HIV-1 Nef Promotes Survival of TF-1 Macrophages by Inducing Bcl-XL Expression in an Extracellular Signal-regulated Kinase-dependent Manner*

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The Nef protein of human immunodeficiency virus-1 (HIV-1) is essential for the progression from human and simian immunodeficiency virus infection to full-blown AIDS. Recent studies indicate that Nef generates anti-apoptotic signals in HIV-infected T cells, suppressing cell death early in infection to allow productive viral replication. Previous work from our laboratory has shown that Nef also promotes proliferation of myeloid cells through a signal transducer and activator of transcription 3-dependent pathway. Here we demonstrate that Nef suppresses cell death induced by cytokine deprivation in the human macrophage precursor cell line, TF-1. Nef selectively induced up-regulation of Bcl-XL, an anti-apoptotic gene that is also regulated by granulocyte/macrophage-colony stimulating factor in this cell line. Activation of the extracellular signal-regulated kinase (Erk) mitogen-activated protein kinase pathway also correlated with the survival of TF-1/Nef cells. Using the selective mitogen-activated protein kinase kinase inhibitor PD98059, we found that Nef-induced Erk signaling is essential for Bcl-XL up-regulation and cell survival. In contrast, expression of Bcl-XL and TF-1 survival was not affected by dominant-negative signal transducer and activator of transcription 3. These data suggest that Nef produces survival signals in myeloid cells through Erk-mediated Bcl-XL induction, a pathway distinct from Nef survival pathways recently reported in T lymphocytes.

Nef is an accessory protein unique to simian and human immunodeficiency viruses (HIV) and is an essential AIDS progression factor (1, 2). Nef plays a crucial role in the maintenance of high viral loads and subsequent development of AIDS-like disease in Rhesus monkeys infected with simian immunodeficiency virus (3). Similarly, long-term survivors of AIDS are often infected with Nef-defective HIV strains (4, 5).

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1 The abbreviations used are: HIV, human immunodeficiency virus; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; AP, activator protein; Stat, signal transducer and activator of transcription; PI3K, phosphatidylinositol 3’-kinase; Mek, MAPK kinase; GM-CSF, granulocyte/macrophage-colony stimulating factor; ER, estrogen receptor; 4-HT, 4-hydroxytamoxifen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SIE, sis-inducible element.

Recent development of a transgenic mouse model for AIDS demonstrated that expression of Nef alone is sufficient to cause AIDS-like pathology (6, 7). Although these and other studies implicate Nef as a major progression factor in AIDS, the mechanism by which it contributes to HIV replication and pathogenesis is still unclear.

Nef is a 25–30 kDa cytosolic protein with a N-terminus myristoylation signal and no known catalytic activity. Instead, Nef associates with diverse cellular proteins and modifies their functions in host cells (1, 8). Nef down-modulates cell surface CD4 and major histocompatibility complex I receptors, which contributes to evasion of host immune surveillance. Protein kinases and other signaling molecules have also been found to interact with Nef. For example, Nef binds directly to multiple Src family kinases, including Hck, Lck, and Fyn (8, 9). Genetic evidence suggests that Nef-Hck interaction may be essential for AIDS progression (7). Nef also interacts with several Ser/Thr kinases, including p21-activated protein kinase, protein kinase C, and the Erk mitogen-activated protein kinases (MAPKs) (8, 9). Nef was shown to increase Erk1/2 activity initiated by T-cell receptor stimulation in primary CD4+ T cells obtained from peripheral blood (10). Enhanced Erk activity may be responsible for increased T-cell activation by Nef, which facilitates HIV replication in this target cell type. Erk is also involved in induction of the activator protein-1 (AP-1) transcription factor by Nef in macrophages, another major target cell for HIV infection (11). The induction of AP-1 may contribute to the activated phenotype of Nef-transfected and HIV-1-infected macrophages.

Several recent reports have suggested that Nef plays a role in longevity of HIV-infected T cells by affecting mediators of apoptosis. Death receptor-mediated apoptosis, which is critical to the host cell immune reaction, is blocked by Nef through apoptosis signal regulating kinase 1 inhibition (12). In addition, Nef suppresses T-cell apoptosis initiated by serum starvation or HIV replication. In this case, Nef was shown to induce serine phosphorylation of Bad, the mitochondrial pro-apoptotic mediator, through a previously described p21-activated protein kinase known to associate with Nef (13).

Recent work from our laboratory has shown that Nef promotes cytokine-independent proliferation of the macrophage precursor cell line, TF-1, through a mechanism that requires the signal transducer and activator of transcription 3 (Stat3) transcription factor (14). In the present report, we show that Nef suppresses apoptosis in this cell line by selectively up-regulating the mitochondrial anti-apoptosis gene, Bcl-XL (15). Experiments with pharmacological inhibitors indicate that cell survival and Bcl-XL induction by Nef are dependent on the Erk MAPK pathway but independent of phosphatidylinositol 3’-kinase (PI3K) and Akt activation. Surprisingly, dominant-neg-
ative Stat3 did not impact Nef-induced cell survival or Bcl-XL induction, despite the identification of Bcl-XL as a Stat3 target gene in other systems (16, 17). These results show, for the first time, that Nef generates anti-apoptotic signals in cells of the myelomonocytic lineage, through a pathway distinct from that observed in T cells.

**MATERIALS AND METHODS**

**Materials**—The anti-Nef antibody (EH-1, mouse monoclonal) was generously provided by the NIH AIDS Research and Reference Reagent Program. Anti-Erk1/2 and anti-phospho-Erk antibodies were purchased from Santa Cruz Biotechnology. The anti-Stat3, anti-Akt, and phospho-Akt antibodies were obtained from Cellular Signaling. The anti-phosphoStat3 antibody to p-Tyr-705 was purchased from Upstate Biotechnology. The MAPK kinase (Mek)-specific inhibitor, PD98059, and the PI3K inhibitor, LY294002, were purchased from Calbiochem.

**Cell Culture**—The human myeloid leukemia cell line TF-1 (18) was obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 50 µg/ml gentamicin, and 1 ng/ml of human granulocyte/macrophage-colony stimulating factor (GM-CSF) (Calbiochem).

Nef, Nef-ER, and Stat3YF Expression Constructs—To create the Nef-estrogen receptor fusion construct, the coding sequence of HIV-1 Nef (SF2 strain) was amplified by polymerase chain reaction and subcloned into the mammalian expression vector, pCDNA3.1 (InVitrogen). The coding sequence of the murine ER ligand-binding domain (amino acids 281 to 599) was amplified by polymerase chain reaction from the genomic DNA of MLY294002 and subcloned downstream and in-frame of the Nef C terminus. The resulting constructs were used to generate pCDNA3.1-Nef-ER. Nef and Nef-ER constructs were transfected into the human myelogenic cell line TF-1, and the resulting constructs were used to generate pCDNA3.1-Nef-ER. Nef and Nef-ER constructs were then subcloned into the retroviral expression vector pSReMSVtkneo, which carries a G418 resistance marker (21). The resulting constructs were used to generate high-titer stocks of recombinant retroviruses by co-transfection of 293T cells (22) with an amphotropic packaging vector as described (14). The Stat3 dominant-negative mutant carries a G418 resistance marker (21). The resulting constructs were used to create recombinant retrovirus for infection of TF-1/Nef-ER cells as described elsewhere (14).

**Cell Lysis and Western Blot Analysis**—TF-1 cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 1% Triton X-100, 1 mM sodium deoxycholate, and 0.1% SDS) supplemented with the protease inhibitors aprotonin (25 µg/ml), leupeptin (25 µg/ml), and phenylmethylsulfonyl fluoride (1 mM) and the phosphatase inhibitors NaF (10 mM), and Na3VO4 (1 mM). TF-1 cell lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4°C. To detect phosphorylation of Erk and Akt, cell lysates were immunoprecipitated with anti-Erk1, Erk2, or Akt antibodies, respectively. Immunoprecipitates were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes and blotted with phospho-specific antibodies to the active forms of Erk1/2 or Akt. Immunoreactive bands were visualized with secondary antibodies conjugated to horseradish peroxidase and detected by chemiluminescence.

**Electrophoretic Mobility Shift Assay**—TF-1 cells (107) were centrifuged and washed once with PBS. Cell lysates containing 30 µg of protein were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blotted with a Nef antibody. The Western blot was probed with an anti-Nef antibody (EH-1, mouse monoclonal), which reacts with the C-terminal domain of Nef. Blots were stripped and probed with an antibody to p-Tyr-705 (Upstate Biotechnology) and an antibody to total Stat3 (Cell Signaling). The membranes were probed with antibodies specific for Erk1/2, phospho-Erk1/2, and Akt, respectively.

**RESULTS**

**HIV-1 Nef Inhibits Apoptosis Induced by Cytokine Deprivation in the Human Myeloid Leukemia Cell Line, TF-1**—Previous work from our laboratory has shown that HIV-1 Nef induces cytokine-independent proliferation of the human macrophage precursor cell line, TF-1 (14). This cell line is dependent on GM-CSF for growth and undergoes apoptosis after cytokine withdrawal. To investigate whether the GM-CSF-independent proliferation induced by Nef results from suppression of apoptosis in TF-1 cells, we first expressed HIV-1 Nef in TF-1 cells by retroviral transduction. Cells were then plated in the presence or absence of GM-CSF, and apoptosis was measured as cell surface Annexin V staining, 4 days later. As shown in Fig. 1, 60% of the control cells infected with empty vector underwent apoptosis upon GM-CSF withdrawal, demonstrating the cytokine requirement for survival of this cell line. In contrast, only 25% of TF-1 cells expressing Nef underwent apoptosis after GM-CSF withdrawal, demonstrating that Nef suppresses apoptosis induced by cytokine deprivation in cells of myeloid lineage.

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**Annexin V F-ITC binding assay**—The TF-1 cells (107) were treated with 200 µM or 10 µM PD98059. After incubation, total cell RNA was isolated from each cell line using the Totally RNA kit (Ambion Inc.) according to the manufacturer’s instructions. To measure expression of Bcl-XL, a multi-probe set containing cDNA templates of nine Bcl-2 family members as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32 as internal controls was used according to the manufacturer’s protocol (BD Biosciences/PharMingen). Brieﬂy, 3P-labeled riboprobes of deﬁned length were generated using T7 RNA polymerase and 50 ng of the DNA template in the presence of 150 µCi of [32P]UTP (New England Nuclear). Template DNA was digested with RNase-free DNase, followed by precipitation of labeled RNA. Five µg of total cellular RNA was mixed with 6.2 µCi of the 3P-labeled riboprobe in hybridization buffer (40 mM 1,4-piperazinediethanesulfonic acid, 1 mM EDTA, 0.4 mM NaCl, and 80% formamide) and incubated for 5 min at 90°C, followed by 12 h at 56°C. The hybridized RNA duplexes were then treated with RNase A and RNase T1, followed by precipitation using proteinase K digestion. RNase-resistant RNA duplexes were extracted with phenol and precipitated by the addition of equal volumes of 4 M ammonium acetate and 2 volumes of ethanol. Labeled RNA samples were resolved on 6% urea denaturing gels and visualized by autoradiography, and the relative RNA signals were quantitated by densitometry. The relative expression level of Bcl-XL was normalized to that obtained with the GAPDH control for each sample.

**Detection of Nef-ER Dimers**—TF-1/Nef-ER cells (107) were treated for 24 h with or without 1 µM 4-HT, followed by incubation in the presence or absence of GM-CSF for 16 h. For cross-linking, cells were washed twice with PBS and incubated in 2 mM DSS for 30 min at room temperature. The reaction was terminated by adding 1 µM Tris-HCl (pH 7.5) and incubating for 15 min. The cells were washed once with PBS and then lysed in 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 25 µg/ml aprotinin, 25 µg/ml leupeptin, 25 µg/ml pepstatin, 1 mM Na3VO4, and 1 mM NaF). The protein concentration of the cell lysate was determined using the Coomassie Plus protein assay reagent (Pierce) and bovine serum albumin as standard. Cell lysates containing 30 µg of protein were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blotted with a Nef antibody.

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Although only 25% of TF-1/Nef cells underwent apoptosis after GM-CSF withdrawal, this number was reduced to 10% in the presence of GM-CSF (Fig. 1). However, immunofluorescent
PI3K Activation—The Erk MAPK pathway is involved in pro-phages through a pathway involving Hck and Erk (11). Other that Nef activates the AP-1 transcription factor in macrofactors and cytokines (28, 29). Previous studies have shown proliferative and survival signaling from a wide variety of growth myeloid cell context.

staining of the Nef-expressing cell population and flow cytometry revealed that only 60–70% of the cells express detectable levels of the Nef protein (data not shown). This heterogeneity in Nef expression in the TF-1 cell population may account for the incomplete survival effect. As described in a later section, experiments with clonal populations of TF-1 cells expressing a conditional form of Nef show responses equivalent to GM-CSF.

Induction of Bcl-X<sub>L</sub> Expression in TF-1 Cells Expressing Nef—Nef-induced proliferation of TF-1 cells requires the Stat3 transcription factor (14), which has been implicated in many growth factor and cytokine signaling pathways controlling cell proliferation, differentiation, and survival (26). Stat3-mediated survival signaling has been linked to Bcl-X<sub>L</sub> expression in tumor cells and other systems (16, 17). Bcl-X<sub>L</sub> is an anti-apoptotic member of the Bcl-2 family of mitochondrial apoptotic regulators (15, 27). The relative levels of the pro- and anti-apoptotic Bcl-2 family proteins are key determinants in the regulation of cell death and survival. Therefore, we investigated Bcl-2 family gene expression in Nef-expressing TF-1 cells using an RNase protection assay. As shown in Fig. 2, Bcl-X<sub>L</sub> transcript levels from TF-1/Nef cells were selectively increased 2.5-fold relative to control cells in the absence of GM-CSF. The steady-state levels of Bcl-X<sub>L</sub> transcripts in TF-1/Nef cells are very close to those observed in parental cells grown in the presence of GM-CSF, suggesting that the level of Bcl-X<sub>L</sub> gene expression maintained by Nef is sufficient to support survival. In contrast, expression of other Bcl-2 family members was unaffected by Nef, including the genes encoding the anti-apoptotic proteins Bcl-2, Bcl-w, Mcl-1, and Bfl-1, and the pro-apoptotic proteins Bax, Bak, Bad, and Bik. These results identify Bcl-X<sub>L</sub> as a candidate downstream target gene that may contribute to the anti-apoptotic effects of Nef in this myeloid cell context.

Nef Survival Signaling in TF-1 Cells Requires MAPK but Not PI3K Activation—The Erk MAPK pathway is involved in proliferative and survival signaling from a wide variety of growth factors and cytokines (28, 29). Previous studies have shown that Nef activates the AP-1 transcription factor in macrophages through a pathway involving Hck and Erk (11). Other work has shown that Erk activation is involved in the GM-CSF-induced proliferation of TF-1 cells (30), suggesting that Nef may mimic this aspect of GM-CSF signaling in this cell line. To test the requirement for Erk activation in Nef-induced cellular survival, we treated TF-1/Nef cells as well as control cells with the Mek inhibitor PD98059 (23) in the presence or absence of GM-CSF. Apoptosis was measured as Annexin V staining 4 days after treatment. As shown in Fig. 3A, PD98059 completely reversed Nef-induced cell survival. In contrast, PD98059 produced only a partial reversal of GM-CSF-induced TF-1 cell survival, indicating that the Nef but not the GM-CSF survival signal is highly dependent upon Erk activation.

To test the ability of Nef to activate Erk/MAPK signaling in TF-1 cells, control and Nef-expressing cells were incubated in
the presence or absence of GM-CSF for 16 h. Erk1 and Erk2 were then immunoprecipitated, followed by immunoblotting with phospho-specific antibodies. As shown in Fig. 3B, Nef expression dramatically increased both Erk1 and Erk2 activation in the absence of GM-CSF. The extent of Nef-induced Erk activation was similar to that observed with GM-CSF treatment. PD98059 suppressed Erk1/2 activation by both Nef and GM-CSF. Control immunoblots for Erk proteins displayed no differences, demonstrating that the Nef-mediated increase in Erk1/2 activity was not attributable to changes in protein levels.

Recent work has shown that anti-apoptotic signaling by HIV-1 Nef involves the PI3K pathway in T cells (13). To examine whether PI3K also contributes to Nef survival signaling in myeloid cells, we tested the effect of a specific PI3K inhibitor, LY294002 (24), on cell survival induced by Nef in the TF-1 model system. In contrast to the Mek inhibitor, LY294002 suppressed Erk1/2 activation by both Nef and GM-CSF. Control immunoblots for Erk proteins displayed no differences, demonstrating that the Nef-mediated increase in Erk1/2 activity was not attributable to changes in protein levels.

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vival Signaling—All of the experiments described so far have been conducted with a retrovirally transduced TF-1 cell population in which the expression of Nef varies from one cell to another. In addition, survival of TF-1/Nef cells may be influenced by G418 selection of the infected cell population and not caused by a direct signal from Nef itself. To address these issues, we created a conditionally active Nef protein by fusing it to the hormone binding domain of the ER using a strategy similar to one originally reported by Walk et al. (32). Structural and biochemical studies have shown that Nef can form dimers and higher-order oligomers (33–36), which may be important for some signaling functions (32). One effect of ER fusion is to allow regulated dimerization, which may in turn control the functional activity of Nef. Fusion to ER may also render the protein inactive because of the steric hindrance caused by binding of cellular chaperones, such as heat shock protein 90.

Treatment with the cell-permeable synthetic estrogen 4-HT induces Nef-ER dimer formation, as well as release of bound chaperones and protein stabilization (see below). A similar Nef-ER construct has been shown to induce CD4 down-regulation and to associate with p21-activated protein kinase activity in a 4-HT-dependent fashion (32).

TF-1 cells were infected with Nef-ER retroviruses and cloned by limiting dilution under G418 selection in the presence of GM-CSF. Cell clones were then screened for expression of the Nef-ER fusion protein (∼60 kDa) by anti-Nef immunoblotting of cell lysates. Four of 11 TF-1 cell clones tested strongly positive for Nef-ER expression (Fig 6A). To determine whether Nef-ER activation protects TF-1 cells from apoptosis in response to GM-CSF deprivation, cells were pretreated with 4-HT for 24 h, followed by GM-CSF withdrawal. After 16 h of GM-CSF starvation, cells were analyzed for initiation of apoptosis by Annexin V staining. As shown in Fig. 6B, 4-HT suppressed programmed cell death after cytokine withdrawal in the TF-1/Nef-ER cell lines, confirming that the survival signal is directly driven by Nef and is not a secondary effect of selection. Furthermore, Nef-ER suppressed apoptosis in TF-1 cells in the presence of 4-HT almost as effectively as GM-CSF. This result is in contrast to the partial effect observed in the bulk TF-1/Nef cultures (Fig. 1), which may reflect heterogeneity of Nef expression in this cell population, as described above.

TF-1/Nef-ER cell clone 10 was studied further because its responsiveness to 4-HT in terms of cell survival was indistinguishable from that of GM-CSF. TF-1 cell clones negative for Nef-ER expression were not protected from apoptosis by 4-HT after GM-CSF withdrawal (data not shown).

Inducible Stabilization and Dimerization of Nef-ER in TF-1 Cells—We next investigated the mechanism by which 4-HT...
induced Nef-ER activation in TF-1 cells. On the basis of work in other systems, we expected to observe 4-HT-dependent formation of Nef-ER dimers (32). To test this idea, TF-1 cells were incubated in the presence or absence of 4-HT and GM-CSF, followed by treatment with the homobifunctional cross-linker disuccinimidyl suberate to stabilize the dimers before cell lysis. Nef-ER proteins were then separated by SDS-PAGE, transferred to polyvinylidene difluoride, and probed with a Nef-specific antibody. As shown in Fig. 7, 4-HT-dependent Nef-ER dimerization was detected, regardless of the presence of GM-CSF. Interestingly, the Nef-ER protein level was also higher in the presence of 4-HT, suggesting that ligand binding may stabilize the Nef-ER protein in addition to promoting dimerization. Similar observations have been reported after expression of Nef in T cells (32) and provide an additional level of control over Nef activity.

Cell Survival and Stat3 Activation by Nef-ER Requires Erk Activation—Constitutive expression of Nef in TF-1 cells promotes survival in an Erk-dependent manner (32). To test whether the inducible survival effect of Nef-ER also requires Erk activation, we treated TF-1/Nef-ER cells with the Mek-specific inhibitor PD98059. As shown in Fig. 8, PD98059 completely blocked the protective effect of activated Nef-ER after withdrawal of GM-CSF. In contrast, PD98059 did not affect cell survival in the presence of GM-CSF, indicating that GM-CSF survival signals are not Erk-dependent in this cell line. These results are consistent with those obtained from the TF-1/Nef cell populations (Fig. 3).

Using Stat3 dominant-negative mutants, we observed previously that constitutive Stat3 activation is required for GM-CSF-independent growth of TF-1/Nef cells in soft agar (14). Here we show that Erk signaling is also essential, suggesting a possible connection between the two pathways. To investigate cross-talk between Stat3 and Erk downstream of Nef, we tested the effect of PD98059 on Nef-ER-driven Stat3 signaling. PD98059 was added to control and Nef-ER-expressing cells...
pretreated with 4-HT in the presence or absence of GM-CSF. Stat3 activation was then assayed using an electrophoretic mobility shift (gel-shift) assay with an oligonucleotide probe corresponding to the SIE, which is strongly bound by active Stat3. As shown in Fig. 9, nuclear extracts prepared from 4-HT-treated TF-1/Nef-ER cells revealed strong SIE-binding activity, which agrees with our previous findings in TF-1/Nef cell populations (14). Surprisingly, the levels of the Stat3/SIE complex were substantially reduced in the presence of PD98059, suggesting a requirement for Erk in Stat3 activation by Nef-ER. In contrast, SIE-protein complex formation induced by GM-CSF in control TF-1 cells was unaffected by PD98059 treatment, indicating that the Erk requirement is unique to Nef-dependent Stat3 activation. Competition with unlabeled SIE completely blocked Stat3-SIE complex formation in each case, indicating that this interaction is specific.

To determine whether Stat3 DNA-binding activity induced by Nef correlates with Stat3 tyrosine phosphorylation, nuclear extracts from the control and TF-1/Nef-ER cells were immunoblotted with anti-phospho-Stat3 antibodies. Fig. 9B shows that Stat3 Tyr-705 is strongly phosphorylated after Nef-ER activation by 4-HT, consistent with the gel-shift result. In contrast, nuclear extracts from TF-1/Nef-ER cells treated with PD98059 showed greatly diminished levels of Stat3 Tyr-705 phosphorylation, indicating that Nef-induced tyrosine phosphorylation of Stat3 also requires Erk activation. In contrast, Stat3 Tyr-705 phosphorylation by GM-CSF was unaffected by PD98059. Control immunoblots show that approximately equivalent levels of Stat3 protein were expressed in each culture. Taken together, these results provide strong evidence that Nef-mediated activation of Stat3 requires active Erk, and that the mechanism of Stat3 activation by Nef is distinct from that of GM-CSF.

**DISCUSSION**

Previous work from our laboratory has shown that Nef promotes the cytokine-independent proliferation of the human myeloid progenitor cell line TF-1 through a mechanism dependent upon the Stat3 transcription factor (14). Here we show for the first time that Nef protects TF-1 cells from apoptosis after cytokine withdrawal and selectively up-regulates expression of the anti-apoptotic gene Bcl-XL (Figs. 1 and 2). Several studies have established that Bcl-XL, a member of Bcl-2 family of mitochondrial apoptotic regulators, is directly induced by the Stat3 transcription factor. For example, Catlett-Falcone et al. (16) reported constitutive Stat3 activation and up-regulated Bcl-XL expression in human multiple myeloma cells. Using a dominant-negative form of Stat3 in which the tyrosine phosphorylation site at position 705 is replaced with phenylalanine (Stat3YF), previous studies have shown that this mutant blocks GM-CSF-independent growth of TF-1/Nef cells in soft- agar colony assays, suggesting that Stat3 may contribute to Nef-induced suppression of apoptosis resulting from cytokine withdrawal in this cell line (14). TF-1/Nef-ER cells were infected with the Stat3YF retrovirus or with a control virus carrying only the drug selection marker. Forty-eight h later, 4-HT was added, and cells were washed free of GM-CSF and incubated for an additional 16 h. As shown in Fig. 10A, activation of Stat3 by Nef-ER in the presence of 4-HT or by GM-CSF was dramatically suppressed in cells infected with the Stat3YF retrovirus but not the control virus. Surprisingly, Stat3YF did not affect the suppression of apoptosis by active Nef-ER (Fig. 10B), nor did it impact expression of Bcl-XL (Fig. 10C). These data show that although Nef strongly activates Stat3 in TF-1 cells, this pathway may not be required for Bcl-XL induction or survival signaling.

**FIG. 9.** Inducible activation of Stat3 by Nef-ER requires Erk activity. A, control and TF-1/Nef-ER cells were incubated in the presence or absence of 4-HT for 24 h. Cells were then treated with PD98059 (30 μM) or vehicle for 16 h in the presence or absence of GM-CSF. Nuclear extracts were prepared from each sample and tested for the presence of activated Stat3 by gel-shift analysis with an SIE probe. To control for the specificity of DNA binding, parallel assays were performed in the presence of a 100-fold molar excess of unlabeled SIE (+ Comp). The position of the shifted Stat3/SIE complex is indicated by the arrow. B, nuclear extracts from A were immunoblotted with phospho-specific antibodies to active Stat3. Control immunoblots show equivalent levels of Stat3 protein in each of the nuclear extracts. This experiment was repeated twice with comparable results.

**FIG. 8.** Survival signaling from Nef-ER requires the Erk MAPK pathway in TF-1 cells. A, TF-1 cells expressing Nef-ER (bottom) and parental TF-1 cells (top) were incubated in the presence or absence of 4-HT for 24 h. The cells were then washed to remove GM-CSF and replated in the presence or absence of GM-CSF and 30 μM PD98059 as indicated. Apoptotic cells were stained with Annexin V-FITC and detected by flow cytometry. Results show the mean percentages of apoptotic cells observed in three independent determinations ± S.E.
apoptotic response in this system. Using a similar dominant-negative approach, we tested the requirement for Stat3 in Bcl-X<sub>L</sub> induction and survival signaling by Nef in TF-1 cells. Surprisingly, expression of a well-established dominant-negative mutant of Stat3 (Stat3YF) did not affect Bcl-X<sub>L</sub> up-regulation or suppression of apoptosis by Nef (Fig. 10), suggesting that Nef induces Bcl-X<sub>L</sub> and promotes survival through a Stat3-independent mechanism involving Erk activation (see below). Although Nef-driven survival and Bcl-X<sub>L</sub> expression were not affected by Stat3YF, this mutant was able to completely block cytokine-independent soft-agar colony formation by TF-1/Nef cells in previous studies (14). In light of work presented here, this observation suggests that the Nef-Stat3 pathway may affect other Stat3-dependent responses in myeloid cells, such as adhesion or migration (37).

The Erk kinases are members of the MAPK family and regulate a wide variety of cell functions, including the survival response to cytokines and other soluble factors (28). Relevant to our study is recent work by Kolonics et al. (30), which showed that GM-CSF-induced survival of TF-1 cells correlates with Erk1/2 activation and Bcl-X<sub>L</sub> induction, consistent with our findings. GM-CSF-dependent changes in Bcl-X<sub>L</sub> protein levels in this study correlate closely with changes in the transcript levels reported here (Fig. 2). In related studies, Kinoshita et al. (38) reported that activation of the Ras-MAPK pathway may be essential for anti-apoptotic signaling by the GM-CSF receptor. They found that a mutant GM-CSF receptor uncoupled from MAPK activation was unable to protect transfected Ba/F3 cells from apoptosis in the presence of GM-CSF. Expression of active Ras complemented the signaling defect from the mutant receptor and supported long-term proliferation of the cells, suggesting that GM-CSF prevents apoptosis of some hematopoietic cells by activating Ras-MAPK signaling. Here we show that Nef mimics this effect of GM-CSF, and that Nef-induced Erk activation is required for TF-1 cell survival in cytokine-free medium. Supporting this hypothesis, inhibition of Nef-induced Erk activation with PD98059 completely abolished the survival of Nef-expressing cells after cytokine withdrawal (Figs. 3 and 8). In addition to the role of Erk in the Nef-induced anti-apoptotic effect described here, Erk MAPK activity has been shown to increase transcription from the HIV proviral long terminal repeat and HIV replication in T cells (39, 40). Furthermore, expression of constitutively activated forms of Ras, Raf, or Mek, as well as activation of Erk signaling with serum or phorbol esters, enhanced the infectivity of HIV virions in T cells (41). Conversely, virus infectivity was reduced by treatment of cells with PD98059 or with antisense oligonucleotides directed against Erks (41). Erk MAPKs have also been identified as HIV virion-associated kinases by density gradient fractionation (10). In this study, viral infectivity was increased by stimulation of virion-associated Erk activity with phorbol esters and impaired by specific inhibitors of the MAPK cascade. Taken together, these studies support the conclusion that HIV takes advantage of the Erk MAPK pathway to enhance viral replication as well as promote survival of infected cells, and that HIV Nef plays an essential role in these processes. Results presented here show for the first time that Nef induces Bcl-X<sub>L</sub> as a mechanism for cytokine-independent survival of myeloid cells (Fig. 2). Although Nef-induced Bcl-X<sub>L</sub> expression depends upon Erk activation (Fig. 5), the mechanism by which this MAPK pathway contributes to Bcl-X<sub>L</sub> regulation is less clear. One possibility is that Erks regulate Bcl-X<sub>L</sub> directly through AP-1 induction. Nef has been found to activate the AP-1 transcription factor through Hck and Erk in macrophages (11), and a consensus motif for AP-1 binding has been identified in the promoter region of Bcl-X<sub>L</sub> (42). However, whether AP-1 binds to the Bcl-X<sub>L</sub> promoter region and directly induces transcription of Bcl-X<sub>L</sub> remains to be examined.
Work presented here also suggests that Nef induces cross-talk between the Erk and Stat3 signaling pathways. This hypothesis is supported by the surprising observation that pharmacological inhibition of Erk signaling suppresses the tyrosine phosphorylation and DNA binding activity of Stat3 downstream of Nef-ER (Fig. 9). Erks have been implicated in phosphorylation of Stat3 at Ser-727, which may be required for full transcriptional activity (43) but has a more controversial role in the regulation of DNA binding (44, 45). Using phospho-specific antibodies, we did not detect changes in the phosphorylation status of Stat3 Ser-727 in response to Nef expression in TF-1 cells (data not shown). However, Nef-induced Erk activation may be linked to activation of Rsk and other Ser/Thr kinases related to survival of hematopoietic cells through phosphorylation of cyclic AMP-responsive element binding protein and other transcription factor targets (28). Whether these kinases can also influence the activity of Stat3 by phosphorylation of sites other than Ser-727 remains to be tested. Another possibility is that Nef-induced Erk activation may affect dephosphorylation of active Stat3 by SHP2 or other protein-tyrosine phosphatases. Erk1 has been shown to phosphorylate SHP2 and inhibit its activity in vitro, consistent with this possibility (46).

HIV and other pathogenic viruses have developed protective mechanisms to suppress apoptosis of infected host cells, to allow time for viral replication before cell lysis (2). Recent studies have shown that Nef inhibits death receptor-mediated apoptosis by interacting with apoptosis signal regulating kinase 1 and blocking its activity in T cells (12). More recently, it has been demonstrated that HIV-1 Nef also suppresses apoptosis by stimulating Akt-independent Bad phosphorylation in T lymphocytes (13). In contrast to these studies, we found that Nef-induced survival does not require PI3K activity (Fig. 4) or involve Bad phosphorylation (data not shown) in cells of myeloid lineage. Instead, Nef appears to suppress cell death in this macrophage precursor cell line through a distinct mechanism involving the Erk MAPK pathway and Bcl-XL induction. A growing number of studies implicate HIV-infected macrophages as key elements in AIDS pathogenesis (47). For example, in severe combined immunodeficient mice transplanted with human peripheral blood leukocytes (hu-PBL-SCID mouse model), extensive CD4+ T cell depletion is induced by non-cytopathic, macrophage-tropic strains of HIV. Surprisingly, HIV strains that are highly cytoytic toward T cells in vitro showed little activity in the hu-PBL-SCID mouse model (48). Also, monkeys infected with HIV-1, which does not infect simian monocytes/macrophages, showed no signs of disease and fail to show accelerated T-cell apoptosis (49). The anti-apoptotic signal generated by HIV Nef described here may allow for persistence of HIV-infected macrophages, promoting viral replication and spread to uninfected T cells. Our findings support the hypothesis that Nef may contribute to the establishment and maintenance of HIV reservoirs by conferring a survival advantage on HIV-infected macrophages.

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