HIV-1 Nef-induced FasL induction and bystander killing requires p38 MAPK activation

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The human immunodeficiency virus (HIV) has been reported to target noninfected CD4 and CD8 cells for destruction. This effect is manifested in part through up-regulation of the death receptor Fas ligand (FasL) by HIV-1 negative factor (Nef), leading to bystander damage. However, the signal transduction and transcriptional regulation of this process remains elusive. Here, we provide evidence that p38 mitogen–activated protein kinase (MAPK) is required for this process. Loss-of-function experiments through dominant-negative p38 isoform, p38 siRNA, and chemical inhibitors of p38 activation suggest that p38 is necessary for Nef-induced activator protein-1 (AP-1) activation, as inhibition leads to an attenuation of AP-1–dependent transcription. Furthermore, mutagenesis of the FasL promoter reveals that its AP-1 enhancer element is required for Nef-mediated transcriptional activation. Therefore, a linear pathway for Nef-induced FasL expression that encompasses p38 and AP-1 has been elucidated. Furthermore, chemical inhibition of the p38 pathway attenuates HIV-1–mediated bystander killing of CD8 cells in vitro. (Blood. 2005;106:2059-2068)

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Introduction

Human immunodeficiency virus (HIV) infection typically results in the eradication of the host’s immune system through eventual depletion of its CD4 cells. Although initially controlled, the virus persists and invariably replicates to high titers. In this regard, disregulated apoptosis is considered a major pathogenesis event leading to severe CD4 lymphopenia during HIV-1 infection. HIV-induced apoptosis of host cells has been reported to both involve and not involve the Fas/Fas ligand (FasL) apoptotic pathway. FasL is not present on resting T cells, but activated T cells may undergo apoptosis through the CD95/CD95 ligand (CD95L) pathway.

Specifically, negative factor (Nef) has been reported to induce apoptosis of bystander cells while protecting infected cells. Nef expression in T cells induces FasL expression on the infected cell. This proposed mechanism is also detected in lymph nodes of simian immunodeficiency virus (SIV)–infected monkeys and HIV-infected patients, further suggesting its importance. Concomitantly, Nef expression in T cells induces signals that protect the infected cell from the same Fas-mediated cell death via B-cell lymphoma 2 (Bcl-2)–antagonist of cell death (Bad) phosphorylation and apoptosis signal-regulating kinase 1 (ASK1) inhibition. Ideally, this would allow the virally infected cell to persist in the face of the host immune response by becoming resistant to apoptosis while provoking localized destruction of neighboring cells and effector T cells attempting to mediate viral factory clearance.

Materials and methods

Cell culture

Jurkat, 293T, and the monocyte line U937 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were passaged in RPMI-1640 or Dulbecco modified Eagle medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Cells were maintained at 37°C and 5% CO2. Leukopacks from individual donors were obtained from the CFAR clinical core facilities at the University of Pennsylvania (UPENN) School of Medicine to isolate T cells as well as monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density centrifugation and cultured as described previously. CD14 monocytes were purified from

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PBMCs by positive enrichment using autoMACS (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Monocytes were cultured in complete RPMI-1640 medium with 500 U/mL recombinant human interleukin-4 (rhIL-4) and 1000 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; both from R&D Systems, Minneapolis, MN) per 10^6 cells.4,12

Mitogen-activated protein kinase (MAPK) inhibitor and plasmids

The p38 inhibitors SB203580 and RWJ67657 have been previously described.13,14 The wild-type Nef (pNef) or pNef (PxxPxR) alanine substitution plasmids were amplified by single-round polymerase chain reaction (PCR) using Nef-specific primers or overlap extension PCR and subcloned into the pVax vector (Invivogen, Frederick, MD). The wild-type p38 MAP kinase, dominant-negative p38 (KM mutant; K to M mutation at the adenosine triphosphate (ATP)-binding site), was constructed.15 Human FasL (hFasL) reporter expression vector hFasL-Luc (1.2-kb FasL promoter frame (ORF) of the mRNA sequence. Next to the mRNA sequence, a 7-base loop. The second stretch of uppercase nucleotides is the reverse complement of the adenosine triphosphate [ATP]–binding site), was constructed.15 Human FasL (hFasL) reporter expression vector hFasL-Luc (1.2-kb FasL promoter frame (ORF) of the mRNA sequence. Next to the mRNA sequence, a 7-base loop. The second stretch of uppercase nucleotides is the reverse complement of the adenosine triphosphate [ATP]–binding site), was constructed.15 Human FasL (hFasL) reporter expression vector hFasL-Luc (1.2-kb FasL promoter frame (ORF) of the mRNA sequence. Next to the mRNA sequence, a 7-base loop. The second stretch of uppercase nucleotides is the reverse complement of the adenosine triphosphate [ATP]–binding site), was constructed.15 Human FasL (hFasL) reporter expression vector hFasL-Luc (1.2-kb FasL promoter frame (ORF) of the mRNA sequence. Next to the mRNA sequence, a 7-base loop. The second stretch of uppercase nucleotides is the reverse complement of

Transfection and luciferase reporter assay

Jurkat or U937 (2 x 10^6) cells were washed twice with phosphate-buffered saline (PBS), resuspended in 500 μL Opti-MEM (Gibco) culture medium, and transfected with LipofectAMINE Plus (Gibco). The plasmid pNef (5 μg), with a mixture of 2 μg cytomegalovirus (CMV) vector expressing green fluorescence protein (GFP), was transfected with the FuGENE 6 Reagent (Roche Applied Science, Indianapolis, IN). Cells were washed twice with complete RPMI-1640 and incubated for 48 hours. Transfection efficiencies were assessed by fluorescence-activated cell sorter (FACS) analyses of GFP expression. For determination of AP-1 and Fasl reporter assay, 2.5 μg reporter plasmid (pAP-Luc or hFas-Luc) alone or with pNef (5 μg), mutated pNef with or without inhibitors (1 μM), pNef or pNef plus p38 dominant-negative construct (5 μg) were added to the cells and mixed well as indicated. Electroporation was carried out at 250 V and 960 μF in a Bio-Rad Gene Pulse II (Bio-Rad, Hercules, CA).16 Cells were grown in complete RPMI 1640 medium for 48 hours at 37°C.

Total amounts of DNA and equal molar ratios of promoters were kept constant in all setups by using empty vectors.16 Because of differences in transfection efficiencies, an expression plasmid pCMV β-galactosidase (β-gal) was cotransfected as a transfection efficiency control, and luciferase activities were normalized based on β-gal activity with the β-gal reporter gene assay.16 Cells were harvested, washed 3 times with PBS, and lysed in 100 μL reporter lysis buffer (RBL) according to the manufacturer’s instructions (Roche Applied Science). Cell debris was removed by centrifugation, and the supernatant was used in the luciferase assay using LUMAT-LB9501 (Berthold, Bad Wildbad, Germany).16

HIV-1 pseudotype viruses and infection

The HIV-1 proviral infectious constructs pNL4-3/HSA, pNL4-3/HSA/ΔEnv, or pNL4-3/HSA/ΔEnv/ΔNef and primary clade specific isolates (subtype A-94UG103, subtype B-92US723, subtype C-96NUSG31, and subtype D-92UG001) were obtained through the AIDS Research and Reference Reagent (RRe)-Program, National Institute of Allergy and Infectious Diseases (NIAID), NIH.13 Constructs containing ΔNef were generated by recombinant PCR mutagenesis.15 HIV-1 viral particles were generated by transfection with pNL4-3/HSA alone or cotransfection of pNL4-3/HSA/ΔEnv or pNL4-3/HSA/ΔEnv/ΔNef with vesicular stomatitis virus G (VSV-G) envelope18 by FuGENE 6 transfection in 293T cells. Infection was carried out by incubating the cells with HIV-1 virus at a concentration of 100 tissue-culture infective dose (TCID50)10^6 cells per milliliter. These retroviruses encode as a specific cell-surface marker, the murine CD24 antigen (heat-stable antigen [HSA]), which allows for identification of infected target cells by FACS analysis.12,18 Culture supernatant was collected at 6-, 12-, and 24-hour intervals and assayed for virus production by measuring p24 antigen by enzyme-linked immunosorbent assay (ELISA) kit (Beckman Coulter, Fullerton, CA). Data are presented as mean plus/minus SEM.

Flow cytometry

Cells suspensions (10^6) were washed in PBS (pH 7.2) containing 0.2% bovine serum albumin and 0.1% NaN3. Cells were stained with 1 to 2 μg of the following monocolonal antibodies (mAb): CD3 fluorescein isothiocyanate (FITC) mouse immunoglobulin G2a (IgG2a), CD4 phycoerythrin (PE) mouse IgG1, CD8 FITC mouse IgG1, CD14 FITC and PE mouse IgG2a (PharMingen, San Diego, CA), and Fasl (NOK-1) mouse IgG1 (eBioScience, San Diego, CA). For intracellular p24 expression, cells were fixed in 1% paraformaldehyde containing 20 μg/mL lyssolecithin (Sigma, St. Louis, MO) for 5 minutes at 4°C. Samples were then washed, permeabilized in 2 mL ice-cold methanol while vortexing, placed on ice for 15 minutes, washed again, and resuspended in 1 mL PBS/0.1% nonidet P-40 (NP-40; Sigma) for 5 minutes at 4°C. Cells were then washed and incubated with 1 μg PE-conjugated K57-7D1 (Coulter) for 15 minutes at 4°C. Cells were washed with PBS and reconstituted in 200 μL PBS and analyzed directly on a Coulter EPICS Flow Cytometer (Coulter) using FlowJo software (TreeStar, San Carlos, CA). All samples were compared with their isotype-matched controls. In the case of dual flow cytometry, individual samples treated with each isotype were used to determine the background levels of autofluorescence.12

Apoptosis, caspase, and Fasl ELISA assays

FACS analysis was performed to identify cells undergoing apoptosis.4 Apoptosis was evaluated by using an annexin-V assay kit (PharMingen) and analyzed directly on a Coulter EPICS Flow Cytometer (Coulter) using FlowJo software. Caspase-3 activity was determined using Caspase-3/CPP32 colorimetric protease assay kit according to the manufacturer’s instructions (MBL, Watertown, MA). Fasl in the supernatant or lysates was quantified by sandwich ELISA with commercial ELISA (R&D Systems) according to the manufacturer’s instructions.

Protein extraction and Western blotting

Cells were washed with ice-cold PBS and lysed with protein lysis buffer (20 mM Tris [tris(hydroxymethyl)aminomethane, pH 7.4], 150 mM NaCl,
were added as described. After 14 hours of incubation of monocytes plus T cells, was added for 90 minutes, before 0.5 OR was added to groups II, III, and IV for 3 hours at 37°C. Anti-FasL mAb was used for the CD8/annexin-V-positive cells (CD8 T cells). The analysis was performed on gated low forward scatter and CD8 T cells and further analyzed for the CD8/annexin-V-positive cells (CD8 T cell population).

**Results**

**HIV infection stimulates p38 phosphorylation**

Activation of p38 kinase following HIV infection in some target cells has been previously demonstrated. Activation was not a result of virus binding to the cell surface but apparently occurs after virus internalization. Western blot analysis was carried out using antibodies specific for either the Thr180 and Thr182 phosphorylated forms of p38 or controls (Figure 1A). We observed that p38 was rapidly phosphorylated following HIV infection in either the cell line or in primary PBMCs, confirming the activation of p38 signaling during HIV infection.

**HIV-1–induced apoptosis is blocked by p38 MAPK inhibitors**

Studies support that active host cell signaling is involved in the induction of apoptosis. A host cell–signaling pathway that may have relevance for bystander apoptosis is the p38 MAP kinase pathway. Genetic studies conducted in mice suggest that MAP kinase kinase 3 (MKK3), an upstream kinase of p38, is required for activation-induced cell death of T cells. Therefore, we examined the role of the p38 pathway in HIV-1–induced apoptosis. Human

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**Figure 1.** p38 MAPK activation is required for HIV-1–related apoptosis. (A) p38 MAPK is activated by HIV-1 infection. Immuno blot analysis of protein extracted from PBMCs or Jurkat T cells infected with mock or NL-4-3 virus. Total cellular protein extract (50 μg) was extracted 24 hours after infection and analyzed by 12% SDS-PAGE. p38 and phospho-p38 MAPK activation was detected by using specific antibodies that recognize phospho-p38 MAPK (top) only when phosphorylated or the p38 MAPK (middle). HIV infection strongly activates phosphorylation in both cell types. The same lysates were blotted with anti-p24 gag (bottom) to monitor viral infection. (B-C) HIV-1–induced apoptosis is blocked by p38 MAPK inhibitor. Human PBMCs were infected with (B) NL-4-3 Wt virus or (C) different HIV-clade specific primary viral isolates. Cells were uninfected (mock) or infected with HIV-1 virus with or without addition of 1 μM p38 inhibitor-I or -II (SB203580 or RWJ67657, respectively). Cells were collected 2 days after infection and stained with annexin-V–FITC, p24 gag–PE and HIV-induced apoptosis analysis was performed. The values indicated frequencies of positive cells of each quadrant. Data were representative of 3 (B) or 2 (C) independent experiments. Both p38 inhibitors block HIV-driven apoptosis. (D) p38 MAP kinase inhibitors block HIV-induced caspase-3 activity. Cell lysates were prepared from the groups described in panel B. Total protein from each cell lysate (100 μg) was used for the colorimetric caspase assay as described in “Materials and methods.” Each column represents the mean ± standard deviation from triplicate samples derived from 1 of 3 independent experiments. All these experiments gave similar results.
PBMCs were infected with NL4-3 virus at a concentration of 100 TCID$_{50}$/10$^6$ cells per milliliter and tested for apoptosis under various conditions, including in the presence or absence of known specific p38 inhibitors designated as inhibitor-I or inhibitor-II (SB203580 or RWJ67657, respectively) at 1 μM as described. Cells were collected 2 days after infection from each treatment group and analyzed for apoptosis induction in HIV-infected cells. HIV-1 infection promoted significant apoptosis in target cells (Figure 1B). Of importance, both p38 inhibitors could block HIV-driven apoptosis. Both inhibitors inhibited apoptosis driven by virus more than 70% at 1-μM concentration. However, p38 inhibitor-II exhibited a greater antiapoptotic potency. To confirm this observation on primary viral isolates, we analyzed the induction of apoptosis by 4 different subtype-divergent HIV viral isolates (Figure 1C). All 4 primary subtype-divergent viruses induced strong and clearly detectable levels of target cell apoptosis in this infection system. Again, both inhibitors were effective at inhibiting apoptosis. We wanted to ensure that the observed apoptosis was also activating caspases. Accordingly, we chose the caspase-3 assay as a downstream marker for all caspase-induced apoptosis, and caspase-3 activation was attenuated by pharmacologic inhibition of p38 with 70% potency with a 1-μM concentration of inhibitor-II (Figure 1D). These results support an essential role for the p38 MAPK pathway in apoptosis induction by diverse isolates of HIV-1 in PBMCs.

HIV-1–mediated FasL up-regulation requires p38 signaling

Previous studies indicate that HIV-infected cells can up-regulate FasL expression and selectively induce the apoptosis of FasL-susceptible T cells from HIV-positive individuals. Recent reports have identified that up-regulation of FasL on HIV-infected cells can be induced by Nef. However, the signaling pathways that are required for this activity remain unclear. Based on the inhibition of apoptosis by p38 inhibitors observed and previous studies implicating Fas/FasL signaling in HIV-induced apoptosis, we decided to examine the relevance of the p38 pathway to HIV-induced FasL expression. We first sought to confirm the induction of FasL by HIV infection. Strong induction of FasL was observed in HIV infection in PBMCs (Figure 2A). Further FasL expression induction was also confirmed using FasL-specific antibody by immunoblot analysis using the infected samples (Figure 2B). This result confirms a relationship between the FasL induction and HIV infection. Next, PBMCs or Jurkat cells were infected with mock or NL4-3 virus in the presence or absence of p38 inhibitor-II (RWJ67657), and FasL expression was analyzed by FACS using FasL-specific antibody. We observed strong inhibition of FasL expression induced by the pharmacologic blockade of p38 signaling (Figure 2C-D). This inhibition appears independent of the cell type used in the experiment. Further, the up-regulation of FasL by individual HIV genes suggests that Nef but not Tat, Vpr, Vpu, or Env is sufficient to activate high-level FasL expression in Jurkat and the monocytic U937 cells (data not shown). This effect was dosage dependent with saturating effects observed at 1 μM.

p38 MAPK is necessary for FasL expression by HIV-1 Nef

To investigate the requirement of p38 activity in Nef-induced FasL expression, we first sought to demonstrate that Nef transfection is sufficient to activate p38 and that this activation can be reversed by a p38 inhibitor. Nef induced a strong increase in phosphorylation of p38 above basal levels (Figure 3A). In contrast, there was no detectable increase in the phosphorylation of p38 in Nef-treated cells in the presence of p38 inhibitor. These data indicate that Nef can induce phosphorylation of the p38 MAP kinase and also that Nef is an activator of this pathway. Next, we studied the p38 requirement for FasL transcription in a promoter-specific fashion. Jurkat cells were transfected with pNef in the presence of cotransfected wild-type p38 (Figure 3B) or dominant-negative p38 constructs (Figure 3C), and activation of a FasL promoter–driven luciferase construct was assayed. The dominant-negative p38 contains a K-to-M mutation at the ATP-binding site, and this molecule has been previously reported to block p38 activity in vitro by protein interference. Nef strongly induced FasL promoter activation in the presence of native p38 (Figure 3B). In contrast, induction of FasL transcription was reduced in cells cotransfected with this dominant-negative mutant p38 and the Nef construct (Figure 3C). To test directly if p38 is required to activate Nef-mediated FasL expression, we sought to develop specific siRNAs that target p38. We targeted the p38-α isoform, which is the dominant isoform expressed in immune cells. These siRNAs...
results were obtained in 3 independent experiments. Transfection efficiency was monitored by cotransfection of a pCMV plasmid encoding GFP, which also served as a marker.

Filled histograms show the FasL expression, and open histograms represent isotype-matched control antibodies. p38 siRNA inhibited Nef-induced FasL induction. Similar expression was suppressed significantly in cells transfected with clone p38-61 and moderately in cells transfected with the other clones compared with control vector.

lysates were extracted 48 hours after transfection, and immunoblotting was performed by using an anti-p38 antibody and an actin antibody as a control for equal loading. p38 expression was suppressed significantly in cells transfected with clone p38-61 and moderately in cells transfected with the other clones compared with control vector transfected.

Jurkat cells were electroporated with 5 μg vector control or p38 siRNA control vector or p38siRNA expression constructs as indicated clones. Cell lysates were extracted 48 hours after transfection, and immunoblotting was performed by using an anti-p38 antibody and an actin antibody as a control for equal loading. p38 expression was suppressed significantly in cells transfected with clone p38-61 and moderately in cells transfected with the other clones compared with control vector transfected.

were studied for their ability to suppress the expression of p38. Four different sets of p38 siRNA oligos were examined. Jurkat cells that were transfected with siRNA vector clone p38-61 demonstrated a dramatic reduction of p38 expression, while the other clones, p38-352, p38-775, and p38-1016, showed only moderate decreases in p38 expression (Figure 3D). Next, Jurkat cells were cotransfected with pNef and siRNA (clone p38-61) and pNef plasmids as indicated. At 48 hours after transfection, the surface levels of FasL expression were determined by flow cytometry using a FasL-specific antibody. Filled histograms show the FasL expression, and open histograms represent isotype-matched control antibodies. p38 siRNA inhibited Nef-induced FasL induction. Similar results were obtained in 3 independent experiments. Transfection efficiency was monitored by cotransfection of a plasmid encoding β-gal and results were normalized to β-gal levels. Similar results were obtained in 3 independent experiments and were reproducible. Nef strongly induced the FasL promoter activation (B). In contrast, induction of FasL transcriptions was reduced in cells cotransfected with dominant-negative mutant p38 (C). (D) Construction and expression of p38 siRNAs. Four different target siRNA expression vectors were constructed as described in “Materials and methods.” Jurkat T cells were transiently transfected with 5 μg siRNA control vector or p38 siRNA expression constructs as indicated clones. Cell lysates were extracted 48 hours after transfection, and immunoblotting was performed by using an anti-p38 antibody and an actin antibody as a control for equal loading. p38 expression was suppressed significantly in cells transfected with clone p38-61 and moderately in cells transfected with the other clones compared with control vector transfected.

(E) FasL expression is significantly reduced by p38 siRNA. Jurkat T cells were electroporated with 5 μg plasmid expressing vector, pNef, or p38 siRNA (clone p38-61) and pNef plasmids as indicated. At 48 hours after transfection, the surface levels of FasL expression were determined by flow cytometry using a FasL-specific antibody. Filled histograms show the FasL expression, and open histograms represent isotype-matched control antibodies. p38 siRNA inhibited Nef-induced FasL induction. Similar results were obtained in 3 independent experiments. Transfection efficiency was monitored by cotransfection of a plasmid encoding GFP, which also served as a marker for gating on transfected cells. (F) p38 siRNA does not affect Nef expression. Total protein extracts were prepared from the groups transfected with vector, pNef, or p38 siRNA plus pNef. Of each protein sample, 50 μg was separated on 12% SDS-PAGE and analyzed by Western blot with a polyclonal antibody against Nef antibody. Immunoblotting was also performed using an antiactin antibody as an internal control.

**HIV-1 Nef-deleted virus fails to induce FasL on human PBMCs**

Next, we wanted to ascertain if p38 is also necessary for FasL induction in HIV-1 infection. Accordingly, we constructed a virus that was devoid of Nef expression to ascertain if there was an effect on FasL in the absence of Nef. Nef-deleted virus was constructed by introducing a frameshift mutation in the Nef ORF, thus eliminating Nef from this virus, and confirmed via proviral transfection into 293T cells via ELISA and Western blotting (Figure 4A-B). To analyze the effect of viral delivery of the nef gene on FasL expression, we used a pseudoviral infection assay for infecting PBMCs with the NL4-3 Wt or NL4-3 Δ Nef–deleted virus in the presence or absence of p38 inhibitor at 1 μM. Flow cytometry analysis of intracytoplasmic p24Gag and FasL levels was determined 4 days after infection, allowing us to follow both markers in infected cells (Figure 4C). A direct correlation was observed between the presence of the nef gene and the ability of the virus to induce FasL expression. The percentage of positive cells is depicted in the upper-right quadrant for each group. Approximately half of the infected (p24Gag positive) cells were FasL positive day 4. The FasL-positive cells constitute a unique separate population of cells that was observed only in the Nef-containing virus infection group. Of interest, viral Nef–mediated FasL expression was suppressed by treatment of the culture with 1 μM p38 inhibitor. These results in conjunction with Figure 3 suggest that Nef is critical for HIV-1–mediated FasL up-regulation. Furthermore, Nef-deleted viruses exhibit strong attenuation of p38 activation (Figure 4D). A modest activation of p38 was also observed in
Nef-deleted viral infections, which could be due to influence from other HIV antigens, including the envelope antigen.24,25 These results extend the association between Nef, FasL induction, and the p38 pathway within an infection setting.

p38-mediated AP-1 activation is required for Nef-induced FasL transcription

AP-1 is an important transcription factor in immune activation and is induced by many stimuli, including growth factors, cytokines, T-cell activators, neurotransmitters, and ultraviolet (UV) irradiation.16,26,27 Eukaryotic cells respond to external stresses and inflammatory factors through the activation of MAPKs, leading to altered transcriptional activity. Specifically, c-Jun N-terminal kinases (JNKs) and p38 MAPK have been implicated in these responses. In most cases, p38 is activated by MKKs 3 and 6 through their phosphorylation.28,29 Of interest, MAPK-activated protein kinases (JNKs) and p38 MAPK have been implicated in these responses. In most cases, p38 is activated by MKKs 3 and 6 through their phosphorylation.28,29 Of interest, MAPK-activated protein kinases (JNKs) and p38 MAPK have been implicated in these responses. In most cases, p38 is activated by MKKs 3 and 6 through their phosphorylation.28,29 Of interest, MAPK-activated protein kinases (JNKs) and p38 MAPK have been implicated in these responses. In most cases, p38 is activated by MKKs 3 and 6 through their phosphorylation.28,29 Of interest, MAPK-activated protein kinases (JNKs) and p38 MAPK have been implicated in these responses. In most cases, p38 is activated by MKKs 3 and 6 through their phosphorylation.28,29

As discussed in the section entitled “p38 MAPK is necessary for FasL expression by HIV-1 Nef,” Nef-induced p38 activation is necessary for the up-regulation of FasL induction in both an infection and a transfection setting. However, the requirement of the AP-1 binding enhancer element for FasL transcriptional activation was unclear, particularly since multiple elements have been proposed to drive FasL induction, including nuclear factor of activated T cell (NF-AT), nuclear factor κB (NF-κB), AP-1, and c-Myc.16,28,30

Accordingly, transfection of Jurkat cells with an AP-1 promoter reporter plasmid in conjunction with Nef induced strong AP-1–dependent transcription of FasL via AP-1.16,28,30 Accordingly, we hypothesized that Nef may exploit this host pathway for inducing FasL during HIV infection.

As discussed in the section entitled “p38 MAPK is necessary for FasL expression by HIV-1 Nef,” Nef-induced p38 activation is necessary for the up-regulation of FasL induction in both an infection and a transfection setting. However, the requirement of the AP-1 binding enhancer element for FasL transcriptional activation was unclear, particularly since multiple elements have been proposed to drive FasL induction, including nuclear factor of activated T cell (NF-AT), nuclear factor κB (NF-κB), AP-1, and c-Myc.16,28,30

Accordingly, transfection of Jurkat cells with an AP-1 promoter reporter plasmid in conjunction with Nef induced strong AP-1–dependent transcription in T cells and was sensitive to p38 inhibitors at 1-μM concentration. This suggests that Nef requires p38 to activate the transcription induced by the AP-1 enhancer elements (Figure 5A). To test whether these observations were related, we mutated the AP-1 enhancer element from the hFasL promoter plasmid by site-directed mutagenesis.16 Cotransfection with Nef failed to activate the AP-1mut promoter, indicating that the activation of AP-1 transcriptional factors by Nef is required for FasL transcription (Figure 5B). However, transfection of Jurkat cells with the hFasL, AP-1mut expression vector in conjunction with pRelA (a subunit of NF-κB) did induce FasL transcription (Figure 5C), indicating that mutation of AP-1 sites does not affect the ability of other transcriptional factors to induce FasL transcription. Transfection of the AP-1 reporter plasmid into Jurkat cells and subsequent infection with NL4-3 WT or NL4-3 ΔNef virus suggest that Nef is necessary for optimal AP-1–dependent transcription induced by HIV-1 (Figure 5D). Collectively, these data show that Nef-induced FasL expression requires p38, which drives the FasL promoter through AP-1 induction.

The PxxP domain of Nef is required for FasL transcription and p38 activation

Previous experiments suggest that Nef possesses various protein-binding domains responsible for its activity.31 Of particular interest is the PxxP motif in Nef, which is a binding site for Src homology 3 (SH3)–mediated protein–protein interactions. We next examined if the PxxP region of Nef is required for phosphorylation of p38 in T cell- or in monocyte cell lines by mutating prolines to alanines, which retain Nef structure but block PxxP–associated activity.10,31-34 The PxxP mutation did not stimulate phosphorylation of p21-related kinase family (Pak) in T cells or hematopoietic cell kinase (Hck) phosphorylation in monocytic cell lines (data not shown). Next, Jurkat T cells and monocyte U937 cell lines were transfected with expression vectors for pNef or pNefPxxP. The Nef containing the PxxP deletion did not stimulate phosphorylation of p38 in either T cells or monocytes (Figure 6A). These data suggest that Nef activation of p38 requires the PxxP domain of Nef.

We next examined FasL induction in T cells or monocytes...
transfected with pNef in the presence or absence of p38 inhibitor or PxxP-mutated Nef. While Nef strongly induced FasL on both T cells as well as monocytes (Figure 6B), mutation of the PxxP domain severely diminished FasL induction, indicating that the PxxP domain of Nef plays an essential role in Nef-driven FasL expression. These results were verified by FasL promoter reporter assays (Figure 6C-D). Collectively, these results illustrate that Nef activates FasL through its PxxP domain in monocytes and T cells. Furthermore, this activation appears to be upstream of Pak activation in T cells and of Hck activation in monocytes (data not shown). This activation in either cell phenotype eventually converges at p38, resulting in its activation, which is required for Nef-mediated AP-1 induction of FasL expression.

Inhibition of p38 is sufficient to prevent HIV-1–induced bystander killing of CD8 T cells

FasL is up-regulated in HIV-infected macrophages and T cells. Such FasL-positive cells have been hypothesized to be able to induce Fas-mediated apoptosis of CD8 effector T cells.3-5 Accumulating evidence indicates that antigen-presenting cells such as macrophages play a key role in the elimination of activated effector T cells.3,4,35 Accordingly, we next investigated the role of p38 activation of HIV-infected macrophages to induce bystander apoptosis of CD8 T cells.

CD14 and CD8 cell populations were isolated from HIV-1–negative PBMCs (Figure 7Ai) by cell-negative immune selection.

Figure 6. PxxP domain of Nef is required for the FasL induction. (A) Loss of the PxxP domain of Nef significantly diminishes the phosphorylation of p38. Western blot analysis of extracts derived from Jurkat or U937 cells transfected with either the expression vector for wild-type Nef or the amino acid–mutated Nef. Cell extracts were prepared 48 hours after transfection as described in “Materials and methods” and subjected to 12% SDS-PAGE followed by PVDF membrane transfer and analyzed by Western blotting using specific p38 and phospho-p38 antibodies as indicated. Note, the amino acid–mutated Nef (pNef(PxxP)) failed to induce phosphorylation of p38. (B) Comparison of FasL induction by wild-type Nef versus amino acid–mutated Nef. Jurkat T cells or U937 cells were electroporated with 5 μg wild-type Nef, amino acid–mutated Nef, or wild-type Nef plus p38 inhibitor (1 μM). At 48 hours after transfection, the surface levels of FasL were determined by flow cytometry by staining with a FasL–specific antibody. Transfection efficiency was monitored by cotransfection of a pCMV plasmid encoding GFP. Thick line histograms show the indicated surface markers, and filled histograms represent the isotype-matched control antibodies. Similar results were obtained in 3 independent experiments. (C-D) The PxxP domain of Nef (amino-acid mutated) is essential for induction of FasL promoter activity in T cell or monocytic cells. Jurkat T cells or U937 cells were transiently transfected with 5 μg hFasL–Luc promoter plasmid, wild-type Nef plus hFasL–Luc promoter plasmid, or amino acid–mutated Nef plus hFasL–Luc promoter plasmid as indicated. Luciferase activity in whole-cell lysates was assayed after 12 to 18 hours and is shown as the mean value ± SEM. Transfection efficiency was monitored by cotransfection of a plasmid encoding β-gal and results were normalized to β-gal levels. Similar results were obtained in 3 independent experiments.
We then examined CD8 T-cell apoptosis after 12 hours of incubation with CD14 macrophages that were infected with the NL4-3 pseudovirus, which encoded VSV-G envelope, which had been activated through treatment with latex beads to stimulate phagocytosis. Annexin-V staining on the gated CD8 cells shows that 22.1% of the cells were induced to undergo apoptosis (Figure 7Aii). Uninfected macrophages, even when activated by polystyrene beads, induced a negligible level of apoptosis in the CD8 T-cell population under any of the conditions tested (Figure 7B, top panels). Also, the ΔNef virus did not induce bystander killing under these conditions (Figure 7B, middle panels). In contrast, HIV-infected macrophages stimulated with latex beads drove apoptosis in the CD8 T cells (Figure 7B, bottom panels). The addition of a neutralizing anti-FasL mAb to the cell culture completely blocked the induction of apoptosis, suggesting its required role. Of interest, p38 blockade was almost as effective as anti-FasL antibody at preventing apoptosis, but there was clearly a residual level of apoptosis that was resistant to the inhibitor. This is consistent with prior published reports suggesting that other HIV antigens through p38-independent mechanisms can play a role in bystander apoptosis. These findings clearly illustrate that HIV-infected macrophages can induce Fas/FasL-mediated apoptosis of CD8 T cells during HIV infection, and inhibition of p38 is sufficient to prevent much of the resulting bystander apoptosis.

Discussion

Hallmarks of HIV-1 infection include the destruction of T cells and the suppression of cellular immune responses in vivo. A mechanism that has been previously reported is through the bystander killing of CD8 effector T cells, the cell population directly responsible for immune clearance and controlling viral load. Indirect cell killing has been proposed to involve the up-regulation of FasL-inducing apoptosis of effector cytotoxic T lymphocytes (CTLs) as they approach viral-harboring CD4 T cells and macrophages. Furthermore, lamina propria of SIV-infected rhesus macaques has been shown to harbor massive apoptosis of viral-specific memory T cells through the activation of the Fas-FasL pathway. Hence this strategy results in a significant advantage for HIV in evasion of immune recognition and viral clearance.

A recent report identified that Nef binds to the CD3ξ chain of the TCR complex, and this interaction is important for the induction of FasL expression. The effect required the PxxP domain, which consequently stimulated the transcriptional activation of FasL. Additional evidence suggests that Nef coprecipitates with the Nef-associated kinase (NAK), a member of the Pak in T cells. NAK is activated via the small guanosine triphosphatases
(GTPases) CDC42 and Rac1 through Vav, and moreover, Pak1 and Pak2 are implicated in this activation. 11,12 Although it is believed that activation of FasL transcription functions through the TCR-CD3 complex mediated by the CD3 ε chain, 12,38,40 the downstream signals and their transcriptional regulation required for this activation have not been determined.

The results presented here indicate that p38 MAPK activation is necessary for T-cell and macrophage activation and eventual FasL transcription in various viral subtypes. Previous work suggests that multiple factors may be sufficient to induce FasL expression. 16,26,27 Accordingly, we suggest that the AP-1 enhancer is required for Nef to induce FasL transcription. Further, p38 is also required for AP-1 activation, suggesting a linear pathway that involves p38 activation and its subsequent AP-1 activation. Recently, Biggs et al.11 identified that AP-1 can be induced by Nef via extracellular signal-related kinase 1/2 (ERK1/2) MAPK signaling events. Our results suggest that p38-like ERK1/2 is required for AP-1 activation, and that this leads to a transcriptional up-regulation of FasL. Our evidence suggests that p38 MAPK is important for FasL-mediated killing and specifically targets AP-1 transcriptional factors for this effect. Thus our data extend these recent observations and link the AP-1 pathway with p38 activation and bystander killing.

Previous studies indicate that HIV infection of macrophages can induce FasL expression, which drives bystander killing of CD4+ T cells. 4,11 Additionally, macrophages also drive FasL up-regulation to induce bystander killing of HIV-specific CD8+ T cells. 33 The destruction of the CD8 T cells by the FasL pathway is likely a significant damper for the cell-mediated immune response, which ultimately could limit immune clearance. 4,5,7 We have also observed blockade of apoptosis using primary viral isolates.

Our results suggest that Nef is critical for efficient p38 activation and AP-1–driven transcription induced by HIV-1 infection. However, residual p38 phosphorylation and AP-1–mediated transcription can be observed in Nef-deleted viruses (Figure 5D), suggesting that other factors such as Env or other HIV accessory genes may also be capable of activating p38 and AP-1–driven transcription. 24,25 On the other hand, Env is neither required nor sufficient to up-regulate FasL (data not shown), suggesting that either the inputs from Env are not sufficiently robust to drive FasL transcription or Nef is sufficient to activate another undetermined pathway that is concomitantly required with p38 to drive FasL transcription (data not shown), and overexpression fails to augment Nef-induced FasL transcription (Figure 3B).

In conclusion, our work identifies a linear signaling pathway that is necessary for HIV-1 Nef-induced bystander killing. We show that Nef activates and requires p38 MAPK activation and its AP-1 enhancer target within the FasL promoter to stimulate its transcription. Through loss-of-function experiments, we were able to delineate which factors are activated versus required for regulating FasL transcription. We suggest that this finding has important applications for the development of novel HIV therapeutics, as anti-p38 compounds could target a vital pathogenic function of HIV-1.

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References

6. Gandhi RT, Chen BK, Straus SE, Dale JK, Leonard JM, Baltimore D. HIV-1 directly kills CD4+ T cells. 4,11 Additionally, macrophages also drive FasL up-regulation to induce bystander killing of HIV-specific CD8+ T cells. 33 The destruction of the CD8 T cells by the FasL pathway is likely a significant damper for the cell-mediated immune response, which ultimately could limit immune clearance. 4,5,7 We have also observed blockade of apoptosis using primary viral isolates.

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HIV-1 Nef-induced FasL induction and bystander killing requires p38 MAPK activation

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