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NF-κB Protects HIV-1-Infected Myeloid Cells from Apoptosis

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HIV-1 infection of primary monocytic cells and myeloid cell lines results in sustained NF-κB activation. Recently, NF-κB induction has been shown to play a role in protecting cells from programmed cell death. In the present study, we sought to investigate whether constitutive NF-κB activity in chronically HIV-1-infected promonocytic U937 (U9-IIIB) and myeloblastic PLB-985 (PLB-IIIB) cells affects apoptotic signaling. TNFα and cycloheximide caused infected cells to undergo apoptosis more rapidly than parental U937 and PLB-985 cells. Inhibition of TNFα-induced NF-κB activation using the antioxidant *N*-acetylcysteine (NAC) resulted in increased apoptosis in both U937 and U9-IIIB cells, while preactivation of NF-κB with the non-apoptotic inducer IL-1β caused a relative decrease in apoptosis. Inhibition of constitutive NF-κB activity in U9-IIIB and PLB-IIIB cells also induced apoptosis, suggesting that NF-κB protects cells from a persistent apoptotic signal. TNFα plus NAC treatment resulted in a marked decrease in Bcl-2 protein levels in HIV-1-infected cells, coupled with an increase in Bax protein compared to uninfected cells, suggesting that the difference in susceptibility to TNFα-induced apoptosis may relate to the differences in relative levels of Bcl-2 and Bax. The protective role of NF-κB in blocking TNFα- and HIV-1-induced apoptosis was supported by studies in Jurkat T cells engineered to express IκBα repressor mutants (TD-IκB) under the control of a tetracycline-responsive promoter. Cells underwent apoptosis in response to TNFα only when NF-κB activation was inhibited by TD-IκB expression. As was observed for the U9-IIIB cells, TNFα treatment also induced a marked decrease in Bcl-2 protein levels in TD-IκB expressing cells. These experiments demonstrate that apoptotic signaling is perturbed in HIV-1-infected U9-IIIB cells and indicate that NF-κB activation may play an additional protective role against HIV-1-induced apoptosis in myeloid cells.

INTRODUCTION

HIV-1 infection is cytopathic for $CD4^+$ T cells but can result in the chronic infection of $CD4^+$ myeloid cells. The mechanism of myeloid cell protection from HIV-1 infection is unknown. As a consequence of the long-term production of viral particles, chronically infected myeloid cells have been suggested to act as reservoirs of virus, propagating virus spread and disease progression (Perno *et al.,* 1997). HIV-1-induced apoptosis has been proposed as one mechanism to explain the direct cytopathic effects of virus infection, as well as apoptosis of bystander lymphocytes (Maldarelli *et al.,* 1995; Ameisen *et al.,* 1995; Herbein *et al.,* 1998). $CD4^+$ myeloid cells may be more resistant to the induction of apoptosis by HIV-1 infection.

NF-κB plays a central role in the HIV life cycle, facilitating replication at several levels. NF-κB is essential for LTR-driven gene expression (reviewed in Roulston *et al.,* 1995) and has been reported to affect mRNA transport by Rev (Wu *et al.,* 1997). NF-κB interacts with HIV Tat, leading to synergistic activation of HIV gene transcription (Chang *et al.,* 1995; Demarchi *et al.,* 1996). In addition, via its ability to activate proinflammatory cytokines such as TNFα (Lenardo and Baltimore, 1989), NF-κB activation initiates an autostimulatory loop that increases HIV replication (Poli *et al.,* 1990).

The NF-κB family of transcription factors is involved in the transduction of immunological responses, cellular differentiation, and cell growth regulation (reviewed in Roulston *et al.,* 1995; Verma *et al.,* 1995; Baldwin, 1996). NF-κB proteins are found in a latent state in the cytoplasm, complexed to inhibitory IκB proteins. Activation of NF-κB results following exposure to a variety of inducing agents including cytokines, bacterial and viral pathogens, chemotherapeutic compounds, and cell damaging agents. Several DNA binding family members exist: p50 and p52 are synthesized as inactive precursors (p105 and p100, respectively) and are proteolytically cleaved to produce the mature DNA binding proteins; p65 (RelA), c-Rel and RelB contain, in addition to an N-terminal DNA binding domain, C-terminal transactivation domains (Baldwin, 1996). In unstimulated cells, NF-κB proteins are sequestered in the cytoplasm, complexed with the IκB inhibitor molecules which include IκBα, IκBβ, IκBγ, the recently identified IκBε (Whiteside *et al.,* 1997), and the proto-oncogene *bcl-3.* IκB binds to NF-κB, masking nuclear localization sequences and preventing nuclear translocation. Structurally, all IκBs (reviewed in Baldwin, 1996) contain between five and seven 33-amino-acid

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repeats termed the ankyrin motif, which is required for interaction with NF-κB. Phosphorylation of serine residues 32 and 36 targets IκBα for ubiquitin-dependent proteolytic degradation. A large multi-subunit kinase complex that contains the IKK kinase activities required for inducer mediated phosphorylation at Ser-32 and Ser-36 was identified recently (Chen *et al.,* 1996; Régnier *et al.,* 1997; DiDonato *et al.,* 1997; Woronicz *et al.,* 1997; Mercurio *et al.,* 1997); regulation of this kinase complex remains to be elucidated.

Chronic HIV-1 infection of U937 promonocytic cells (termed U9-IIIB) or the myelomonoblastic PLB-985 (termed PLB-IIIB) (Roulston *et al.,* 1992) leads to constitutive NF-κB expression (Bachelerie *et al.,* 1991; Roulston *et al.,* 1992, 1993, 1995), increased proteasome-mediated turnover of IκBα, and elevated expression of NF-κB1, NF-κB2, and c-Rel proteins (McElhinny *et al.,* 1995; DeLuca *et al.,* 1996). This modulation of intracellular NF-κB levels may contribute to enhanced NF-κB-directed gene expression and increased HIV-1 replication. Recently, NF-κB activation was shown to protect cells from several apoptotic stimuli, including TNFα, and inhibition of NF-κB activity rendered resistant cells sensitive to TNFα-induced apoptosis (Van Antwerp *et al.,* 1996; Beg and Baltimore, 1996; Wang *et al.,* 1996; Wu *et al.,* 1996). In particular, RelA(p65) and c-Rel have been implicated as subunits capable of conferring protection. RelA-/- mouse embryonic fibroblasts are sensitive to TNFα toxicity but become resistant when RelA is reexpressed by transfection (Beg and Baltimore, 1996), while microinjection of GST-IκBα or anti-c-Rel affinity-purified antibody induced apoptosis in mouse WEHI 231 cells (Wu *et al.,* 1996).

NF-κB has also been implicated as a mediator of apoptosis (Lin *et al.,* 1995; Abbadie *et al.,* 1993; Grimm *et al.,* 1996; Ivanov *et al.,* 1997; Marianneau *et al.,* 1997; Lipton, 1997) since stimuli that lead to cell apoptosis often lead to NF-κB activation (UV, TNFα, Fas, HIV infection, and various chemotherapeutic agents). Blocking NF-κB induction can prevent apoptosis, although this appears to be cell specific and in several situations, NF-κB can be blocked without preventing apoptosis (Cai *et al.,* 1997; Hsu *et al.,* 1996; Dbaido *et al.,* 1997). The mechanism by which NF-κB protects cells from apoptosis remains to be elucidated, although NF-κB activates several protective genes implicated in anti-apoptotic effects including A20 (Jaattela *et al.,* 1996; Tewari and Dixit, 1996), Bcl-2, c-Myc, p53 (Baichwal and Baeuerle, 1997), and c-IAP2 (Chu *et al.,* 1997). Bcl-2 is a mitochondrial protein that protects against apoptosis in many cell systems; inactivation of Bcl-2 by degradation or phosphorylation permits mitochondrial proteases and cytochrome c to enter the cytoplasm and execute apoptosis (Kluck *et al.,* 1997; Yang *et al.,* 1997). The Bcl-2 family of proteins is composed of pro-apoptotic and protective members (reviewed in Yang and Korsmeyer, 1996; Reed *et al.,* 1996) and the relative levels of these proteins influences whether a cell lives or commits cell "suicide" by apoptosis. The HIV-1 Tat protein has been reported to modulate Bcl-2 and Bax levels, suggesting that modulation of Bcl-2 proteins may be one mechanism used by HIV to induce apoptosis in infected and bystander cells (Zauli *et al.,* 1995; Sastry *et al.,* 1996; Zauli and Gibellini, 1996).

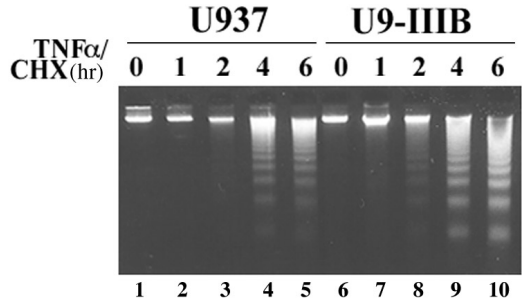


FIG. 1. Chronically HIV-infected U9-IIIB cells undergo apoptosis in response to TNFα/CHX more rapidly than uninfected U937 cells. Cells were treated with the protein synthesis inhibitor cycloheximide (CHX, 100 μg/ml) and tumor necrosis factor α (TNFα, 10 ng/ml) for 0, 1, 2, 4, or 6 h. 2×10^6 cells were lysed and analyzed for DNA fragmentation by electrophoresis and visualized by ethidium bromide staining. Fragmentation was visible after 1–2 h in U9-IIIB (lanes 7–8) cells but not until 4 h in U937 cells (lane 4).

This study sought to determine whether HIV-1 infection of myeloid cells induced alterations in apoptotic signaling pathways and whether NF-κB was involved. Our results demonstrate that constitutive NF-κB activation is required to counteract a persistent apoptotic signal resulting from chronic HIV-1 infection and that NF-κB is important in protecting cells from the cytotoxic effects of TNFα in both the U937/U9-IIIB and PLB-985/PLB-IIIB myeloid and Jurkat T cell lines. Thus, a previously unrecognized role for constitutive NF-κB activation in HIV-1-infected cells is to protect from virus-mediated apoptotic cell death.

RESULTS

Increased apoptosis in HIV-1-infected U937 cells

Experiments presented in this paper derived from three observations: (1) chronic HIV-1 infection of myeloid cells results in constitutive NF-κB DNA binding activity (Bachelerie *et al.,* 1991; Roulston *et al.,* 1992, 1993, 1995); (2) NF-κB has a bipotential role in apoptotic signaling, protective in some cells (Van Antwerp *et al.,* 1996; Beg and Baltimore, 1996; Wang *et al.,* 1996) pro-apoptotic in others (Lin *et al.,* 1995; Jung *et al.,* 1995; Abbadie *et al.,* 1993); and (3) HIV-1 infection is associated with virus-induced apoptosis in susceptible cells (Maldarelli *et al.,* 1995; Ameisen *et al.,* 1995; Herbein *et al.,* 1998). We therefore asked whether perturbation of NF-κB activity in U937/U9-IIIB or PLB-985/PLB-IIIB cells resulted in altered apoptotic signaling. U937 and U9-IIIB cells were incubated in the presence of 10 ng/ml TNFα and 100 μg/ml cycloheximide (CHX) for 0 1, 2, 4 or 6 h and analyzed for DNA fragmentation. Figure 1 illustrates that

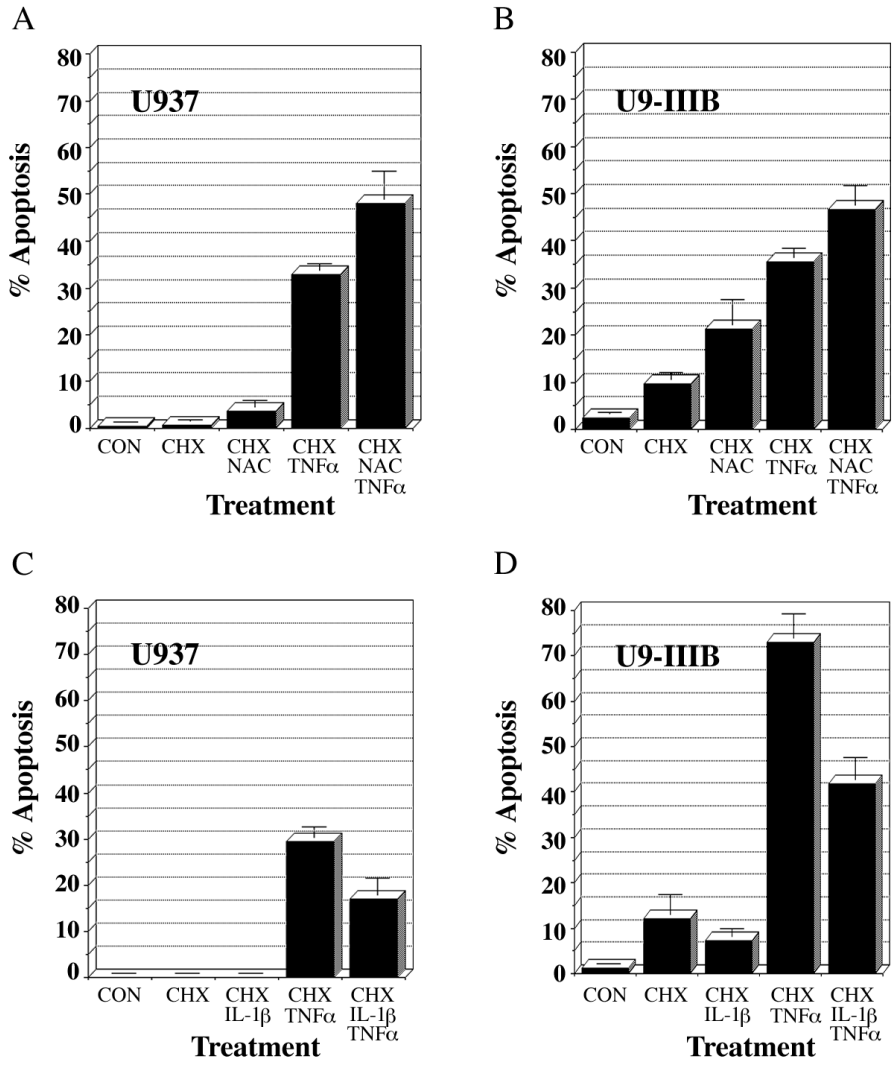


FIG. 2. Inhibition of NF-κB increases — while preactivation of NF-κB decreases — TNFα/CHX-induced apoptosis. U937 (A) and U9-IIIB (B) cells were pretreated with or without the antioxidant *N*-acetylcysteine (NAC, 50 mM) for 1 h or U937 (C) and U9-IIIB (D) cells were pretreated with or without interleukin 1β (IL-1β, 5 ng/ml) for 5 h. Cells were subsequently treated with CHX (50 μg/ml) and/or TNFα (20 ng/ml) for 2 h. Apoptotic cells were measured by TUNEL assay and the total cell numbers determined by Hoescht staining. Percentages of apoptotic cells were calculated with the results plotted for an experiment carried out in triplicate.

U9-IIIB cells exhibited increased sensitivity to TNFα, undergoing detectable DNA fragmentation as early as 1–2 h after TNFα treatment (Fig. 1, lanes 7 and 8) compared to uninfected U937, which do not show visible signs of apoptosis until 4 h (Fig. 1, lane 4). Increasing the concentration of TNFα induced a more rapid induction of apoptosis in both cell types (data not shown). Similar results were obtained using promonocytic PLB-985 cells and HIV-1-infected PLB-IIIB cells. These cells were treated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 0–5 h. Infected cells exhibited DNA laddering after 1 h, while uninfected cells underwent fragmentation beginning at 2 h (data not shown).

Relationship between NF-κB levels and apoptosis

To assess the involvement of NF-κB in apoptotic signaling, U937 and U9-IIIB cells were pretreated with the antioxidant *N*-acetylcysteine (NAC 50 mM) for 1 h and then with TNFα (20 ng/ml) and CHX (50 μg/ml) for 2 h; NAC inhibited NF-κB activation by blocking IκB phosphorylation (Schreck *et al.,* 1991; Staal *et al.,* 1993; Lee *et al.,* 1997). The level of TNFα/CHX induced apoptosis was increased in both U937 and U9-IIIB cells following NAC pretreatment as quantified by the TUNEL assay (Figs. 2A and 2B). TNFα/CHX induced approximately 30% apoptosis in U937 (Figs. 2A and 2C) and this value was increased to 50% by NAC pretreatment. TNFα/CHX treatment induced 35 to 70% apoptosis in U9-IIIB cells (Figs. 2B and 2D); NAC pretreatment resulted in a consistent 20–40% increase in apoptotic cells (Fig. 2B). Interestingly, the combination of NAC/CHX induced significant apoptosis in U9-IIIB, whereas U937 cells were only marginally affected. In addition, CHX alone caused a low level of apoptosis in U9-IIIB cells (~10%), suggesting that a labile

TABLE 1

Effect of NF-κB Activation on TNFα/CHX-Induced Apoptosis in PLB-IIIB Cells

Condition	Percentage of Apoptosis
Untreated	1.3
Cycloheximide	11.9 ± 0.54
NAC + cycloheximide	33.5 ± 2.2
IL-1β + cycloheximide	6.9 ± 0.57
TNFα + cycloheximide	39.8 ± 5.2
NAC + TNFα + cycloheximide	57.5 ± 1.7
IL-1β + TNFα + cycloheximide	27.9 ± 3.4

Note. PLB-IIIB cells were pretreated with IL-1β (10 ng/ml) for 5 h or NAC (50 mM) for 1 h and were subsequently incubated with TNFα (20 ng/ml)/CHX (50 μg/ml) for 2 h. The number of apoptotic cells was quantified by TUNEL assay. Values are the result of an experiment performed in triplicate.

factor may be important for cell survival in HIV-1-infected cells. Again, similar results were obtained using PLB-IIIB cells (Table 1).

To determine if NF-κB preactivation would decrease apoptosis, U937 and U9-IIIB cells were stimulated with IL-1β (5 ng/ml), a noncytotoxic inducer of NF-κB, for 5 h followed by a 2-h incubation with TNFα (20 ng/ml) and CHX (50 μg/ml). In both cell types, addition of IL-1β reduced both TNFα/CHX-induced apoptosis by approximately 40% and the low level of CHX-induced apoptosis (Figs. 2C and 2D), again suggesting that a labile protein induced by constitutive NF-κB, maintained cell survival. Figure 3 illustrates cells positive for apoptosis by TUNEL assay (right panels) within the total cell population (left panels) induced by the treatments described in Fig. 2.

NF-κB DNA binding activity in TNFα-, IL-1β-, and NAC-treated nuclear extracts was assessed by EMSA. Figure 4A illustrates that both TNFα and IL-1β induced NF-κB DNA binding activity in U937 (Fig. 4A, lanes 4, 5, and 7) and U9-IIIB cells (Fig. 4A, lanes 11, 12, and 14) and this level of DNA binding was inhibited by NAC preincubation (Fig. 4A, lanes 6 and 13, respectively). U9-IIIB cells exhibited constitutive NF-κB DNA binding activity (Fig. 4A, lane 8) which was abolished by NAC treatment (Fig. 4A, lane 10); specificity of the complex was confirmed by competition with a 125 molar excess of unlabeled competitor DNA (Fig. 4A, lane 15). Figure 4B illustrates that TNFα/CHX induced IκBα degradation in both U937 and U9-IIIB cells (Fig. 4B, lanes 5, 7 and 12, 14, respectively) and this degradation was inhibited by NAC pretreatment (Fig. 4B, lanes 6 and 13), as recently described (Lee *et al.,* 1997). PLB-IIIB cells showed a similar pattern of IκBα degradation in response to the conditions described above (data not shown).

TNFα alone also induces DNA fragmentation in U937 and U9-IIIB cells, with the latter exhibiting signs of DNA fragmentation earlier than noninfected cells. U937 and U9-IIIB cells were incubated with TNFα (20 ng/ml) and/or NAC (30 mM) for 0 to 48 h and in all cases NAC was added to cells 1 h prior to TNFα addition. U937 cells did not undergo apoptosis when incubated with NAC (Fig. 5A, lane 2) but underwent DNA fragmentation when incubated with TNFα (Fig. 5A, lane 3) or TNFα and NAC (Fig. 5A, lane 4). In contrast, U9-IIIB cells were sensitive to all three treatments, undergoing DNA fragmentation by 8 h after NAC (Fig. 5A, lane 6), TNFα (Fig. 5A, lane 7) or TNFα plus NAC (Fig. 5A, lane 8) addition. Electrophoretic mobility shift analyses (EMSA) of nuclear extracts from TNFα- and/or NAC-treated cells confirmed that NAC inhibited both TNFα-induced NF-κB binding and constitutive NF-κB in HIV-1-infected cells (Fig. 5B). NF-κB binding was detected in TNFα-induced extracts from U937 and U9-IIIB cells (Fig. 5B, lanes 3 and 7, respectively) but was reduced by NAC preincubation (Fig. 5B, lanes 4 and 8). In addition, constitutive NF-κB in U9-IIIB cells (Fig. 5B, lane 5) was blocked by NAC (Fig. 5B, lane 6).

Bcl-2/Bax expression in HIV-infected cells

These results suggest that NF-κB may regulate genes important in protecting cells from apoptosis. One candidate gene is bcl-2, an inhibitor of apoptosis that is known to be NF-κB regulated (Baichwal and Baeurele, 1997). To analyze Bcl-2 expression, U937 and U9-IIIB cells were treated with NAC (30 mM), TNFα (20 ng/ml), or TNFα/NAC for 0, 8, 16, 24, or 48 h and whole cell extracts (50 μg) were examined for Bcl-2 levels. NAC had little effect on Bcl-2 levels in U937 cells but resulted in a decrease in Bcl-2 protein level in U9-IIIB cells (data not shown). TNFα/NAC treatment caused a significant decrease in Bcl-2 protein with time in U9-IIIB cells (Fig. 6A, lanes 6–10) but only a slight decrease in U937 cells (Fig. 6A, lanes 1–5). Bcl-2 protein levels relative to actin levels are plotted as a function of time for TNFα/NAC-treated cells.

Bcl-2 protein levels were approximately equal in U937 and HIV-1-infected U937 cells and therefore could not account for the difference in sensitivity of U937 and U9-IIIB cells to TNFα-induced apoptosis. Previous studies demonstrated that HIV-1 Tat expression resulted in increased Bax expression, a pro-apoptotic member of the Bcl family (Sastry *et al.,* 1996). To determine whether differential expression of Bax contributed to the differences in sensitivity to apoptosis in U9-IIIB cells, extracts were prepared from cells treated with NAC, TNFα, or TNFα/NAC for 24 h. U9-IIIB cells expressed approximately two- to threefold higher levels of Bax compared to U937 cells (Fig. 6B, lanes 1 and 5). NAC treatment did not affect Bax in U937 cells or U9-IIIB cells (Fig. 6B, lanes 2 and 6), while TNFα treatment increased Bax levels approximately two-fold in U937 and U9-IIIB (Fig. 6B, lanes 3 and 7, respectively). NAC only partially inhibited TNFα-induced increase in Bax protein in U937 and U9-IIIB cells (Fig. 6B, lanes 4 and 8).

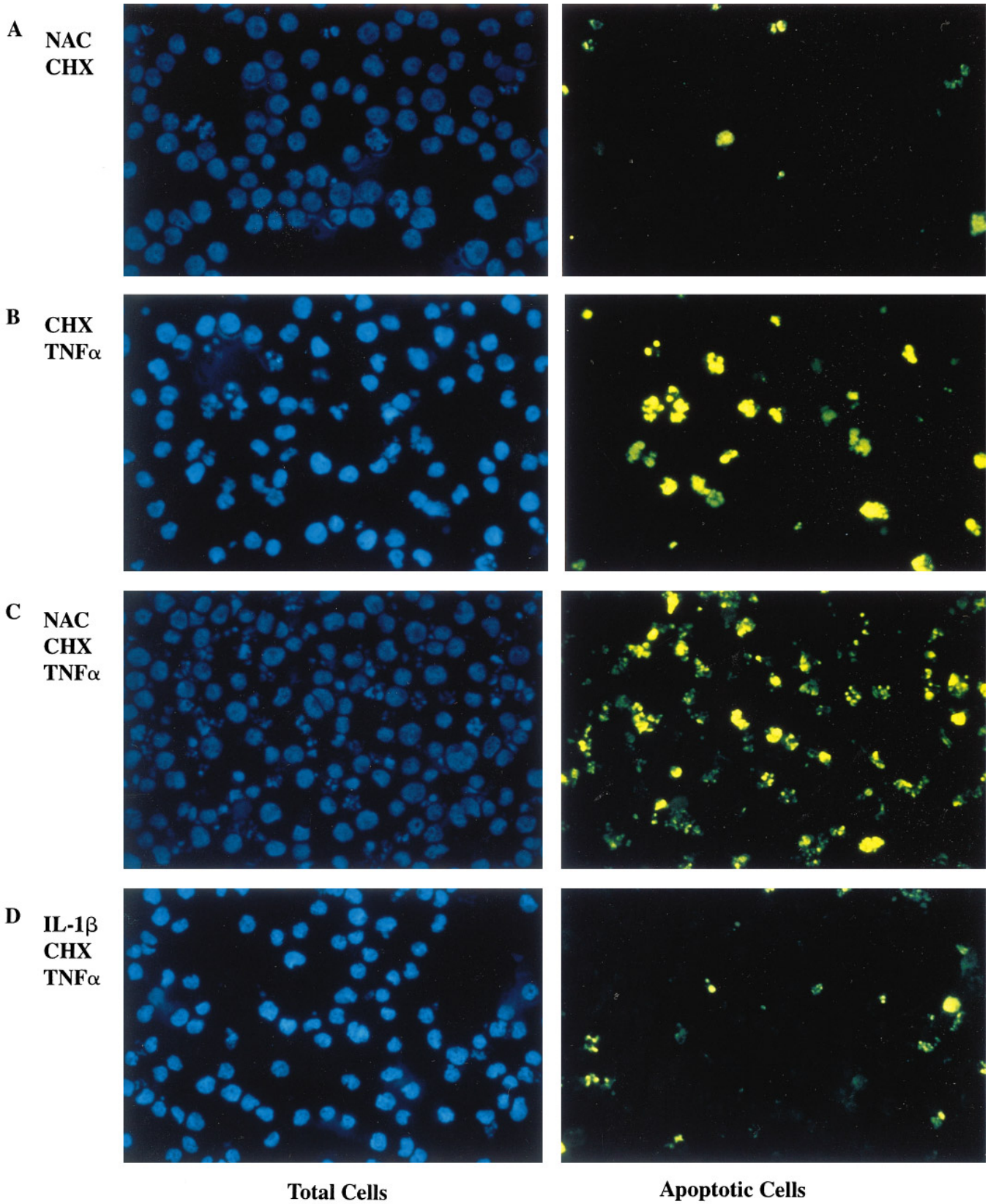


FIG. 3. TUNEL analysis of U937 cells pretreated with NAC or IL-1β followed by TNFα/CHX treatment. U937 cells were treated with NF-κB inducers or inhibitors, fixed, analyzed for TUNEL positivity and photographed (magnification 200×). (A) Cells were pretreated with NAC (50 mM) for 1 h followed by CHX (50 μg/ml) for 2 h. (B) Cells were treated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 2 h. (C) Cells were pretreated with NAC (50 mM) for 1 h and incubated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 2 h. (D) Cells were pretreated with interleukin-1β (IL-1β, 5 ng/ml) for 5 h and stimulated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 2 h. Total cells (on the left) were stained with Hoechst 33342, while apoptotic cells (on the right) were detected using the TUNEL assay (Boehringer Mannheim), as described under Materials and Methods.

Induction of apoptosis in T cells expressing IκBα repressor mutants

The protective role of NF-κB in preventing TNFα-induced apoptosis was verified in Jurkat T cells inducibly expressing nondegrading transdominant forms of IκBα (TD-IκB) under the control of a tetracycline-responsive promoter (Kwon *et al.,* 1998). In this system doxycycline (Dox) addition induces high levels of TD-IκB which effectively block TNFα-induced NF-κB activation (Kwon *et al.,* 1997). Control Jurkat cells (rtTA-Neo) and Jurkat cells expressing the IκB repressors (rtTA-IκB-2N and rtTA-IκB-2NΔ4, mutated at S32/36A) were incubated with or without Dox (1 μg/ml) for 16 h followed by TNFα (20 ng/ml) treatment for 0–24 h. As shown in Fig. 7, TNFα did not induce apoptosis in control rtTA-Neo cells in either the absence or the presence of Dox (Fig. 7A, lanes 1–5 and 6–10). Similarly, no DNA fragmentation was observed in the absence of Dox in the rtTA-IκB-2N and rtTA-IκB-2NΔ4 cells (Figs. 7B and 7C, lanes 1–5). In contrast, rtTA-IκB-2N and rtTA-IκB-2NΔ4 cells displayed DNA fragmentation after Dox induction (expression of the IκB repressors) and TNFα addition (Figs. 7B and 7C, lanes 6–10), demonstrating an increased sensitivity to apoptosis in cells blocked for NF-κB-dependent activity.

Dox induction of TD-IκB repressors was verified by immunoblot analysis (Fig. 8A) and the subsequent inhibition of NF-κB DNA binding activity was observed by EMSA (Fig. 8B). Addition of Dox to rtTA-2N and rtTA-2NΔ4 resulted in increased TD-IκB levels (Fig. 8A, lanes 5, 6, 8, and 9); interestingly, as previously described, activation of the 2NΔ4 mutant resulted in the inhibition of endogenous IκBα, as distinguished from endogenous IκBα using the MAD 10B antibody (Arenzana-Seisdedos *et al.,* 1995) (Fig. 8A, lanes 7–9). Furthermore, TNFα stimulation did not affect TD-IκB (Fig. 8B, lanes 6 and 9). Nuclear extracts from these cells confirmed that NF-κB could be activated in all three cell lines in the absence of Dox (Fig. 8B, lanes 2, 6, and 10). Dox treatment for 24 h led to the complete inhibition of TNFα induced binding activity in cells expressing TD-IκB (Fig. 8B, lanes 8 and 12) but not in control cells (Fig. 8B, lane 4). rtTA2N (Fig. 8C, lanes

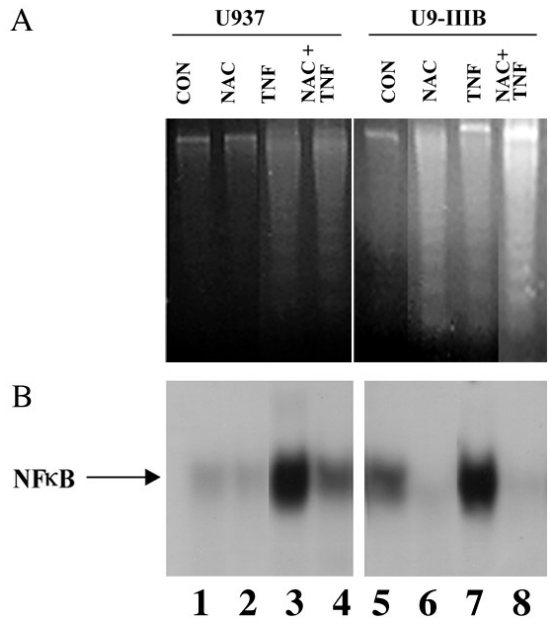
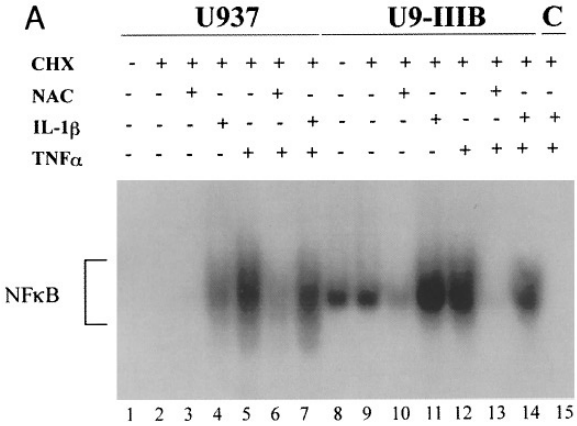


FIG. 5. NAC treatment induces DNA fragmentation in U9-IIIB cells but not in U937 cells. (A) U937 and U9-IIIB cells were treated with NAC (30 mM) or TNFα (20 ng/ml) or both for 24 h. Samples were collected and analyzed for DNA fragmentation. U937 cells undergo apoptosis in response to TNFα (lane 3) or TNFα plus NAC (lane 4). U9-IIIB cells are also sensitive to TNFα (lane 7) or TNFα plus NAC treatment (lane 8) but in addition exhibit DNA fragmentation when treated with only NAC (lane 6). (B) DNA fragmentation correlates with NF-κB binding activity. Nuclear extracts were made from U937 and U9-IIIB cells treated as above. NAC inhibits constitutive NF-κB activity in U9-IIIB cells (lane 6) as well as TNFα induced binding in both cell lines (lanes 4 and 8).



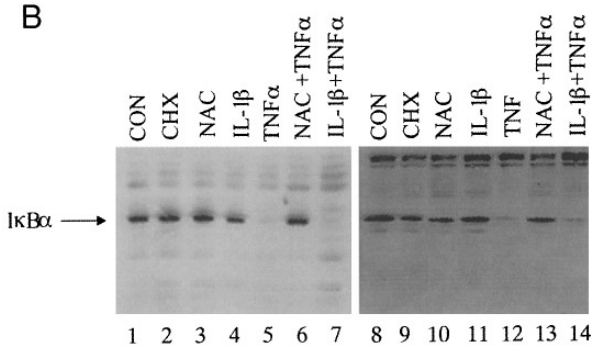


FIG. 4. Analysis of NF-κB DNA binding activity and IκBα protein levels. (A) Nuclear extracts from samples treated as in Fig. 2 were analyzed for NF-κB activity using a ^{32}P-labeled probe containing two NF-κB sites from the IFN-β promoter. U9-IIIB cells exhibit constitutive NF-κB binding activity (lane 8) that is increased by TNFα and IL-1β (lanes 11, 12, and 14). U937 cells exhibit binding activity upon TNFα and/or IL-1β stimulation (lanes 4, 5, and 7). NAC pretreatment inhibited both the constitutive binding activity in U9-IIIB cells (lane 10) as well as TNFα-induced NF-κB binding (lanes 6 and 13). (B) Whole cell extracts were made from cells treated as described in Fig. 2 and protein (30 μg) was separated by SDS–PAGE and blotted for IκBα protein. IκBα is degraded by TNFα in both U937 (lanes 5 and 7) and U9-IIIB cells (lanes 12 and 14) and this degradation is blocked by pretreatment with NAC (lanes 6 and 13, respectively). Equal amounts of protein were confirmed by actin staining (data not shown).

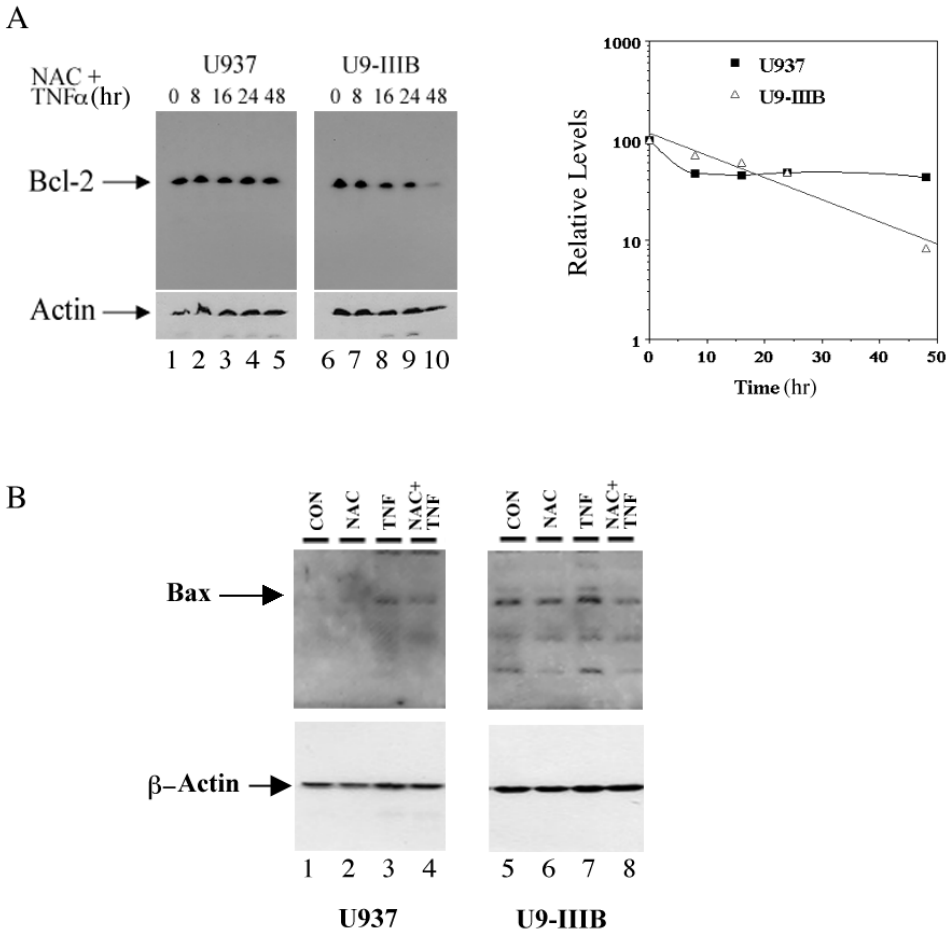


FIG. 6. Bcl-2 and Bax protein levels in U937 and U9-IIIB cells. U937 and U9-IIIB cells were treated with TNFα (20 ng/ml) and NAC (30 mM) for 0, 8, 16, 24, or 48 h. Whole cell extracts were electrophoresed and blotted for Bcl-2 and β-actin protein levels. The ratio of Bcl-2 to β-actin was calculated and plotted on a semi-log scale. (A) Bcl-2 levels dramatically decrease in NAC/TNFα-treated U9-IIIB cells while showing only a modest decrease in U937. (B) The 24-h time points were analyzed for Bax levels by Western blot analysis. U937 cells (left panel) express barely detectable levels of Bax protein, which increases upon TNFα induction (lane 3). U9-IIIB cells (right panel) express a higher level of Bax protein (lane 5) that is further augmented by TNFα treatment (lane 7).

7–12) and rtTA2NΔ4 (Fig. 8C, lanes 13–18) cells induced to express the TD-IκB repressors also downregulated Bcl-2 protein levels upon prolonged TNFα treatment, indicating that bcl-2 expression is controlled by NF-κB induction. Bcl-2 levels in control cells were not affected (Fig. 8C, lanes 1–6) and equal protein was confirmed by actin staining (data not shown). rtTA-Jurkat cells were subsequently treated with combinations of NAC, CHX, and TNFα in the absence of TD-IκB induction to determine if inhibition of NF-κB by NAC rather than TD-IκB expression produced similar results. As shown in Table 2, TNFα/CHX treatment induced apoptosis which was further augmented by NAC pretreatment, as was described above for myeloid cells.

DISCUSSION

Macrophages and cells of the myeloid lineage are permissive for HIV-1 infection but infection is generally not cytolytic, leading to the concept that cells of the myeloid lineage may serve as viral reservoirs capable of transmitting virus to $CD4^+$ T cells. The inherent phagocytic properties of monocytes and macrophages, coupled with their ability to migrate throughout the body, likely contribute to the extensive dissemination of virus observed in HIV-1 infection (reviewed in Perno *et al.,* 1997).

NF-κB has been implicated in the regulation of stimulus-induced apoptosis, inducing cell death in some circumstances (Baeuerle and Baltimore, 1996; Lin *et al.,* 1995; Jung *et al.,* 1995; Abbadie *et al.,* 1993) and protecting in others (Van Antwerp *et al.,* 1996; Beg and Baltimore, 1996; Wang *et al.,* 1996). The results of the present study indicate that TNFα induces apoptosis in U937 and U9-IIIB cells and the extent of cytotoxicity is inversely correlated with the level of NF-κB activation. Inhibition of NF-κB activation using the anti-oxidant NAC increased apoptosis in both infected and noninfected cells. Conversely, deliberate activation of NF-κB by the noncyto-

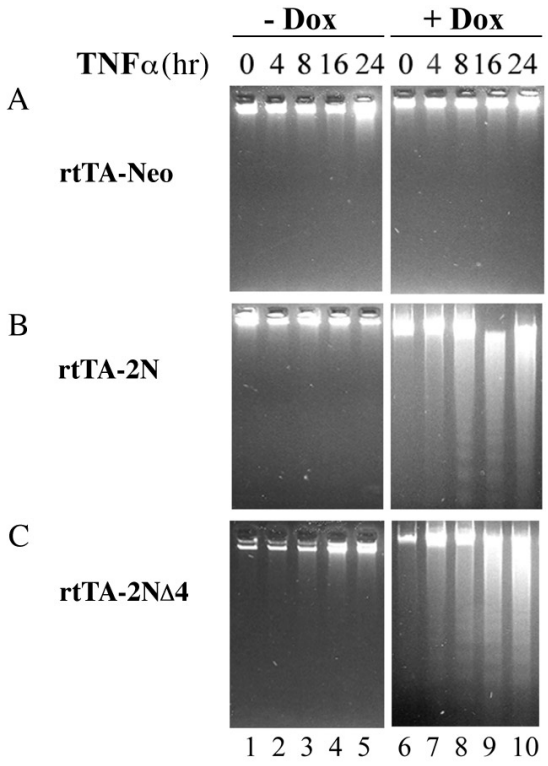


FIG. 7. TD-IκB expression induces sensitivity to TNFα-signaled apoptosis. Jurkat T cells inducibly expressing IκBα mutated in two critical serine residues, abolishing inducer-mediated degradation, were treated with TNFα (20 ng/ml) for 0, 4, 8, 16, or 24 h. IκBα was induced prior to TNFα treatment with doxycycline (Dox 1 μg/ml) for 16 h. TNFα treatment does not induce DNA fragmentation in control rtTA-Neo (A) cells but does in rtTA-2N (B) and rtTA-2NΔ4 (C) which express transdominant IκBα (compare lanes 1–5 and 6–10).

toxic inducer IL-1β partially rescued cells from TNFα/CHX-induced apoptosis. In T cells, NF-κB antagonized TNFα-induced apoptosis and conversely its inhibition exacerbated TNFα cytotoxicity.

Interestingly, NAC incubation alone or CHX/NAC-induced HIV-1-infected cells to undergo apoptosis. HIV-1-infected monocytic cells may receive a persistent apoptotic signal that is countered by the maintenance of a redox-sensitive, NF-κB-induced, anti-apoptotic protein. Although HIV-1-infected cells constitutively express NF-κB, they are still more sensitive to TNFα-mediated cytotoxicity. This may suggest that HIV-1 infection propels an apoptotic pathway in myeloid lineage cells that is countered by NF-κB activation. Cells are therefore more sensitive to further cytotoxic insults since an apoptotic pathway is ready to be executed. The effect of NAC on chronically infected U9-IIIB cells may be similar to its effect on TNFα-induced apoptosis. Productive HIV-1 in-

TABLE 2

NAC Inhibition of TNFα-Induced NF-κB Activation Augments Apoptosis in Jurkat Cell Lines

Condition	rtTA-Neo	rtTA-2NΔ4
Control	0.52	8.2
Cycloheximide	14.1 ± 0.54	17.5 ± 1.8
NAC + cycloheximide	14.4 ± 1.3	15.2 ± 1.2
TNFα + cycloheximide	22.0 ± 1.2	30.6 ± 2.1
NAC + TNFα + cycloheximide	54.5 ± 8.7	45.0 ± 4.5

Note. Jurkat rtTA-Neo, rtTA-2N, and rtTA-2NΔ4 cells were pretreated with NAC (30 mM) for 1 h and stimulated with TNFα(30 ng/ml)/CHX (50 μg/ml) for 2 h. Tunel assay was used to quantitate the number of apoptotic cells. Values are the average percentage of apoptosis of two experiments.

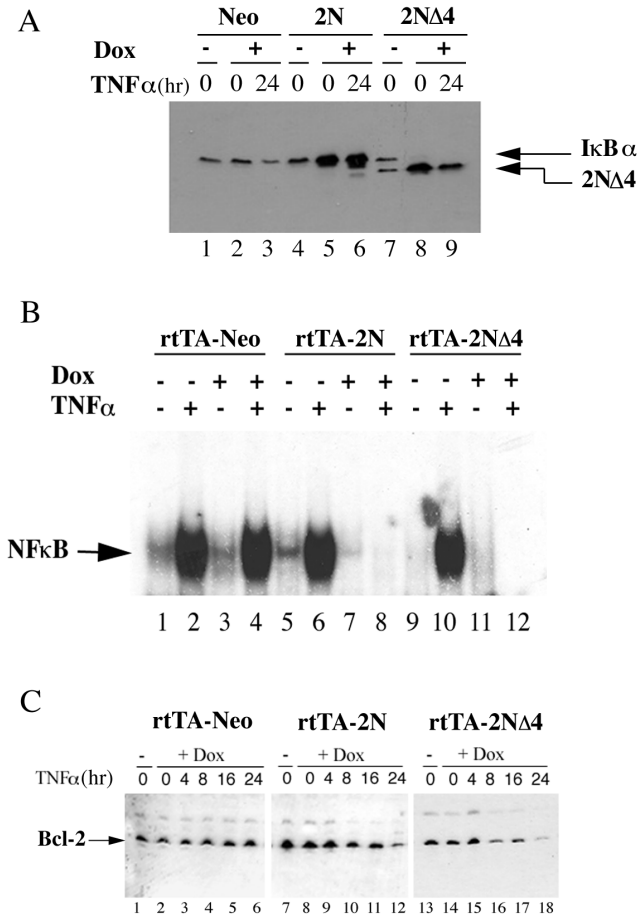


FIG. 8. TD-IκBα is resistant to TNFα-induced degradation and leads to decreased Bcl-2 expression in TNFα-treated cells. Jurkat rtTA-Neo, rtTA-2N, and rtTA-2NΔ4 cells were incubated with or without Dox (1 μg/ml) for 24 h to induce expression of the IκBα mutants. Cells were subsequently treated with TNFα (20 ng/ml) for 24 h. (A) Western blot analysis of whole cell extracts was performed using an N-terminal IκBα mAb which identifies both endogenous IκBα and the C-terminal truncated 2NΔ4 (lower band). IκBα levels were unchanged by Dox addition to control rtTA-Neo cells (compare lanes 1 and 2) or by TNFα addition for 24 h (lane 3). Dox induction of rtTA-2N and rtTA-2NΔ4 cells led to increased IκBα expression (lanes 5 and 8) which was not degraded upon TNFα stimulation (lanes 6 and 9). (B) Electrophoretic mobility shift assay (EMSA) analysis illustrated that NF-κB activation by TNFα was blocked in Dox-treated cells expressing the TD-IκBα (lanes 8 and 12) but not in control rtTA-Neo cells (lane 4). (C) Western blot analysis of Bcl-2 levels also revealed that Dox-treated Jurkat rtTA-2N (lanes 7–12) and rtTA-2NΔ4 (lanes 13–18) but not rtTA-Neo (lanes 1–6) cells decreased Bcl-2 levels with increased TNFα incubation.

fection leads to increased TNFα expression, which may stimulate cells by an autocrine regulatory mechanism. Constitutive expression of TNFα mRNA is present in U9-IIIB but not U937 cells and can be induced to higher levels in HIV-1-infected cells (Lacoste *et al.,* 1990). Also, U9-IIIB cells strongly upregulate IL-1β gene transcription and protein production in response to PMA stimulation, while U937 cells do not express detectable IL-1β mRNA (D'Addario *et al.,* 1992). Increased TNFα and/or IL-1β levels would activate NF-κB and protect cells from HIV-1-induced apoptosis. Alternatively, HIV-1 infection may affect another apoptotic pathway that is sensitive to NF-κB inhibition.

NF-κB activation potently stimulates HIV-1 replication by inducing LTR-driven gene expression (reviewed in Roulston *et al.,* 1995) and is responsible for the robust viral replication seen upon TNFα stimulation. NF-κB is also responsible for the activation of numerous cellular genes, including immunoregulatory molecules and cytokines. Activation of NF-κB by various stimuli including TNFα, which transduce their activation signal through radical oxygen intermediates (ROI), can be inhibited by pretreating cells with the antioxidant NAC. NAC is thought to inhibit signaling directly through its capacity as a ROI scavenger as well as through its ability to replenish cellular glutathione (GSH) levels (Staal *et al.,* 1993). Replenishing GSH strongly suppressed HIV-1 replication in human macrophages (Garaci *et al.,* 1997), as did NAC addition in latently infected cell lines (Roederer *et al.,* 1991) and PBMC (Roederer *et al.,* 1990). Others have found that NAC did not affect HIV-1 replication in chronically infected promonocytic U937 cells (Aillet *et al.,* 1994) or actually stimulated HIV-1 virus replication in monocyte-derived macrophages (Nottet *et al.,* 1997).

Murine macrophages and fibroblasts deficient in the prototypical transactivating NF-κB subunit RelA are highly sensitive to TNFα-induced apoptosis (Beg and Baltimore, 1996) and reintroduction of RelA repressed the toxic effects of TNFα. Membrane interaction with antibodies against IgM in WEHI 231 B cells also induces apoptosis that is exacerbated by the inhibition of NF-κB (Wu *et al.,* 1996). Ectopic expression of c-Rel rescued these cells from anti-IgM-induced apoptosis, while microinjection with a GST-IκBα fusion protein or anti-c-Rel antibody induced cell death. Interestingly, we previously reported dramatic increases in c-Rel protein levels in both U937 and U9-IIIB cells upon TNFα stimulation (DeLuca *et al.,* 1996). It will be interesting to determine if c-Rel is involved in protecting these cells from apoptosis.

The anti-apoptotic effects of NF-κB are supported by experiments in Jurkat cells which are engineered to inducibly express transdominant IκBα repressors (TD-IκB) (Kwon *et al.,* 1998). Normally resistant to TNFα toxicity, Jurkat cells treated with TNFα underwent rapid apoptosis when NF-κB induction was abolished by the induction of TD-IκB. Similar results were obtained by Verma and colleagues (Van Antwerp *et al.,* 1996), who examined TNFα-induced apoptosis in cells constitutively overexpressing a transdominant IκBα mutant harboring the Ser-32/36-Ala modification in the signal response domain. Both the sensitivity and the kinetics of TNFα-induced apoptosis were enhanced in the cell lines tested (Van Antwerp *et al.,* 1996). Together with the previous studies, our results indicate that S32/36A mutations are sufficient to confer sensitivity to TNFα-induced apoptosis. Additionally, Jurkat cells pretreated with NAC, in the absence of TD-IκB expression, exhibited heightened sensitivity to TNFα/CHX-induced apoptosis. These results suggest that the effect of NAC on TNFα-induced apoptosis in HIV-1-infected and noninfected U937 cells is due to inhibition of NF-κB activation.

Various NF-κB-regulated anti-apoptotic gene products are known to protect cells from programmed cell death, including the zinc finger protein A20 (Jaattela *et al.,* 1996; Tewari and Dixit, 1996) and members of the Bcl-2 family (reviewed in Yang and Korsmeyer, 1996; Reed *et al.,* 1996). Expression of A20 in Jurkat cells expressing a transdominant IκBα mutant did not restore protection from TNFα-induced apoptosis (Van Antwerp *et al.,* 1996), suggesting that it is either not the target of NF-κB transactivation or that it acts in cooperation with other anti-apoptotic genes.

Bcl-2 expression counters apoptotic signaling by a vast array of inducers in many cell models (reviewed in Yang and Korsmeyer, 1996). Several Bcl-2 proteins have been identified in mammals including Bcl-xL, Bcl-w, and Mcl-1, which inhibit apoptosis, and Bax, Bik, Bak, Bad, and Bcl-xs, which activate apoptosis. Cell fate is determined by the relative ratio of anti- and pro-apoptotic members. Decreased Bcl-2 levels have been reported to account for the induction of apoptosis by HIV-1 infection in some cell systems (Strack *et al.,* 1996; DeRossi *et al.,* 1994). Although Bcl-2 levels were comparable in infected and noninfected cells, the level of Bax was elevated in HIV-1-infected U937 cells, suggesting that increased Bax expression may contribute to the rapid induction of apoptosis observed in HIV-1-infected cells. Bcl-2 expression decreased in TNFα-treated Jurkat cells which were deficient in NF-κB induction because of TD-IκB expression but not in cells in which NF-κB could be activated. Bcl-2 levels were also modulated by TNFα and NAC treatments in U937 and U9-IIIB, although the decreases were not dramatic. This was particularly true in noninfected cells, suggesting that other NF-κB-regulated proteins may be important in protecting these cells from TNFα-induced apoptosis. During the review of this work, c-IAP2 was identified as an NF-κB-regulated gene involved in the suppression of TNFα-induced cell death (Chu *et al.,* 1997). It will be interesting to determine if c-IAP2 expression is dysregulated in HIV-1-infected myeloid cells. Macrophages and cells of the monocyte lineage produce TNFα in response to viral challenge and are important perpetrators of viral spread in HIV infection. Understanding the basis of monocyte resistance to HIV-1-induced cytopathic effects

may be crucial to limiting viral dissemination and battling the devastation of AIDS.

MATERIALS AND METHODS

Cell culture

Promonocytic U937 and HIV-1 infected U9-IIIB cells, as well as myelomonoblastic PLB-985 and HIV-1-infected PLB-IIIB cells (infected with HIV strain IIIB) were maintained in RPMI 1640 (GIBCO, Life Technologies Inc., Grand Island, NY) supplemented with 5% Fetal Clone (Hyclone, Logan, Utah), 2 mM L-glutamine, and 20 μg/ml gentamicin (Schering Canada, Pointe Claire, Quebec). Jurkat T cells stably expressing Dox-inducible transdominant mutants of IκBα (rtTA Jurkat 2N, containing an IκBα mutated in at S32A/S36A; rtTA Jurkat 2NΔ4, containing an IκBα mutated at S32A/S36A and 22-aa C-terminal truncation) or the empty Neo vector (rtTA-Neo-Jurkat) were previously described (Kwon *et al.,* 1998). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 20 μg/ml gentamicin. The transgenes were induced by the addition of Dox (1 μg/ml) to the culture medium for a minimum of 16 h. Cells were seeded at a density of 0.5×10^6 cells/ml for all experiments and treated with various inducers as indicated in the figure legends: 10–30 ng/ml of recombinant human TNFα (0.1% BSA in PBS, R&D Systems), 5–10 ng/ml IL-1β (0.1% BSA in PBS, R&D Systems), 30–50 mM *N*-acetylcysteine (PBS, pH 7.5, Boehringer Mannheim), and 50–100 μg/ml cycloheximide (Sigma).

Western blot analysis

Whole cell extracts were prepared by resuspension in NP-40 lysis buffer (10 mM Tris–HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5 mM PMSF, and 0.01 mg/ml each of leupeptin, pepstatin, and aprotinin). After incubation on ice for 10 min, cellular debris was removed by a 10-min centrifugation at 4°C. Cell extracts (20–100 mg) were resolved by SDS–PAGE and then transferred to a nitrocellulose membrane. Membranes were blocked for a minimum of 2 h in 5% skim milk and incubated overnight in 5% milk containing anti-peptide monoclonal or polyclonal antisera (dilutions ranged from 1:250 to 1:1000). Monoclonal IκBα antiserum MAD-10B was a kind gift from Ron Hay (Jaffray *et al.,* 1995). Antiserum AR20, which recognizes the N-terminus of IκBα (aa 2–16), was prepared as described previously (Pepin *et al.,* 1994). Monoclonal anti-Bcl-2 and polyclonal anti-Bax were purchased from Santa Cruz Inc. and monoclonal anti β-actin was purchased from Boehringer Mannheim. The membranes were rinsed four times in PBS and incubated with a secondary antibody, FITC-conjugated goat anti-rabbit, or rabbit anti-mouse (1:1000, Amersham) for 1 h at room temperature and then rinsed again four times in PBS. The ECL–Western blotting detection system (NEN Life Sciences) was used according to manufacturer's instructions to visualize the specific signals. Autoradiograms were scanned by laser scanner and quantified using NIH Image 1.60 software. Bcl-2:actin ratios are presented relative to levels in unstimulated cells. Values were plotted on a semi-log scale and the best fit curves were determined. The experiments were repeated a minimum of three times and representative autoradiograms are shown.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from untreated cells or cells treated for varying times with one or a combination of the following inducers: TNFα (20 ng/ml), *N*-acetylcysteine (50 mM), IL-1β (5 ng/ml), and cycloheximide (50 μg/ml). Briefly, cells were washed in Buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol (DTT); and 0.5 mM phenylmethylsulfonyl flouride (PMSF)) and were resuspended in Buffer A containing 0.1% NP-40. Cells were then chilled on ice for 10 min before being centrifuged at 10,000 *g.* Pellets were then resuspended in Buffer B (20 mM HEPES, pH 7.9; 25% glycerol; 0.42 M $NaCl_2$; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT; 0.5 mM PMSF; 5 μg/ml leupeptin; 5 μg/ml pepstatin; 0.5 mM spermidine; 0.15 mM spermine; and 5 μg/ml aprotinin). Samples were incubated on ice for 15 min before being centrifuged at 10,000 *g.* Nuclear extract supernatants were diluted with Buffer C (20 mM HEPES, pH 7.9; 20% glycerol; 0.2 mM EDTA; 50 mM KCl; 0.5 mM DTT; and 0.5 mM PMSF). Nuclear extracts were subjected to EMSA using a ^{32}P-labeled probe corresponding to the PRDII region of the IFN-β promoter (5′-GGAAATTCCGGGAAATTCC-3′) as described (Arai *et al.,* 1990). The resulting protein–DNA complexes were resolved by a 5% Tris–glycine gel and exposed to X-ray film. To demonstrate the specificity of protein–DNA complex formation, a 125-fold molar excess of unlabeled oligonucleotide was added to the nuclear extract before adding the labeled probe.

DNA fragmentation

Following treatments, $\sim 2 \times 10^6$ cells were pelleted, washed with phosphate-buffered saline (PBS), resuspended in 250 μl of lysis buffer (20 mM Tris–HCl, pH 7.5; 10 mM borate; 0.25% NP-40; 0.1 mg/ml RNase), and incubated for 1 h at 37°C. Proteinase K was added to a final concentration of 1 mg/ml and extracts were incubated for an additional hour. Samples were separated on a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide and visualized by UV illumination.

TUNEL analysis

Apoptosis was quantified using an *in situ* cell death detection kit (Boehringer Mannheim). Approximately 1×10^6 cells were centrifuged, washed once with PBS, and resuspended in 20 μl of PBS. Cells were plated on a multichamber slide, air dried, and fixed with 4% parafor-

maldehyde for 30 min at room temperature. Slides were rinsed twice with PBS and incubated for 2 min at 4°C in permeabilization solution (0.1% Triton; 0.1% sodium citrate), rinsed with PBS, and incubated with fluorescein-labeled TUNEL reaction mixture for 1 h at 37°C in a humid, darkened chamber. Slides were again rinsed with PBS, incubated with the nuclear dye Hoescht 33342 (0.4 ng/ml) to stain all nuclei, washed with PBS, and embedded in mounting solution (10 mM Tris–HCL, pH 8.8; 0.1 M propylgallate in glycerol). Samples were analyzed by fluorescence microscopy and the percentage of apoptotic cells was determined by counting a minimum of 350 nuclei (blue filter) and the corresponding TUNEL-positive cells (green filter).

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