultures of HBMECs were treated with IFN-γ (2 ng/ml) for up to 24 h. The mRNA were isolated and RT-PCR analyses were performed using CCR1, CCR2, CCR3, CCR5, CXCR4, and β-actin primers as described in Materials and Methods. Note that HBMECs treated with IFN-γ exhibited higher levels of CCR3 and CCR5, whereas β-actin was equally amplified from all samples. In contrast, IFN-γ treatment resulted in decreased CCR2 expression and had no effect on CXCR4 expression. Results are representative of three independent experiments.

Role of MAPK in gp120- and IFN-γ–induced cytotoxicity of HBMECs

Stimulation of HBMECs with gp120 in the presence of IFN-γ induced tyrosine phosphorylation of several proteins including proteins of approximate molecular weights of 38 and 42 to 44 kDa (data not shown), suggestive of possible involvement of p38 and extracellular signal-regulated kinase (ERK)1/2, respectively. We, therefore, determined whether mitogen-activated protein kinases (MAPKs) are involved in gp120-mediated cytotoxicity. HBMECs were treated with MAPK kinase 1 (MEK1)/ERK inhibitor, PD98059, or p38 MAPK inhibitor, SB202190, before the addition of IFN-γ and gp120. Supernatants were collected and tested for lactate dehydrogenase (LDH) release. As shown in Figure 5, SB202190 abolished gp120- and IFN-γ–mediated cytotoxicity of HBMECs. In contrast, the MEK1/ERK inhibitor PD98059 had no effect on gp120-mediated cytotoxicity in HBMECs (Figure 5).

We next examined whether p38 MAPK activation occurs in HBMECs in response to IFN-γ and

Table 1 Primer sequences for chemokine receptors used for RT-PCR analyses in this study

Receptor	Forward	Reverse
CCR1	GCAACTCCGTGCCAGAAGGTGA	CCAAATGATGATGCTGGTGATGACACCA
CCR2	CAACATGCTGGTCGTCCTCATC	AGAAGCAAACACAGCCACCAACC
CCR3	CAGGGAGAAGTGAAATGACAACCTC	TCCTCTGGGTAAAGAGCACTGCAA
CCR5	GGTGGTGACAAGTGTGATCACTTGG	TCGGGAGCCTCTTGCTGGAAA
CXCR4	AATCTTCCTGCCCACCATCTACTCC	GCGGTCACAGATATATCTGTCATCTGCC

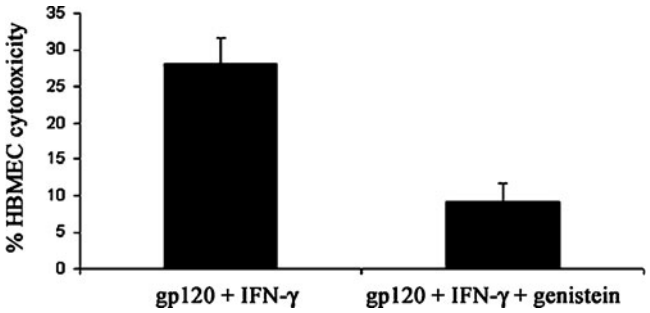

Figure 4 HIV-1 gp120-induced HBMEC cytotoxicity involves tyrosine phosphorylation signaling pathways. Gp120 and IFN-γ were incubated with primary HBMECs with and without the prior treatment of HBMECs with genistein, a protein tyrosine kinase inhibitor (25 μM), for 45 min. Cultures were incubated for 72 h. At the end of incubation period, supernatants were collected and cytotoxicity determined as described in Materials and Methods. Note that genistein treatment inhibited (>60%) gp120-mediated cytotoxicity. Results represent means of three independent experiments. Error bars represent standard deviation.

gp120 from both T-tropic and M-tropic gp120. Cell lysates were prepared and equal amounts of proteins were immunoprecipitated using p38 MAPK antibody and Western blotted using phospho-p38 MAPK antibody. As shown in Figure 6, within 30 min, gp120 (both T-tropic and M-tropic strains) in the presence of IFN-γ stimulated p38 MAPK activation (i.e., increased phsopho-p38 MAPK bands) compared to bovine serum albumin (BSA) or IFN-γ–treated HBMEC. The total amounts of immunoprecipitated p38 MAPK were similar among BSA-, IFN-γ–, or gp120/IFN-γ–treated HBMECs. These findings indicate that gp120 in the presence of IFN-γ stimulated p38 MAPK in HBMECs.

Of interest, preincubation of HBMEC with anti-CD4 antibody inhibited p38 MAPK activation from both

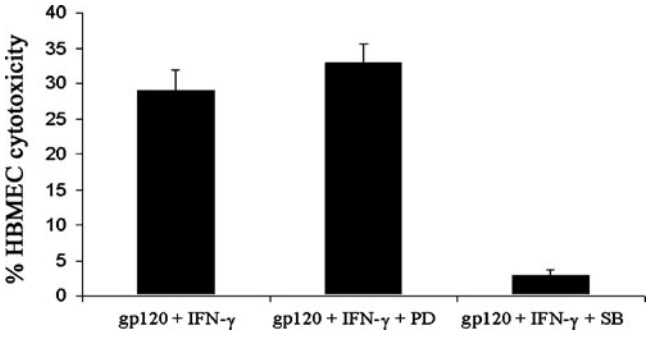

Figure 5 HIV-1 gp120-mediated HBMEC cytotoxicity is blocked by p38 MAPK inhibitor but not by MEK1/ERK inhibitor. Gp120 and IFN-γ were incubated with HBMECs with or without the prior treatment of p38 MAPK inhibitor, SB202190 (10 μM) or MEK1/ERK inhibitor, PD98059 (10 to 50 μM) for 45 min. Cultures were incubated for up to 72 h, supernatants collected, and LDH release determined as described in Figure 1. Note that p38 MAPK inhibitor but not MEK1/ERK inhibitor blocks gp120-mediated cytotoxicity. Results represent means of three independent experiments. Error bars represent standard deviation.

M-tropic and T-tropic strains of HIV-1 (Figure 6). In addition, M-tropic gp120-mediated p38 MAPK activation was inhibited when cells were treated with RANTES. In contrast, T-tropic gp120-mediated p38 MAPK activation was not inhibited by stromal cell–derived factor (SDF)-1α. Taken together, these findings suggest that gp120-/IFN-γ–mediated cytotoxicity of HBMECs involves p38 MAPK via M- and T-tropic gp120 interactions with CD4 and chemokine receptors, particularly for M-tropic gp120.

Identification of gp120 regions involved in HBMEC cytotoxicity

Viral-host cell interaction is a critical step for understanding the molecular mechanisms of HIV-1 pathogenesis. HIV-1 envelop glycoprotein gp120 has been shown to interact with CD4 on host cells (McDougal *et al,* 1986; Helseth *et al,* 1990). These interactions lead to gp120 conformational changes especially in the third variable (V3) region of the gp120, which binds to chemokine receptors on the host cells (Cheng-Mayer *et al,* 1990; O'Brian *et al,* 1990). Our earlier findings suggest the possible involvement of CD4 and chemokine receptors in gp120-/IFN-γ–mediated cytotoxicity of HBMECs. To determine the potential roles of CD4 and chemokine binding regions of gp120 in gp120/IFN-γ–mediated cytotoxicity of HBMECs, assays were performed using peptides representing different regions of gp120 as indicated in Tables 2 and 3. We determined that peptides 703, 1587, 1963, 1990 (representing CD4 binding regions of gp120 as indicated in Table 2) were able to inhibit gp120/IFN-γ–mediated cytotoxicity (>90% inhibition) of HBMECs derived from children (Table 2). Similar findings were obtained for gp120 derived from T-tropic and M-tropic strains of HIV-1. Also, peptides representing V3 loop of gp120, i.e., regions that are involved in binding to chemokine receptor (e.g., 864, 1830, 1834, 1965, as indicated in Tables 2 and 3) inhibited gp120/IFN-γ–mediated cytotoxicity (>90% inhibition) (Table 3). Peptide 864 represents sequences from both MN and IIIB strains of T-tropic HIV-1, with 22 amino acids residues from MN strain plus two amino acids (QR sequence) from IIIB inserted before the GPG tripeptide.

In addition to CD4 and chemokine receptor binding regions of gp120, peptides representing other regions of gp120, as indicated in Tables 2 and 3, such as peptide 747 (derived from β3, β4 regions), peptide 1937 (derived from loop V1/V2, β3), and peptide 1988 (derived from α2, loopE), inhibited gp120/IFN-γ–induced cytotoxicity of HBMEC (Table 3). Of interest, sequence of the peptide 1988 was derived from outer membrane domains of gp120.

As indicated above, CD4 binding regions as well as chemokine receptor binding regions (V3 loop) of gp120 are shown to be involved in gp120/IFN-γ–induced cytotoxicity of HBMECs as shown by the demonstration that peptides representing those binding regions of gp120 inhibited gp120/

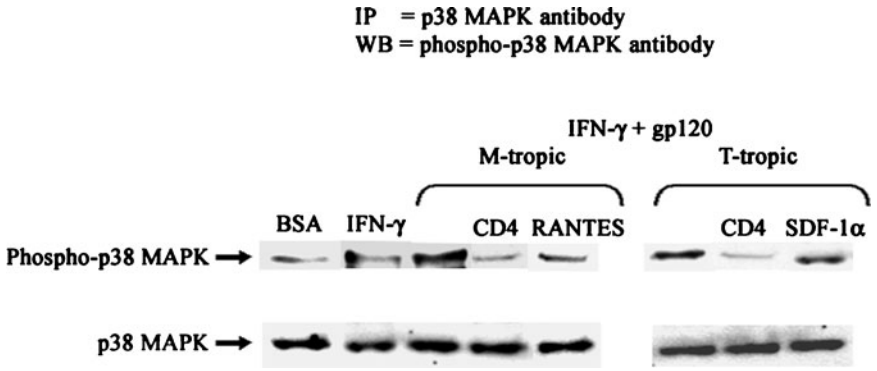

Figure 6 HIV-1 gp120 in the presence of IFN-γ promotes p38 MAPK phosphorylation in CD4-dependent manner. Confluent cultures of HBMECs were pretreated with IFN-γ for 24 h before stimulation with gp120 for 30 min in the presence or absence of anti-CD4 antibody and chemokines, RANTES and SDF-1α. The cell lysates were prepared and immunoprecipated with p38 MAPK antibody, followed by immunoblotting using phospho-p38 MAPK antibody (Cell Signaling Technology). HBMECs treated with BSA or IFN-γ alone were used as a control. Note that p38 MAPK activation in response to IFN-γ/M- and T-tropic gp120 was completely blocked by anti-CD4 antibody, and p38 MAPK activation in response to IFN-γ/M-tropic gp120 was reduced by RANTES, whereas SDF-1α had no effect on p38 MAPK activation in response to IFN-γ/T-tropic gp120.

IFN-γ–induced cytotoxicity of HBMECs. To further confirm these findings, we performed inhibition assays with anti-CD4 monoclonal antibody, RANTES and SDF-1α. We determined that anti-CD4 antibody inhibited gp120/IFN-γ–mediated cytotoxicity from both T-tropic as well as M-tropic strains of HIV-1 (Figure 7). RANTES, a β-chemokine, blocked cytotoxicity (>70%) induced by IFN-γ/gp120 from M-tropic strains of HIV-1, but had no effect on cytotoxicity induced by IFN-γ/gp120 from T-tropic strains of HIV-1 (Figure 7). In contrast, SDF1α, a α-chemokine, failed to inhibit cytotoxicity induced by IFN-γ/gp120 from both M- and T-tropic strains of HIV-1. Similarly, the small molecule inhibitors of CXCR4 (T140, TN140, and TC140) failed to inhibit HBMEC cytotoxicity induced by IFN-γ/T-tropic gp120 (data not shown). Taken together, these findings suggest the involvement of CD4 and chemokine receptors in gp120/IFN-γ–mediated cytotoxicity of HBMECs. This was shown by the inhibition of cytotoxicity with anti-CD4 antibody and RANTES as well as peptides representing chemokine receptor binding regions of gp120 derived from M-tropic HIV-1 strains. This concept is also supported by the demonstration

Table 2 Peptides representing various regions of gp120

Peptide	Peptides representing CD4 binding region Amino acids	Region
1931	121–140	CD4 (β2, loop V1/V2)
1963	271–290	CD4 (loop D, β11, β12)
1587	350–378	CD4 (loop E, β14, β15, β16, α3)
1990	351–370	CD4 (β14, β15, β16, α3)
703	419–438	CD4 (β19, β20, β21)
Peptide	**Peptides representing chemokine receptor binding region Amino acid sequence**	**Region**
864	YNKRKRIHIQRGPGRAFYTTKNII(C)	V3 loop
1830	KSIHIGPGRAFYTTG	V3 loop
1834	KSIYIGPGRAFHTTG	V3 loop
1835	TSITIGPGQVFYRTG	V3 loop
1836	KGIRIGPGRAVYAAE	V3 loop
1965	VHLNESVQINCTRPNYNKRK	V3 loop
Peptide	**Peptides representing non-CD4, non–chemokine receptor binding region Amino acids**	**Region**
1921	27–35	N-terminus
1922	31–50	N-terminus
1928	91–100	β1,α1
1937	181–200	Loop V1/V2, β3
747	200–217	β3,β4
1988	331–350	α2,loop E

Table 3 Effects of peptides on HIV-1 gp120 plus IFN-γ–mediated HBMEC cytotoxicity

No.	Peptides + gp120 + IFN-γ	Viral isolate	Sequence	% Cytotoxicity
		gp120 + IFN-γ		27.8 ± 3.8
1	702	Z3	TGNITLPCRIKQVVRTWQG	17.25 ± 5.5
2	703	CDC42	TGDIITLPCRIKQIINRWQV	3.2 ± 1.9
3	747	HXB2	(C)VITQACPKVSFEPIPIHY	4.8 ± 2.8
4	864	MN/IIIB	YNKRKRIHIQRGPGRAFYTTKNII(C)	3.1 ± 0.8
4	1587	IIIB	REQFGNNKTIIFKQSSGGDPEIVTHSFNC	<1
5	1588	IIIB	CTDLKNDTNTNSSSGRMIMEKGEIKNC	13.5 ± 4.3
6	1590	IIIB	TRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAH	23 ± 5.8
7	1830	North American consensus	KSIHIGPGRAFYTTG	<1
8	1831	Subtype B	KSIPMGPGKAFYATG	31.4 ± 4.3
9	1832	MN	KRIHIGPGRAFYTTK	29 ± 6.8
10	1833	RF	KSITKGPGRVIYATG	11.5 ± 3.5
11	1834	SF2	KSIYIGPGRAFHTTG	<1
12	1835	Subtype E	TSITIGPGQVFYRTG	6.8 ± 0.8
13	1836	Subtype B	KGIRIGPGRAVYAAE	7.5 ± 2.8
14	1837	North American consensus	CKSIHIGPGRAFYTTGC	12.4 ± 4.4
15	1838	Subtype E	CTSITIGPGQVFYRTGC	10.5 ± 3.6
16	1839	Subtype B	CKGIRIGPGRAVYAAEC	11.4 ± 2.3
17	1840	MN	TRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAH	32.7 ± 7.3
18	1841	MN	CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC	27 ± 4.2
19	1920	MN	LLMICSAT	25 ± 4.6
20	1921	MN	CSATEKLWV	9.5 ± 2.4
21	1922	MN	EKLWVTVYYGVPVWKEATTT	3.6 ± 1.4
22	1927	MN	ELVNVTENFNMWK	17.4 ± 5.7
23	1928	MN	NFNMWKNNMVEQMHEDIISL	6.5 ± 2.8
24	1930	MN	WDQSLKPCVKLTPLCVTLNC	10.4 ± 3.6
25	1931	MN	LTPLCVTLNCTDLRNTTNTN	7.1 ± 3.5
26	1933	MN	NSTANNNSNSEGTIKGGEMK	11.7 ± 5.1
27	1934	MN	EGTIKGGEMKNCSFNITTSI	22.5 ± 3.7
29	1936	MN	RDKMQKEYALLYKLDIVSIV	22.5 ± 4.4
30	1937	MN	LYKLDIVSIDNDSTSYRLIS	4.3 ± 0.5
31	1938	MN	NDSTSYRLISCNTSV	16 ± 3.9
32	1956	MN	CNTSVITQACPKISFEPIPI	17.2 ± 5.8
33	1957	MN	PKISFEPIPIHYCAPAGFAI	32 ± 8.3
34	1959	MN	LKCNDKKFSGKGSCKNVSTV	34.6 ± 6.3
35	1960	MN	KGSCKNVSTVQCTHGIRPVV	24.4 ± 4.2
36	1962	MN	STQLLLNGSLAEEEVVIRSE	23.6 ± 3.5
37	1963	MN	AEEEVVIRSENFTDNAKTII	<1
38	1965	MN	VHLNESVQINCTRPNYNKRK	<1
39	1985	MN	CTRPNYNKRKRIHIGPGRAF	21.1 ± 5.9
40	1988	MN	RQAHCNISRAKWNDTLRQIV	3.1 ± 0.4
41	1990	MN	SKLKEQFKNKTIVFNQSSGG	1.2 ± 0.7
42	1992	MN	DPEIVMHSFNCGGEF	14.8 ± 3.4
43	1994	MN	SPLFNSTWNGNNTWNNTTGF	33.6 ± 4.2
44	2010	MN	GLLLTRDGGKDTDTNDTEIF	16.5 ± 3.3

that anti-CD4 antibody and RANTES inhibited M-tropic gp120/ IFN-γ–mediated p38 MAPK activation in HBMECs.

Discussion

In this study, we studied the interactions of the HIV-1 envelope glycoprotein gp120 with HBMECs, which constitute the blood-brain barrier. It is interesting to note that gp120 did not produce cytotoxicity of HBMECs derived from adults. These findings are consistent with those of previous studies (Moses *et al,* 1993; Moses and Nelson, 1994) and may be explained by the absence of CD4 on the blood-brain barrier endothelium derived from adults (Moses *et al,* 1993; Moses and Nelson, 1994; Stins *et al,* 2001). CD4 is a well-known receptor for gp120. In contrast, gp120-induced cytotoxicity of HBMECs derived from children in a CD4-dependent manner. This is further supported by demonstrations that HBMECs derived from children express CD4 (Stins *et al,* 2001) and that monoclonal anti-CD4 antibody abolished gp120 (from T-tropic or M-tropic)-mediated HBMEC cytotoxicity. However, priming of HBMECs with IFN-γ is critical for gp120-mediated HBMEC cytotoxicity. Overall, these findings suggests that although gp120 binding to CD4 is a primary step, it is not sufficient to induce host cell damage and to this end, chemokine receptors may play important role(s).

Despite the involvement of CD4, we showed that the priming of HBMECs with IFN-γ is required for

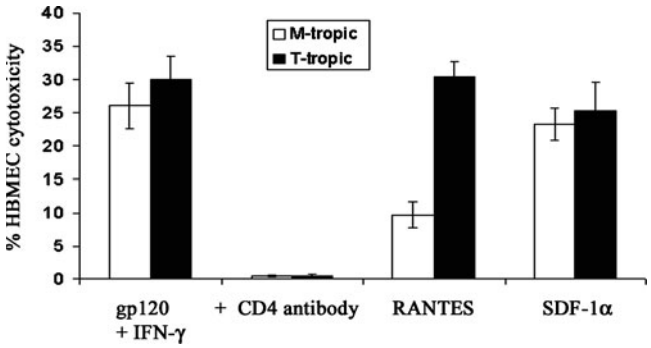

Figure 7 HIV-1 gp120-induced HBMEC cytotoxicity is abolished in the presence of anti-CD4 antibody. Cytotoxicity assays were performed in the presence of anti-CD4 antibody (10 μg/ml), RANTES (40 μg/ml), or SDF-1α (40 μg/ml). Supernatants were collected following 72-h incubation and LDH release was measured as described in Materials and Methods. Note that anti-CD4 antibody completely abolished gp120-mediated HBMEC cytotoxicity in response to both M- and T-tropic gp120. However, RANTES partially inhibited M-tropic gp120-mediated HBMEC cytotoxicity but had no effect on T-tropic gp120-mediated cytotoxicity. In contrast, SDF-1α had no effect on M- or T-tropic gp120-mediated cytotoxicity. Results represent means of three independent experiments. Error bars represent standard deviation.

gp120-mediated HBMEC cytotoxicity. The interactions of gp120 with host cell CD4 (CD4 provides a primary docking site for gp120) leads to conformational changes in gp120, exposing V3 loop epitope that interacts with the chemokine receptors, preferably CCR5 in the case of M-tropic strains of HIV-1 and CXCR4 for T-tropic strains of HIV-1. The chemokine receptors CCR5 and CXCR4 belong to the superfamily of seven-transmembrane G protein–coupled receptors. To shed light on the requirement of IFN-γ priming, we determined the effects of IFN-γ on the transcriptional regulation of chemokine receptors. We showed that CCR3 and CCR5 are upregulated with IFN-γ treatment but had no effects on CXCR4, thus suggesting a possible role for these receptors in gp120-mediated cytotoxicity. Of note, IFN-γ treatment abolished the expression of CCR2. Given that HBMEC cytotoxicity is observed in the presence of IFN-γ, it is unlikely that CCR2 plays a role in mediating HBMEC cytotoxicity, but may act in HBMEC survival mechanisms in response to gp120/IFN-γ. Future studies are in progress to address these issues. To determine the role of chemokine receptor(s) in gp120/IFN-γ–mediated cytotoxicity of HBMECs, cytotoxicity assays were performed in the presence of the CC chemokines RANTES and SDF-1α (ligand for CXCR4). As expected, RANTES blocked HBMEC cytotoxicity of gp120 derived from M-tropic strains of HIV-1, whereas it had no effect on HBMEC cytotoxicity in response to gp120 from T-tropic strains of HIV-1. However, SDF-1α had no effect on HBMEC cytotoxicity induced by gp120 derived from M- or T-tropic strains of HIV-1. These findings suggest the possible involvement of non-CXCR4 in T-tropic gp120-mediated cytotoxicity. In support, previous studies have shown that gp120 derived from T-tropic strains of HIV-1 not only interact with CD4 and chemokine receptors but also with cell surface heparan sulfate proteoglycans (HSPGs) and that signaling is primarily generated by gp120-CD4 interaction, while HSPGs/CXCR4 may play a partial role in a differential signaling outcome (reviewed in Popik and Pitha, 2000). In contrast, binding of M-tropic gp120 may trigger signaling through both CD4 and CCR5 receptors (Popik and Pitha, 2000). Overall, our findings showed that gp120, in the presence of IFN-γ induced cytotoxicity of HBMEC derived from children but not from adults. These findings differ from those of previous studies, which demonstrated that gp120 induced brain endothelial cell death without the requirement of IFN-γ (Kanmogne *et al,* 2002). At present, the basis of these differences remains unknown; however, the method of isolating HBMECs, source of brain capillary endothelium, and age of brain capillary endothelium may explain these discrepancies. Studies are in progress to clarify this issue.

We next investigated the mechanisms that are involved with gp120/IFN-γ–mediated HBMEC cytotoxicity. The cell death is known to be dependent on host cell signaling, and proteins that regulate cell fate are known to involve tyrosine as well as serine/threonine phosphorylations for intracellular signaling (Mutoh *et al,* 1999; Mizukami *et al,* 2001). Consistent with this, we showed that genistein, a protein tyrosine kinase inhibitor, blocked gp120-mediated HBMEC cytotoxicity. Furthermore, SB202190, a p38 MAPK inhibitor, blocked gp120-mediated HBMEC cytotoxicity and also gp120-induced phosphorylation of p38 MAPK. Studies are in progress to determine the effector molecules downstream of MAPK, including caspases, in the gp120-mediated HBMEC cytotoxicity.

We used peptides representing CD4 binding region of gp120, chemokine receptor binding region of gp120, as well as other regions of gp120, as indicated in Tables 2 and 3. Peptide studies further support our findings that CD4 and chemokine receptors are involved in gp120/IFN-γ—mediated HBMEC cytotoxicity, i.e., peptides representing CD4 binding region and chemokine receptor binding region of gp120 are inhibitory for HBMEC cytotoxicity. An additional interesting finding is our demonstration that peptides representing non-CD4 and non–chemokine receptor binding regions of gp120 inhibited gp120/IFN-γ–mediated cytotoxicity, such as β3, β4, loop V1/V2, α2, and loop E regions of gp120. Of interest, peptide 1988 was derived from extracellular domain of gp120. At present, the mechanisms of inhibition of gp120/IFN-γ–mediated HBMEC cytotoxicity by these peptides are not clear. It is, however, important to note that cytotoxicity is a delayed event (i.e., up to 72 h) and it is possible that the interactions of peptides with HBMEC may result in signaling events that

interfere with cytotoxicity. Studies are in progress to elucidate the mechanisms associated with inhibition of HBMEC cytotoxicity by these peptides.

In conclusion, we showed that gp120 (from both T-tropic and M-tropic strains of HIV-1) in the presence of IFN-γ produced cytotoxicity of HBMEC derived from children, which involves p38 MAPK activation via several gp120-HBMEC interactions.

Materials and methods

Gp120 and gp120 peptides
Peptides representing sequences from different regions of gp120 were obtained from the National Institute of Health AIDS Research and Reference Reagent Program. Sequences of the peptides and their viral isolates are described in Tables 2 and 3.

Cell cultures
Human brain microvascular endothelial cells (HBMECs) were isolated from brain tissues from children (<13 years old) and adults as previously described (Stins *et al,* 1997). Briefly, brain specimens were homogenized in Dulbecco's modified Eagle medium (DMEM) containing 2% fetal bovine serum (DMEM-S). The homogenate was centrifuged in 15% dextran in DMEM-S for 10 min at $10{,}000 \times g$. The pellet containing crude microvessels was further digested in a solution containing 1 mg/ml collagenase/dispase in DMEM-S for 1 h at 37°C. Microvascular capillaries were isolated by adsorption to a column of glass beads and washing off the beads. Viability of the microvessels tested by trypan blue exclusion test. HBMECs were isolated and purified by fluorescence-activated cell sorting (FACS) and tested for endothelial characteristics, such as expression of endothelial markers, factor VIII, carbonic anhydrase IV and uptake of acetylated low density lipoprotein (AcLDL), indicating endothelial origin, as well as for γ-glutamyltranspeptidase, indicating brain origin, resulting in >99% pure cultures as previously described (Stins *et al,* 1997). HBMECs were routinely grown on rat tail collagen-coated dishes in RPMI medium containing 10% heat inactivated fetal bovine serum, 10% Nu-Serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), nonessential amino acids, and vitamins (Stins *et al,* 1997).

Cytotoxicity assays
To determine whether gp120 is cytotoxic to HBMECs, gp120 from both T-tropic (MN) and M-tropic (JRFL) strains of HIV-1 were used for cytotoxicity assays as previously described (Sissons *et al,* 2005). Briefly, HBMECs were grown to confluency in 24-well plates and then incubated with gp120 (0.5 μg/ml, both from M-tropic and T-tropic strains of HIV-1) and IFN-γ (2 ng/ml; R & D Systems, Minneapolis, MN) in experimental medium (5% heat inactivated fetal bovine serum, 2 mM glutamine, penicillin [100 U/ml], streptomycin [100 μg/ml], and nonessential amino acids) at 37°C in 5% CO_2 incubator for up to 72 h. After this incubation, supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release using cytotoxicity detection kit (Roche, Indianapolis, IN) and converted into percent cytotoxicity as follows: ((sample value − control value)/(total LDH release − control value)) × 100 = % cytotoxicity. Control values were obtained from cells incubated with medium alone. Total LDH release was determined from HBMECs treated with 1 % Triton-X-100 for 15 min at 37°C. Results represent mean (± standard deviation) from three independent experiments.

Inhibition of cytotoxicity
In an attempt to identify gp120 regions responsible for interactions with HBMECs leading to their cytotoxicity, the following experiments were performed. First, peptides of approximate 15 to 30 amino acids representing different regions of gp120 (Tables 2 and 3) were obtained and used in cytotoxicity assays. Briefly, peptides were added to confluent HBMEC monolayers for 30 min prior to the addition of gp120 and IFN-γ. The gp120 proteins derived from both T-tropic and M-tropic strains of HIV-1 were used. Cells were incubated for up to 72 h. Supernatants were collected and LDH release was determined and converted into percentage cytotoxicity as described above.

HIV-1 infection is initiated by binding of gp120 to its principal cellular receptor, CD4 and a chemokine receptor, i.e., CC chemokine receptors in case of M-tropic strains of HIV-1 and CXC chemokine receptors in case of T-tropic strains of HIV-1, which function as coreceptors (Berger *et al,* 1999). To determine the role of CD4 binding region and chemokine receptor binding region in gp120-induced HBMEC cytotoxicity, anti-CD4 monoclonal antibody (10 μg/ml; Dako, Carpinteria, CA) and chemokines such as RANTES, which specifically binds to CC family of chemokine receptors, and stromal cell–derived factor-1 alpha (SDF-1α), which specifically binds to CXC family of chemokine receptors (40 μg/ml; obtained from National Institute of Health AIDS Research and Reference Reagent Program), were used in cytotoxicity assays. Following incubation for 72 h, supernatants were collected and LDH release was determined and converted into percentage cytotoxicity as described above.

Chemokine receptors are members of the seven-transmembrane G protein–coupled receptors family and are shown to involve several signaling transduction pathways (Del Corno *et al,* 2001). To determine the role of tyrosine signaling on gp120-mediated cytotoxicity, HBMECs were treated with the tyrosine kinase inhibitor genistein, the mitogen-activated protein kinase kinase (MEK1/ERK) inhibitor PD98059, or the p38 mitogen-activated protein kinase

(p38 MAPK) inhibitor SB202190 before the addition of gp120 and IFN-γ.

RT-PCR analysis of CCR1, CCR2b, CCR3, CCR5, and CXCR4 expression in HBMECs

IFN-γ has been shown to regulate chemokine receptor expression in monocytoid U937 cells (Zella *et al,* 1998). To determine the involvement of IFN-γ in gp120-mediated HBMEC cytotoxicity, mRNA levels of CCR1, CCR2B, CCR3, CCR5, and CXCR4 were examined with and without the treatment of IFN-γ by RT-PCR analyses using primers specific for chemokine receptors (Table 1). Briefly, total RNA was extracted from HBMEC cultures using RNAqueous-Midi (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The mRNA fraction was purified from total RNA using GenElute mRNA miniprep kit (Sigma, St. Louis, MO, USA). The mRNA preparations were treated with DNaseI (Ambion) for 30 min to further remove any traces of DNA. Following this treatment, the enzyme and the divalent cations were removed using DNase Inactivation Reagent (Ambion). The amount and the quality of the mRNA were verified by measuring the absorbance at 260 and 280 nm.

Oligo (dT) primed reverse transcription of RNA was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen); 200 ng of mRNA were used in each reaction. Subsequent PCR amplifications of CCR1, CCR2B, CCR3, CCR5, and CXCR4 were performed from 2 μl of each cDNA using primers listed in Table 1 (Zella *et al,* 1998). The PCR program consisted of one preincubation at 94°C for 2 min and 40 cycles at 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. β-Actin amplification was successively performed from 2 μl of each PCR reaction using commercial primers (QuantumRNA β-actin Internal Standards; Ambion). The PCR reactions consisted of one preincubation 94°C for 2 min and 20 cycles at 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. All PCR reactions were performed using a Robocycler Gradient 40 with heated lid (Stratagene, La Jolla, CA) in 50 μl of 1 $\times$ PCR Buffer, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 200 μM of dNTP, and 1 U of Taq DNA polymerase (Qiagen, Valencia, CA). Amplification mixtures were analyzed by agarose gel electrophoresis and bands were compared on a Gel Doc 1000 using Quantity One 4.1 software (Bio-Rad, Hercules, CA).

Immunoprecipitation and immunoblotting

Confluent HBMECs were treated with and without IFN-γ in experimental medium for 24 h. Following this treatment, cells were stimulated with gp120 (0.5 μg/ml; from both T-tropic and M-tropic strains of HIV-1) for the indicated periods and rinsed with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM Na_3VO_4. The monolayers were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 0.1% sodium dodecyl sulfate [SDS], 0.5% Na deoxycholate, 10 mM Na pyrophosphate, 25 mM β-glycerophosphate, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Cell lysates were centrifuged at 16,000 $\times$ *g* at 4°C and supernatant quantified for protein concentration (Bio-Rad). Equal amounts of protein (500 μg) were incubated with anti-phosphotyrosine antibody 4G10 (Upstate Biotech, Lake Placid, NY) overnight at 4°C for immunoprecipitation and lysates were incubated for 1 h with Protein A-agarose (Invitrogen). The samples were washed four times with lysis buffer without sodium deoxycholate. Samples were eluted by boiling in SDS sample buffer containing 10% β-mercaptoethanol (Sigma) and separated by SDS–polyacrylamide gel electrophoresis (PAGE). Samples were then electrophoretically transferred onto pure nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were blocked in TBST (25 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at room temperature for 1 h. Blots were probed with appropriate antibody overnight at 4°C, washed with TBST, and incubated with horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) for 1 h at room temperature. Finally, blots were washed with TBST and immune complexes were visualized through enhanced chemiluminescence (Amersham, La Jolla, CA).

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