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Glycoprotein 120 Binding to CXCR4 Causes p38-Dependent Primary T Cell Death That Is Facilitated by, but Does Not Require Cell-Associated CD4

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HIV-1 infection causes the depletion of host CD4 T cells through direct and indirect (bystander) mechanisms. Although HIV Env has been implicated in apoptosis of uninfected CD4 T cells via gp120 binding to either CD4 and/or the chemokine receptor 4 (CXCR4), conflicting data exist concerning the molecular mechanisms involved. Using primary human CD4 T cells, we demonstrate that gp120 binding to CD4 T cells activates proapoptotic p38, but does not activate antiapoptotic Akt. Because ligation of the CD4 receptor alone or the CXCR4 receptor alone causes p38 activation and apoptosis, we used the soluble inhibitors, soluble CD4 (sCD4) or AMD3100, to delineate the role of CD4 and CXCR4 receptors, respectively, in gp120-induced p38 activation and death. sCD4 alone augments gp120-induced death, suggesting that CXCR4 signaling is principally responsible. Supporting that model, AMD3100 reduces death caused by gp120 or by gp120/sCD4. Finally, prevention of gp120-CXCR4 interaction with 12G5 Abs blocks p38 activation and apoptosis, whereas inhibition of CD4-gp120 interaction with Leu-3a has no effect. Consequently, we conclude that gp120 interaction with CXCR4 is required for gp120 apoptotic effects in primary human T cells. The Journal of Immunology, 2007, 178: 4846–4853.
CD4 T cells were incubated with gp120IIIB (10 μg/ml) or SDF-1α (125 nM) for 1 h at 4°C, and then stimulated at 37°C for the indicated times. Activation of p38 and Akt was analyzed by immunoblotting with anti-phospho-p38 (T180/Y182) and anti-phospho-S473Akt. Total amount of p38 and Akt was detected with anti-p38 and anti-Akt Abs. B. Primary human CD4 T cells were incubated with the indicated doses of soluble rHIV gp120IIIB for 1 h at 4°C and then stimulated at 37°C for 1 min. Control cells were treated with 10 μg/ml BSA. Activation of p38 was analyzed by immunoblotting, as described above. C. Primary CD4 T cells were incubated with BSA or 1 μg/ml gp120IIIB for 1 h at 4°C. To detect binding of gp120, cells were fixed with 2% paraformaldehyde and stained with AlexaFlour 647-labeled anti-gp120 Abs. D–F. CD4 T cells were pretreated with either 1 μM SB203580 or vehicle (DMSO) for 20 min at 37°C. Then SB203580- or control-treated CD4 T cells were incubated with gp120IIIB (10 μg/ml; F) or BSA (10 μg/ml; E) for 1 h on ice. Thereafter, cells were incubated at 37°C for 1 min (or were stimulated with SDF-1α for 1 min at 37°C) (D), permeabilized, and analyzed for phospho-p38 (T180/Y182). G. Purified CD4 T cells from a healthy donor or two HIV-positive individuals (patient 1: viral load 111,000 copies/ml; CD4 cell count 383/μl; patient 2: viral load 3,540 copies/ml; CD4 cell count 182/μl) were analyzed for phospho-p38 by immunoblot.

Materials and Methods

Cell culture and reagents

CD4 T cells were isolated from the blood of healthy volunteer blood donors or HIV-positive patients by using RosetteSep CD4 enrichment mixture in accordance with the manufacturer’s protocol (StemCell Technologies). The remaining cell population was repeatedly found to be 98% CD4 T cells, as determined by flow cytometry. CD4 T cells were maintained in RPMI 1640, supplemented with 10% FBS (Invitrogen Life Technologies), 2 mM L-glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 0.5 × 10^6 cells/ml. CD4 T cells, used in the various experiments, were stimulated with PHA (5 mg/ml) for 2 h, and then cells were washed twice with RPMI 1640 and maintained in medium supplemented with 100 U/ml IL-2 for 24–36 h.

CD4 T cells were incubated with HIV-1 X4 gp120IIIB or HIV-1 R5 gp120 YU2 (Immuno Diagnostics) or gp120IIIB and gp120 YU2 pre-treated with soluble CD4 (sCD4) (1:2 ratio; Immuno Diagnostics) at concentrations of 10 μg/ml/2 × 10^6 cells for 1 h at 4°C. Similarly, CD4 T cells were treated with Leu-3a, 12G5, and M183 Abs for 30 min at 4°C, and then cells were incubated at 37°C for the indicated times. Primary 92 Ug20.9 Env was donated by C. Cicala (National Institutes of Health, Bethesda, MD) (35) and used at 20 nM.

OKT3 Abs were obtained from Ortho Biotech, and anti-CD4 (Leu-3a) and isotype mouse IgG controls were purchased from BD Biosciences. Anti-CXCR4 12G5 and stromal cell-derived factor (SDF)-1α were purchased from R&D Systems, and anti-CCR5 M183 Ab was obtained from the National Institutes of Health AIDS Research and Reference program. Anti-p38 (C-20), anti-Lck (3A5) were purchased from Santa Cruz Biotechnology. Anti-p38 (T180/Y182) (D7), anti-phospho-Akt (Ser473), anti-phospho-Src (Y416), and anti-Akt were purchased from Cell Signaling Technology. AlexaFlour 488-labeled-phospho-p38 (T180/Y182) Abs were purchased from BD Transduction Laboratories, and AlexaFlour 647-labeled anti-gp120 Abs were purchased from Immuno Diagnostics. AMD3100 (National Institutes of Health AIDS Research and Reference program) was used at 2 μM for 1 h at 37°C. SB203580 was purchased from Calbiochem. Leupeptin, aprotinin, and pepstatin A were obtained from Boehringer Mannheim.

Cell extract preparation and immunoblotting

To obtain total cellular proteins, cells were washed with cold PBS, resuspended in a modified whole cell extract buffer (36) (40 mM Tris-HCl (pH 8), 0.3 M NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 10 mM NaF, 10 mM paranitrophenyl phosphate, 10 mM β-glycerophosphate, 300 μM sodium orthovanadate, 1 mM DTT, 2 μM PMSF, 10 μg/ml aproatinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin), and centrifuged at 12,000 × g for 15 min at 4°C. The resultant supernatant contained total cellular protein. The amount of cellular protein present in the clarified supernatant was calculated by using the Bio-Rad protein assay.

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2 Abbreviations used in this paper: sCD4, soluble CD4; SDF-1α, stromal cell-derived factor.
For Western immunoblots, equal amounts of whole cell extract were loaded and separated by 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Immunoblotting was performed with specific Abs and visualized by using the ECL Western blotting detection kit (Amersham).

Cell death analysis and flow cytometry
CD4 T cells were untreated or preincubated with specific inhibitors and then stimulated with either BSA or HIV gp120IIIB, and cell death was analyzed using the Cell Death Detection ELISA kit (Roche Diagnostics), following the manufacturer’s instructions. Cell death was confirmed by measuring the decrease of live cells by using CellTiter-Glo Luminescence Cell Viability Assay (Promega). All experiments were performed at least three times in duplicate and presented as means with SDs.

Phospho-p38 and gp120 immunostaining
Primary CD4 T cells were incubated with BSA control or 1 μg/ml HIV gp120IIIB for 1 h at 4°C. To detect binding of gp120, cells were fixed with 2% paraformaldehyde and stained with AlexaFlour 647-labeled anti-gp120 Abs. For phospho-p38 staining, CD4 T cells were incubated with gp120IIIB for 1 h on ice. Thereafter, cells were left on ice or incubated at 37°C for 2 min to induce stimulation. Incubation with SDF-1α for 2 min at 37°C was included as a positive control. After the indicated stimulation, cells were fixed and permeabilized using FIX AND PERM (Caltag Laboratories) and stained with AlexaFlour 488-labeled phospho-p38 (T180/Y182) Abs.

Results
gp120 induces p38, but not Akt phosphorylation in human primary CD4 T cells
We have demonstrated previously that ligation of CXCR4 with its natural ligand, SDF-1α, induces p38 phosphorylation on residues Thr180 and Tyr182 that is counteracted by activation of the anti-apoptotic protein, Akt (37). We speculated that HIV X4 Env-triggered death of CD4 T cells might result in p38 activation in the absence of Akt activation, consequently causing death. Primary human CD4 T cells were treated with either soluble gp120 (IIIB) or SDF-1α, and p38 phosphorylation was analyzed by immunoblotting with anti-phospho-p38 and anti-phospho-S473 Akt Abs. Total amount of p38 and Akt was detected with anti-p38 and anti-Akt Abs. B, Purified primary human CD4 T cells were incubated with HIV gp120IIIB for 1 μM for 20 min and then stimulated with either BSA control (10 μg/ml), rHIV gp120IIIB (10 μg/ml), or plate-bound anti-CD4 (10 μg/ml) for 10 min as a positive control.

FIGURE 3. Both CD4 and CXCR4 signaling activate p38 and induce p38-dependent apoptosis of primary human CD4 T cells. A, Purified primary human CD4 T cells were treated with 10 μg/ml Leu-3a (anti-CD4), 10 μg/ml 12G5 (anti-CXCR4), or 10 μg/ml isotype control Abs on ice for 30 min, and then cross-linked with plate-bound goat anti-mouse IgG for the indicated times. As a positive control for Akt activation, CD4 T cells were stimulated with SDF-1α (125 nM) for 1 min at 37°C. Activation of p38 and Akt was assessed with anti-phospho-p38 and anti-phospho-S473 Akt Abs. Total amount of phospho-S473 Akt and phospho-p38 was measured using the Cell Death Detection ELISA kit (Roche Diagnostics), following the manufacturer’s instructions. Cell death was confirmed by measuring the decrease of live cells by using CellTiter-Glo Luminescence Cell Viability Assay (Promega). All experiments were performed at least three times in duplicate and presented as means with SDs.
We next confirmed that gp120 binding to primary CD4 T cells was required for induction of p38 phosphorylation by flow cytometry (38). First, gp120 binding to primary CD4 T cells was confirmed using anti-gp120 Abs (Fig. 1C). CD4 T cells treated with gp120 were then assessed for p38 phosphorylation (Fig. 1F), which occurred as efficiently as SDF-1/H9251 induction of phospho-p38 (Fig. 1D). We used the p38 inhibitor SB203580 (39), which does not alter basal phosphorylation of p38 (Fig. 1E), to assess the specificity of gp120 phosphorylation of p38, demonstrating that SB203580 inhibits gp120-induced phosphorylation of p38 (Fig. 1F). Finally, we observed increased p38 phosphorylation in in vitro HIV-infected primary human CD4 T cells (data not shown) and in purified CD4 T cells isolated from HIV-infected patients (Fig. 1G). Altogether, these data indicated that gp120 binding to CD4 T cells can result in p38 phosphorylation and that p38 activation is seen following experimental HIV infection, and in freshly isolated CD4 T cells from HIV-infected patients.

**gp120 induces cell death of human primary CD4 T cells in p38-dependent manner**

To study whether gp120-mediated p38 activation is a necessary event for gp120-induced CD4 T cell death, we used the specific p38 inhibitor, SB203580 (39). As shown in Fig. 2, A and B, two different strains of soluble gp120 (a laboratory adapted strain, or IIIB, and a patient-derived primary HIV strain, 92Ug20.9) induced CD4 T cell death that was inhibited by 1 μM SB203580 pretreatment, a dose that selectively inhibits p38 phosphorylation (40). This dose of SB203580 efficiently inhibited p38 activation induced by either gp120 stimulation (Fig. 2, A and B) or OKT3 cross-linking as a positive control (Fig. 2C) (41).

Ligation of either the CD4 or CXCR4 receptor induces p38 phosphorylation and causes apoptosis

Having established that gp120 treatment causes p38 phosphorylation, which is required for gp120-induced apoptosis, we next sought to determine whether gp120 binding to the CXCR4 or to the CD4 receptor is responsible for the observed p38 phosphorylation. To discern the differential signaling defects, human primary CD4 T cells were cross-linked with either anti-CD4 Abs (Leu-3a) or anti-CXCR4 (12G5) Abs, which bind to the same regions of the respective receptors as gp120 does (42–44). Cross-linking of either CD4 or CXCR4 induces p38 phosphorylation in primary human CD4 T cells, yet no Akt phosphorylation (Fig. 3A). Because p38 phosphorylation in the absence of Akt phosphorylation should lead to apoptosis, we tested whether 12G5 or Leu-3a treatment could result in p38-dependent primary CD4 T cell death. Primary human CD4 T cells were pretreated or not with 1 μM SB203580, and then cross-linked with either Leu-3a or 12G5 Abs. CD4 and CXCR4 cross-linking alone led to p38 phosphorylation (Fig. 3B) and to CD4 T cell apoptosis (Fig. 3C), both of which were inhibited by SB203580. Therefore, CD4 ligation alone or CXCR4 ligation...
alone can independently trigger apoptosis of CD4 T cells in a p38-dependent manner.

**Inhibition of CXCR4 signaling is required to block p38-mediated apoptosis induced by gp120**

To further isolate CXCR4- and CD4-specific events following gp120 binding to CD4 T cells, we opted to block the interaction of gp120 with CD4, CXCR4, or both. First, we used gp120 (IIIB) preincubated with sCD4 (gp120/sCD4), which prevents further binding to CD4, and exposes the CXCR4-binding motif (45, 46). Second, to block gp120 interaction with CXCR4, we used a specific inhibitor of this interaction, AMD3100 (47). Finally, to prevent gp120 interaction with both CD4 and CXCR4, we used gp120/sCD4 in the presence of AMD3100. As expected, pretreatment of gp120 with sCD4 significantly reduced binding of gp120 to cells (Fig. 4A). In contrast, AMD3100 did not significantly alter gp120 binding to CD4 T cells (presumably by allowing binding to CD4), although the activity of AMD3100 was confirmed by its ability to inhibit SDF-1α-induced chemotaxis (Fig. 4B).

Next, we analyzed p38 phosphorylation in primary human CD4 T cells following stimulation with either gp120 or gp120/sCD4 in the presence or absence of AMD3100. Consistent with our previous observation, gp120 stimulation alone induced a strong p38 phosphorylation (as well as Lck activation, demonstrating that the CD4 receptor was triggered), which decreased over time. In contrast, gp120/sCD4 induced a more prolonged p38 phosphorylation, yet failed to induce Lck phosphorylation, indicating that sCD4 efficiently blocked gp120 interaction with CD4. Pretreatment with AMD3100 only partially decreased gp120-induced p38 phosphorylation, most likely representing CD4-induced effects on p38. However, gp120/sCD4 in the presence of AMD3100 decreased, but did not completely abrogate p38 phosphorylation (Fig. 4C).

We next questioned whether blocking gp120 interaction with either CD4 or CXCR4 alone, or blocking both, could prevent CD4 T cell death. Human primary CD4 T cells were treated with either gp120 or gp120/sCD4 in the presence or absence of AMD3100. Treatment of primary CD4 T cells with gp120 alone caused low-level CD4 death that was significantly increased by pretreatment of gp120 with sCD4, suggesting that exposing the CXCR4-binding motif in gp120 by sCD4 pretreatment augments CXCR4 interaction. AMD3100 pretreatment reduced death caused by gp120, as well as death caused by gp120/sCD4, yet the magnitude of death reduction was greatest in cells pretreated with AMD3100 and gp120/sCD4 (Fig. 4D).

Inability of AMD3100 to completely block p38 activation and apoptosis induced by gp120/sCD4 prompted us to further examine the individual roles of CD4 and CXCR4 in gp120-induced CD4 T cell death. First, we blocked CD4 or CXCR4 interactions with gp120 by pretreatment of CD4 T cells with soluble Leu-3a (to block CD4) or soluble 12G5 (to block CXCR4) before gp120 treatment. As shown on Fig. 5A, prevention of gp120 interaction with CXCR4 decreased p38 phosphorylation. In contrast, Leu-3a pretreatment did not reduce gp120-induced p38 phosphorylation, whereas it efficiently blocked Lck phosphorylation, indicating that Leu-3a efficiently inhibited the CD4-gp120 interaction. Second, we tested whether HIV-1 R5 gp120 YU2 can induce p38 phosphorylation (Fig. 5B), and we found gp120 R5 induced p38 phosphorylation (13.7-fold) through CCR5, because pretreatment with anti-CCR5 M183 Abs, but not with anti-CXCR4 sCD4, blocked gp120 R5-induced p38 phosphorylation. Finally, we tested whether gp120IIIB-induced CD4 T cell death was inhibited by Leu-3a or 12G5. Neither sCD4 nor Leu-3a reduced gp120-induced death. However, gp120-induced death was inhibited by pretreatment with 12G5. Altogether, our data indicate that, although both CD4 and CXCR4 signaling can independently induce p38 activation and CD4 T cell death, gp120 binding to CD4 T cells preferentially triggers CXCR4 to activate p38, leading to apoptosis of CD4 T cells.

**Discussion**

A variety of mechanisms has been proposed to explain how HIV-1 Env induces apoptosis of T cells, including gp120:CD4- and/or gp120:CXCR4-initiated apoptosis signaling pathways. Each of these receptors is capable of causing p38 activation, yet which of these two receptors drives both p38 and apoptosis downstream of gp120 ligation is unknown. In the current study, we have inhibited CD4, CXCR4, or both to define the involvement of each receptor in p38 activation and the obligate role of p38 for gp120-induced apoptosis.
Activation of p38 MAPK pathway has been implicated in multiple cellular processes, including cytokine expression, proliferation (reviewed in Ref. 48), cell survival (49), and apoptosis. Indeed, p38 activation can induce apoptosis of CD8 T cells (50), epithelial cells (25), and neurons (30); however, its role in apoptosis of primary CD4 cells is less understood. Results in the current report, as well as our previous findings (23), suggest that in the absence of antiapoptotic Akt signaling, p38 activation results in CD4 T cell death, yet when Akt is activated in parallel (for example by SDF-1α), cell death is prevented.

Our finding that gp120 ligation of CD4 T cells results in p38 activation, but fails to induce Akt or ERK (data not shown), is in agreement with other reports that gp120 induces p38 activation in cell lines (26, 27), neurons (29, 30), and B cells (51). Furthermore, the finding of activated p38 in the CD4 T cells from untreated HIV patients (Fig. 1G) concurs with observations of activated p38 in lymph node biopsies from untreated HIV-1 patients (52). However, other reports in primary CD4 T cells suggest that gp120 ligation to CXCR4 can activate Akt, ERK, and Ca²⁺ mobilization (53, 54); however, those studies used gp120 X4 at concentrations of 10–20 nM to induce calcium mobilization and modest Akt activation (54), or very high concentrations of gp120 × 4 (200 nM), which is close to its Ki₅₀ for CXCR4 (55) to induce activation of Akt and ERK (53). We observed only very weak Akt and ERK activation at 85 nM gp120IIIB (data not shown), yet gp120 at 4.2 nM (Fig. 1B) induced only p38, but not Akt. This raises the intriguing possibility that the thresholds for p38 and Akt activation by gp120 may differ by 100-fold and may be mediated by distinct gp120 binding domains within CXCR4. Therefore, Akt activation may require high concentrations (close to Ki₅₀) of gp120, whereas p38 phosphorylation may be mediated by lower amounts.

The fact that both CD4 and CXCR4 can independently activate p38 and initiate apoptosis indicates that CD4- and CXCR4-mediated apoptosis share similar features. Indeed, both CD4 and CXCR4 induce Gₛₐ, and caspase-independent apoptosis in human PBLs, involving reduction of mitochondria membrane potential and loss of membrane asymmetry (18). Previously, the signals downstream of receptor ligation have remained incompletely characterized. Our results indicate that either CD4 alone or CXCR4 alone can induce p38 phosphorylation and apoptosis. Moreover, following gp120 treatment, both CD4 and CXCR4 can contribute in p38 activation, which is a necessary event for CD4 T cell apoptosis. Several interesting features of p38 activation by gp120 are underscored by our results. First, gp120-induced p38 activation peaks at 1 min and persists up to 10 min. These kinetics of p38 activation may explain a controversial in p38 activation by gp120, which has been observed previously at early (25), but not late times after gp120 stimulation (28). Second, both CD4 and CXCR4 independently induce p38 phosphorylation and apoptosis following stimulation with agonistic plate-bound Abs. Finally, whereas gp120 binding to CD4 and CXCR4 results in transient p38 activation, ligation of gp120 to CXCR4 alone induces more persistent p38 activation and a higher degree of CD4 T cell death (Figs. 4D and 5C). It is unclear why gp120 ligation of both receptors individually results in transient p38 activation. This could be due to cross-talk between receptors, resulting in desensitization of CXCR4 signaling by CD4 (56, 57). The fact that inhibition of CD4-gp120 interaction with either sCD4 or Leu-3a Ab resulted in inhibition of Lck phosphorylation at Y394 (Figs. 4C and 5A), which correlated with higher CD4 T cell death, suggests that CD4-mediated Lck activation might oppose CXCR4-induced apoptosis. Because gp120 ligation of CD4 activates Lck (58), it is possible that activated Lck induces CXCR4 down-regulation (56) or Lck might activate the tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 (59), which negatively regulates CXCR4 signaling (60). These possibilities are worthy of direct experimentation.

Observations that interference with CXCR4 binding by gp120 inhibits apoptosis, whereas the addition of sCD4 enhances gp120 killing, suggest a model in which gp120 killing of CD4 T cells requires CXCR4-dependent signals that are facilitated by CD4 interaction, presumably via a steric mechanism.

Our observation that p38 activation results in CD4 T cell apoptosis is in agreement with previous findings that p38 activation following SDF-1α ligation of CXCR4 can lead to cell death if Akt is not activated concurrently (37). In this study, we demonstrate that gp120 binding to either CD4 or CXCR4 each fails to activate Akt in primary human CD4 T cells, similar to the previous reports that gp120 does not activate Akt in cell lines (29, 61). Recent studies indicate that the phosphorylation of BH₃-only proteins by Akt or by p38/JNK results in divergent effects on proapoptotic activity of these proteins. Although Akt-mediated phosphorylation of Bad at S112 and S136 (62–64), Bax at S184 (64), and Bim at S87 (62) inactivates these proteins by causing their retention in cytoplasm through association with 14-3-3 proteins (63), phosphorylation by JNK/p38 can increase the proapoptotic activity of Bax and Bim (65–67). Therefore, proapoptotic Bax, Bim, and Bad may represent the point in which Akt-mediated prosurvival and p38-mediated proapoptotic pathways converge. Which of the BH₃-only Bcl-2 family proteins is the target for p38 action following gp120 ligation to CD4 T cells is under current investigation.

Disclosures
The authors have no financial conflict of interest.

References


42. Chanh, T. C., G. R. Dressman, and R. C. Kennedy. 1997. Monoclonal anti-


44. Alvarado-Kristenson, M., F. Melander, K. Leanderson, L. Ronnstrand, C. Wernstedt, and T. Andersson. 2004. p38 MAPK signals survival by phos-


