# HIV-1 Tat potentiates TNF-induced NF-κB activation and cytotoxicity by altering the cellular redox state

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This study demonstrates that human immunodeficiency virus type 1 (HIV-1) Tat protein amplifies the activity of tumor necrosis factor (TNF), a cytokine that stimulates HIV-1 replication through activation of NF-κB. In HeLa cells stably transfected with the HIV-1 *tat* gene (HeLa-tat cells), expression of the Tat protein enhanced both TNF-induced activation of NF-κB and TNF-mediated cytotoxicity. A similar potentiation of TNF effects was observed in Jurkat T cells and HeLa cells treated with soluble Tat protein. TNF-mediated activation of NF-κB and cytotoxicity involves the intracellular formation of reactive oxygen intermediates. Therefore, Tat-mediated effects on the cellular redox state were analyzed. In both T cells and HeLa cells HIV-1 Tat suppressed the expression of Mn-dependent superoxide dismutase (Mn-SOD), a mitochondrial enzyme that is part of the cellular defense system against oxidative stress. Thus, Mn-SOD RNA protein levels and activity were markedly reduced in the presence of Tat. Decreased Mn-SOD expression was associated with decreased levels of glutathione and a lower ratio of reduced:oxidized glutathione. A truncated Tat protein (Tat$_{1-72}$), known to transactivate the HIV-1 long terminal repeat (LTR), no longer affected Mn-SOD expression, the cellular redox state or TNF-mediated cytotoxicity. Thus, our experiments demonstrate that the C-terminal region of HIV-1 Tat is required to suppress Mn-SOD expression and to induce pro-oxidative conditions reflected by a drop in reduced glutathione (GSH) and the GSH:oxidized GSH (GSSG) ratio. They further imply a distinct mechanism of Mn-SOD suppression as compared with HIV-1 LTR transactivation by Tat. Taken together, our data suggest that Tat expressed in HIV-1-infected cells and Tat taken up by non-infected cells modulates TNF activity by altering the cellular redox state. These findings may be relevant for HIV-1 replication and for T cell depletion in acquired immune deficiency syndrome.
*Key words*: cytotoxicity/Mn-SOD/NF-κB activation/Tat/TNF

## Introduction

The Tat protein from human immunodeficiency virus type 1 (HIV-1) is essential for efficient HIV-1 gene expression. Tat acts by binding to a transactivation response element (TAR), an RNA stem−loop structure located close to the 5′ end of HIV-1 transcripts (Berkhout *et al.*, 1989; Dingwall *et al.*, 1989). Tat increases the rate of transcription (for reviews see Sharp and Marciniak, 1989; Cullen, 1990) by either enhanced transcriptional initiation (Hauber *et al.*, 1987; Southgate and Green, 1991) or increased transcriptional elongation (Laspia *et al.*, 1990; Feinberg *et al.*, 1991; for a review see Frankel, 1992), or a combination of both processes. Tat exhibits immuno-suppressive properties and inhibits antigen-induced T cell proliferation (Viscidi *et al.*, 1989; Meyaard *et al.*, 1992). Furthermore, the expression of a number of cellular genes and proteins is influenced by HIV-1 Tat. Thus, lymphotoxin, tumor necrosis factor (TNF), interleukin (IL)-2 and IL-6 genes (Buonaguro *et al.*, 1992; Scala *et al.*, 1994; Westendorp *et al.*, 1994) are transactivated by Tat, and collagen expression in glioblastoma cells is increased (Taylor *et al.*, 1992). However, Tat was also shown to suppress promoter activity of major histo-compatibility complex (MHC) class I genes (Howcroft *et al.*, 1993), to decrease IL-2 receptor expression in Jurkat T cells (Purvis *et al.*, 1992) and to down-regulate TNF receptor surface expression in Raji B lymphoblastoid cells (Poesik *et al.*, 1992). Finally, HIV-1 Tat is secreted by HIV-1-infected cells and can be taken up rapidly by other cells (Cullen, 1986; Frankel and Pabo, 1988; Marcuzzi *et al.*, 1992; Westendorp *et al.*, 1994). Thus, Tat can potentially influence cellular gene expression in infected and non-infected cells.

TNF was found to amplify HIV-1 expression in chronically infected T lymphocytes, monocyte cell lines and primary monocytes (Folks *et al.*, 1989; Mellors *et al.*, 1991). It stimulates HIV-1 replication via activation of the transcription factor NF-κB, which binds to the κB enhancer motifs in the long terminal repeat (LTR; Duh *et al.*, 1989; Osborn *et al.*, 1989). In unstimulated cells, NF-κB is present in the cytoplasm in an inactive form complexed with its inhibitory subunit IκB (Baeuerle and Baltimore, 1988). When cells are stimulated with TNF, IkB rapidly dissociates from the inactive complex. This process involves the activation of certain protein kinases and proteases (Henkel *et al.*, 1993) and allows translocation of NF-κB into the nucleus and subsequent binding to its DNA motifs (Baeuerle, 1991; for a review see Blank *et al.*, 1992).

It has been shown previously that activation of NF-κB in response to TNF and other agents is mediated by pro-oxidative conditions (Schreck *et al.*, 1991; Schulze-Osthoff *et al.*, 1993). Thus, exogenous reducing agents, such

as *N*-acetyl-cysteine and glutathione, suppressed TNF-induced activation of NF-κB (Staal *et al.*, 1990; Mihm *et al.*, 1991) and HIV-1 expression in infected Molt-4 T leukemia cells (Roederer *et al.*, 1990).

HIV-1 infection is associated with a substantial decrease in $CD4^+$ T lymphocytes (Meyaard *et al.*, 1992). It has been proposed that $CD4^+$ T cells are depleted by apoptosis (Ameisen and Capron, 1991). According to one model, T cells are primed to undergo apoptosis upon crosslinking of CD4 by gp120 (Banda *et al.*, 1992; Gougeon and Montagnier, 1993). Subsequent activation by conventional antigens or superantigens induces apoptosis in these T cells (Ameisen and Capron, 1991). Both TNF and lymphotoxin play a central role in the course of the HIV-1 infection and acquired immune deficiency syndrome (AIDS). High levels of TNF have been observed in sera (Zauli *et al.*, 1992) and in supernatants of cultured monocytes (Wright *et al.*, 1988), peripheral blood mono-nuclear cells (Vyakarnam *et al.*, 1991) and alveolar macro-phages from AIDS patients (Krishnan *et al.*, 1990). It has also been reported that an abnormal cellular redox state contributes to apoptosis of $CD4^+$ T cells (Dröge *et al.*, 1992), and that TNF, by generating oxidative stress, may be one of the mediators causing lysis of HIV-1-infected $CD4^+$ T cells (Huber *et al.*, 1992). However, it is unclear what events render cells susceptible to the cytotoxic action of TNF during HIV-1 infection.

In this study we examined the ability of HIV-1 Tat protein to modulate TNF activity. It was found that HIV-1 Tat potentiates TNF-induced NF-κB activation and TNF-mediated cytotoxicity by down-regulation of Mn-dependent superoxide dismutase (Mn-SOD), shifting the cellular redox state towards pro-oxidative conditions. Thus HIV-1 Tat might provide the link between viral infection and the observed oxidative stress and consequent $CD4^+$ T cell depletion in AIDS.

## Results

### Expression of HIV-1 Tat potentiates TNF-induced activation of NF-κB and TNF-mediated cytotoxicity

To explore the effects of endogenous tat expression on TNF-induced activation of NF-κB and TNF-mediated cytotoxicity, HeLa cells stably transfected with a *tat* expression vector were employed. Expression of Tat protein was evident from a 1200-fold increase of HIV LTR-directed reporter gene expression in the transfected compared with the wild-type HeLa cells (data not shown). To monitor NF-κB activation, parental cells and HeLa-tat cells were exposed to TNF (10 ng/ml). After 30 min, cells were lyzed and analyzed in electrophoretic mobility shift assays (EMSA) using an NF-κB-specific $^{32}$P-labeled oligonucleotide. As shown in Figure 1, constitutive and TNF-induced NF-κB DNA binding was significantly elevated in HeLa-tat cells in comparison with parental cells. Since NF-κB was reported previously to be inhibited by antioxidants (Schreck *et al.*, 1991; Schulze-Osthoff *et al.*, 1993), we further studied the effect of the antioxidant butylated hydroxyanisole (BHA) on Tat-modulated NF-κB activation. Parental cells pretreated with 500 μM BHA revealed a significant decrease of TNF-induced NF-κB DNA binding (Figure 1A, lane 4). Similarly, in HeLa-tat cells activation of NF-κB in response to TNF was strongly
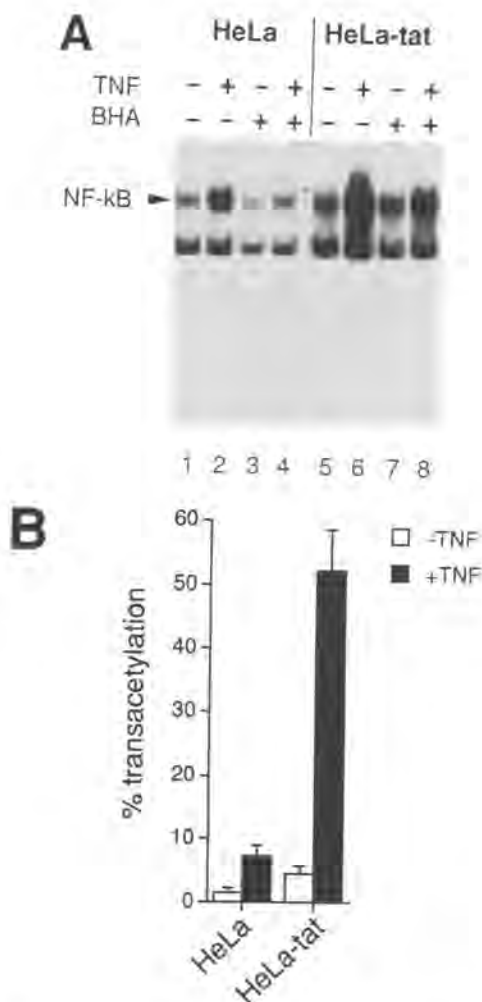


Fig. 1. TNF-induced activation of NF-κB in HeLa-tat and parental cells. (A) NF-κB binding activity. HeLa-tat and parental cells ($1 \times 10^6$ each) were cultured in 6-well microtiter plates overnight. Then BHA (500 μM) and subsequently TNF (10 ng/ml) were added and incubation continued for 30 min. Extracts were prepared and analyzed for NF-κB binding activity in EMSA. (B) NF-κB-dependent transactivation of gene expression. HeLa cells or HeLa-tat cells were transiently transfected with a $4 \times$ NF-κB/CAT construct. NF-κB activity in the presence or absence of TNF (10 ng/ml) was quantified as percent transacetylation. Mean values and standard deviations of triplicate samples are given.

inhibited by BHA (Figure 1A, lane 8), but was still stronger than in the parental cells (Figure 1A, lane 4).

To provide evidence that increased NF-κB binding activity was also reflected by increased NF-κB-mediated transcriptional activation, we transiently transfected HeLa cells and HeLa-tat cells with a reporter gene construct carrying four copies of the NF-κB binding sequence in front of the CAT gene. As shown in Figure 1B, constitutive and TNF-induced (10 ng/ml) NF-κB transactivating activities were increased several-fold in HeLa-tat cells as compared with the parental HeLa cells. The influence of the expression of Tat on TNF-induced cytotoxicity was investigated next. HeLa and HeLa-tat cells were assayed for TNF sensitivity. As shown in Figure 2A, HeLa-tat cells were more susceptible to the cytotoxic activity of TNF than the parental cell line. To kill 50% of HeLa-tat cells, ~100 times less TNF was required than for the
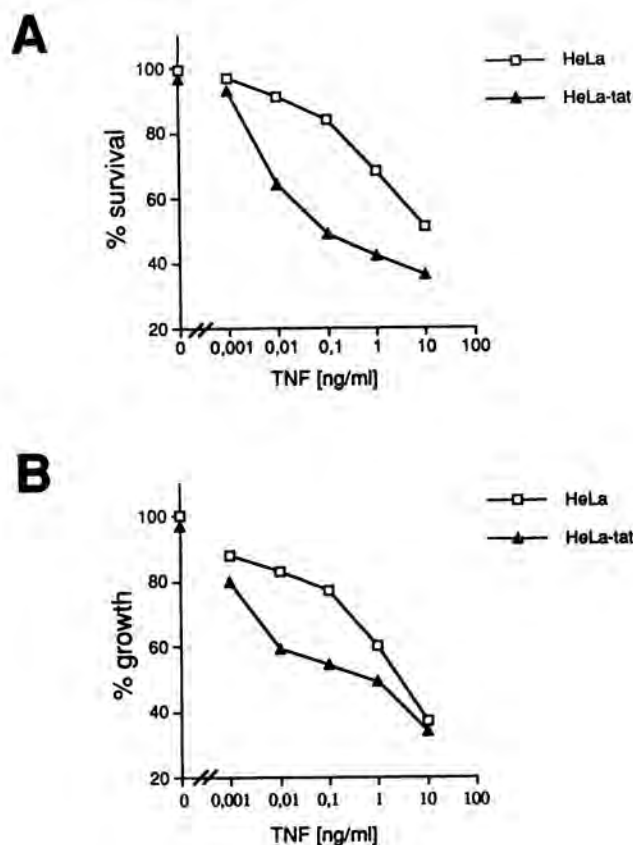
**Fig. 2.** Survival and growth inhibition of HeLa-tat and parental cells in response to TNF. (A) Survival of cells. HeLa-tat and HeLa parental cells were seeded in 96-well plates at a density of $10^4$ cells/well. After 16 h, cell cultures were incubated with TNF and incubation was continued for 16 h in the presence of actinomycin D (1 µg/ml). Survival was assayed by the reduction of MTT and calculated as the percentage of the staining value of TNF-treated and untreated cultures. (B) Growth inhibition. HeLa-tat cells and parental cells were seeded and exposed to TNF as described above. Cultures were incubated for a 20 h period. At 6 h before harvest, each culture received 0.5 µCi [³H]thymidine. Cells were then lyzed with 1% SDS, 0.1 N HCl and radioactivity was counted. Percent growth was calculated as the percentage of [³H]thymidine incorporation of TNF-treated and untreated cultures. 100% growth corresponds to 73 795 c.p.m.



**Fig. 3.** TNF-induced activation of NF-κB in HeLa cells (**A**) and Jurkat T cells (**B**) after treatment with sTat protein. (A) $1 \times 10^5$ HeLa cells were grown in 35 mm Petri dishes, washed with PBS and scraped off the plastic surface in 0.5 ml PBS containing 1 µg/ml sTat at 4°C. Then 2 ml of culture medium were added and the incubation continued. After 16 h, TNF was added (0.1, 1.0 and 10.0 ng/ml). After a further 30 min of incubation, cell extracts were prepared and analyzed for NF-κB binding activity in EMSA. (B) $1 \times 10^5$ Jurkat T cells were incubated with 1 µg/ml sTat which was added to the medium for 16 h. Subsequently, cells were treated with TNF (10 ng/ml) and processed as in (A).

parental cells. Similarly, HeLa-tat cells were considerably more susceptible to growth inhibition by TNF (Figure 2B).

### Effect of synthetic Tat (sTat) on TNF activities

It has been shown previously that extracellular Tat is present in the supernatant of HIV-1-infected cells in culture (Zauli *et al.*, 1992). Furthermore, HIV-1 Tat can enter and disturb non-infected cells *in vitro* and also possibly *in vivo*. Therefore, to investigate whether the observed effects in *tat*-transfected cells were caused by clonal heterogeneity, experiments with exogenously added Tat protein were performed. Tat was synthesized as a full-length protein (Kraft *et al.*, 1994). Control experiments using cells transfected with an HIV-1 LTR reporter construct confirmed that incubation with sTat had transactivating activity (data not shown). HeLa cells were preincubated with 1 µg/ml sTat for 24 h and then exposed to various concentrations of TNF for 30 min. As shown in Figure 3A, the addition of sTat protein significantly enhanced TNF-induced activation of NF-κB. However, Tat treatment alone did not result in enhanced NF-κB binding activity
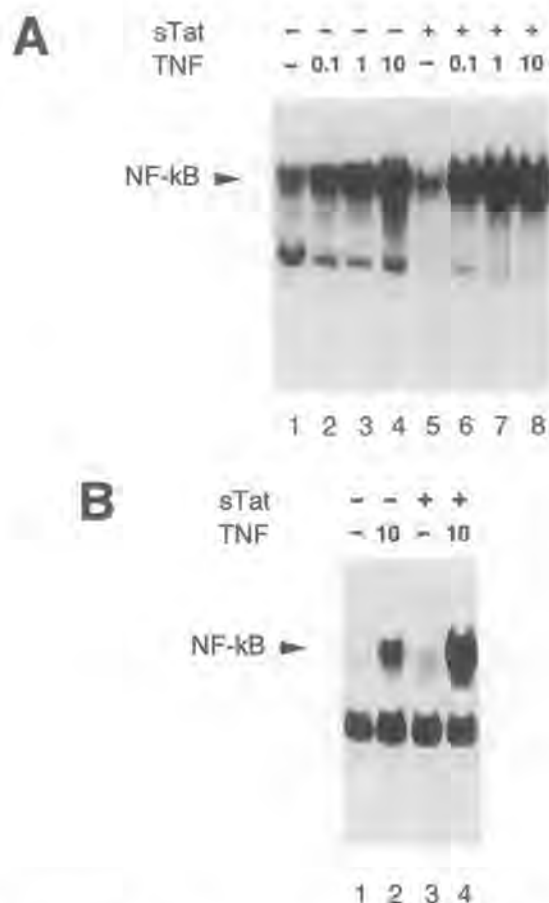
(Figure 3A). Thus, the higher constitutive NF-κB activity in HeLa-tat cells compared with HeLa cells may be due to clonal heterogeneity and not be directly caused by Tat. To investigate whether the Tat effects observed in HeLa cells are also seen in T cells, we performed similar experiments in Jurkat T cells and found an equally strong potentiation of NF-κB activity (Figure 3B).

In accordance with the previous experiments, treatment with sTat also enhanced the cytotoxic effects of TNF in HeLa cells (Figure 4A). Maximal potentiating effects were observed at TNF concentrations between 0.01 and 1.00 ng/ml and 10 µg/ml of sTat protein. Jurkat T cells, like other T cell lines, are resistant against the cytotoxic effect of TNF, but exhibit growth inhibition in response to TNF treatment. We therefore investigated the effect of sTat on TNF-induced proliferation inhibition in Jurkat T cells (Figure 4B). The results clearly demonstrate a potentiation of TNF-induced growth inhibition by Tat, even at concentrations where TNF alone had only small inhibitory effects.
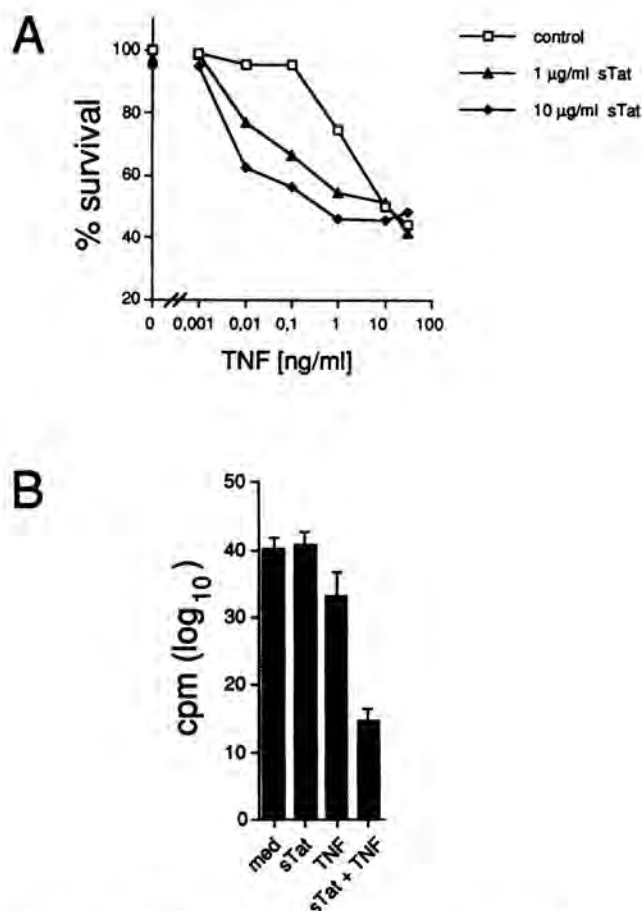
**Fig. 4.** Effect of sTat on TNF-induced cytotoxicity and growth inhibition. (**A**) HeLa cells were cultured in 96 microtiter wells ($10^4$ cells/well) and incubated with 1 or 10 µg/ml sTat. After 16 h cultures were incubated with TNF. Incubation was continued in the presence of actinomycin D (1 µg/ml). Survival was assayed by the reduction of MTT as described in the legend to Figure 1. (**B**) Jurkat T cells were incubated overnight with 1 µg/ml sTat followed by the addition of 1 ng/ml TNF for a further 40 h, as indicated. [$^3$H]thymidine incorporation was measured after a 6 h pulse. Mean values and standard deviations of triplicate samples are given.

### Effects of endogenously produced and sTat protein on Mn-SOD activity and Mn-SOD-specific mRNA levels

It has been reported that both the gene-inducing and the cytotoxic effect of TNF are mediated by reactive oxygen intermediates originating in the mitochondrial respiratory chain (Schulze-Osthoff *et al.*, 1992, 1993). Important enzymes counteracting oxidative stress are SODs which convert superoxide anion ($O_2^-$) to hydrogen peroxide ($H_2O_2$). Mn-SOD, an enzyme located in the mitochondrial matrix, was shown to be induced by various agents conferring oxidative stress to cells, whereas Cu,Zn-containing SOD (Cu,Zn-SOD) was shown to be constitutively expressed (Hassan, 1988). Moreover, the overexpression of Mn-SOD was shown to protect cells against TNF cytotoxicity (Wong *et al.*, 1989). To examine whether SOD activity is involved in the modulation of TNF effects caused by Tat, extracts of parental HeLa and HeLa-tat cells were assayed for Mn-SOD and Cu,Zn-SOD activity. As shown in Figure 5A, in HeLa-tat cells the activity of Mn-SOD was decreased to ~50% of the level measured in parental cells. In contrast, both cell types contained
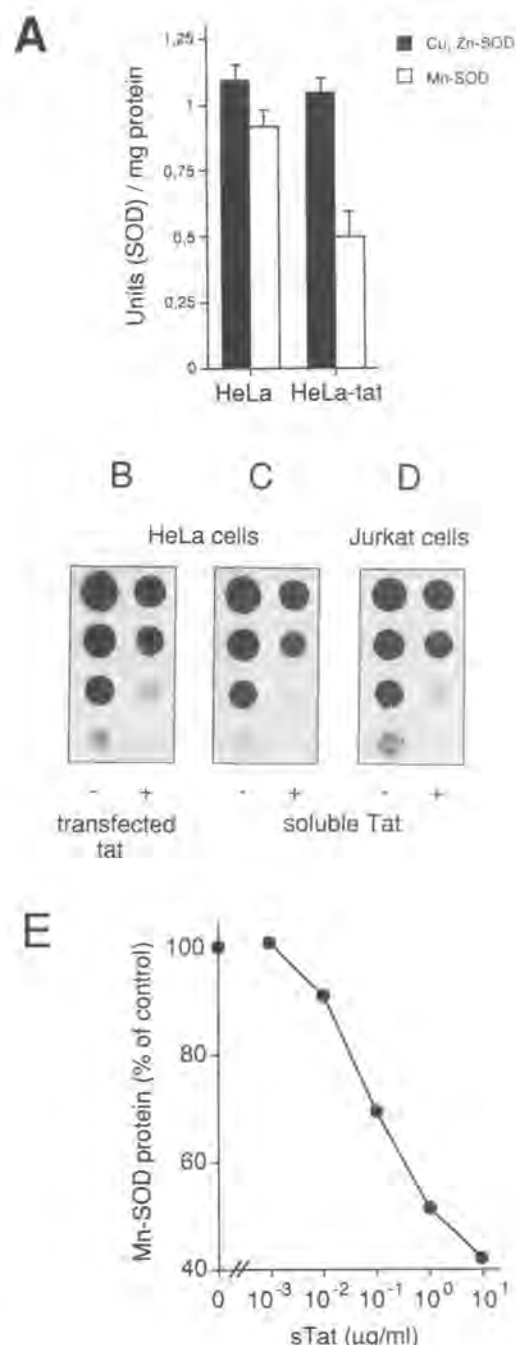


**Fig. 5.** Activities of Mn-SOD and Cu,Zn-SOD, and dot blot analysis of Mn-SOD expression. (**A**) Activities of SODs. Cell extracts of parental HeLa and HeLa-tat cells ($1\times10^6$) were prepared by sonication. The values (given in U/mg of protein) are the means of three independent determinations. (**B**−**D**) Dot blot analysis of Mn-SOD expression. Total cell lysates ($1\times10^6$ cells, equal amounts of protein) were dotted onto a nitrocellulose membrane. The membranes were washed and incubated with a monoclonal antibody against human Mn-SOD, washed again and then incubated for 90 min with peroxidase-labeled goat anti-mouse antibody. Signals were developed employing the enhanced chemoluminescence reaction. (**E**) Dose-dependent suppression of Mn-SOD protein expression by sTat. HeLa cells were scrape-loaded with sTat at the given concentrations and incubated for 18 h before harvest. The cell lysates were used for dot blot analysis, as described in Materials and methods, and quantified by densitometry. Results are given as percent Mn-SOD protein expression of an untreated control. In addition, sTat (10 µg/ml) was inactivated by oxidation. Cell cultures incubated with oxidized Tat showed Mn-SOD activities similar to untreated control cultures (data not shown).
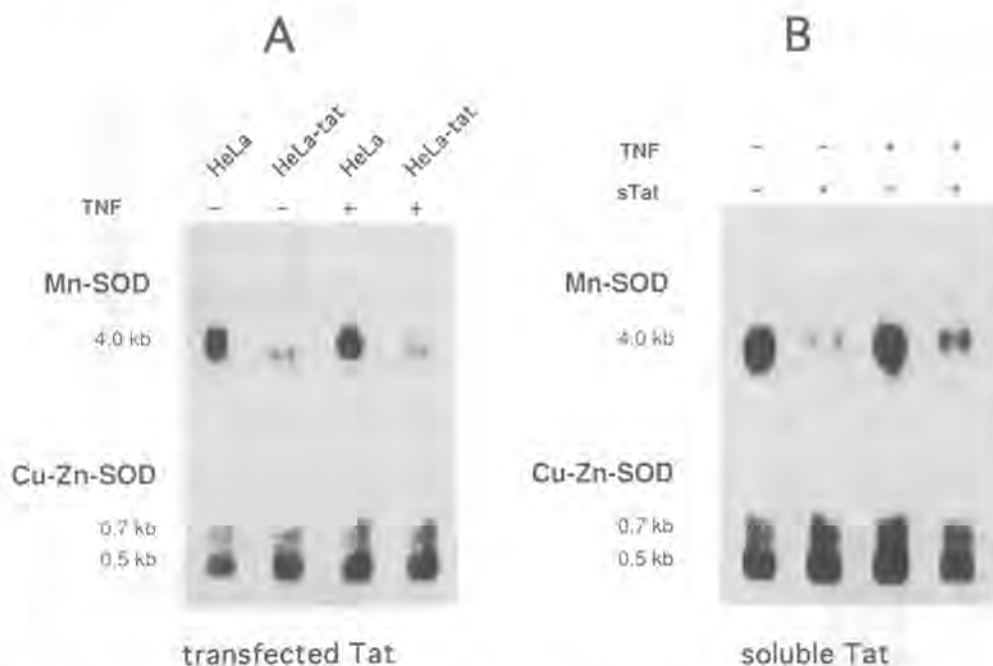
**Fig. 6.** Effect of Tat on Mn-SOD and Cu,Zn-SOD transcription. (A) Expression of SOD mRNA in HeLa-tat and parental cells. $2 \times 10^7$ cells were seeded in tissue flasks and treated with 10 ng/ml TNF as indicated. After 4 h total RNA was isolated. Samples (20 µg RNA) were submitted to electrophoresis on agarose–formaldehyde gels and hybridized with $^{32}$P-labeled cDNA probes coding for human Mn-SOD and Cu,Zn-SOD. Mn-SOD RNA levels of HeLa-tat cells compared with HeLa cells as determined by densitometry were 28 (− TNF) and 33% (+ TNF). (B) Expression of SOD mRNA in HeLa cells after treatment with sTat. HeLa cells were treated for 20 h with sTat protein (1 µg/ml). Then cultures were either left untreated or treated with TNF (10 ng/ml) for 4 h. Total RNA was isolated, subjected to electrophoresis and hybridized with radiolabeled Mn-SOD and Cu,Zn-SOD cDNA probes. Mn-SOD RNA levels of Tat-treated HeLa cells compared with control cells as determined by densitometry were 33 (− TNF) and 32% (+ TNF).

similar activities of Cu,Zn-SOD. This decrease in Mn-SOD activity was found to correspond to reduced protein expression. Dot blot analysis using an antibody against Mn-SOD revealed that Mn-SOD expression was significantly lower in HeLa-tat cells and HeLa cells incubated with sTat cells than in parental HeLa cells or HeLa cells incubated without sTat (Figure 5B and C). Similar effects were observed in Jurkat T cells treated with sTat (Figure 5D). Figure 5E shows that the suppression of Mn-SOD protein expression by sTat is dose-dependent. To determine whether suppressed Mn-SOD activity was caused by a decreased transcription of the Mn-SOD gene, total RNA isolated from both cell types was examined by Northern blot analysis. As shown in Figure 6A, the steady state level of Mn-SOD mRNA was reduced to ~30% of normal levels, whereas mRNA expression of Cu,Zn-SOD revealed no significant changes. The transcription of both enzymes was not altered significantly by treatment with TNF. In addition, a similar reduction in Mn-SOD mRNA expression was also observed when HeLa cells were treated with sTat protein (Figure 6B).

Full-length, two-exon Tat (86 amino acids long, Tat$_{1-86}$) is required to repress MHC class I gene promoter activity (Howcroft *et al.*, 1993), while truncated Tat (Tat$_{1-72}$) is sufficient to transactivate the HIV-1 LTR (Kuppuswamy *et al.*, 1989). To define the structural features of HIV-1 Tat required to suppress Mn-SOD expression, Jurkat T cells were incubated with full-length Tat$_{1-86}$ and truncated Tat$_{1-72}$. As shown in Figure 7, only Tat$_{1-86}$ decreased Mn-SOD expression, whereas Tat$_{1-72}$ had no effect. However, an HIV-1 LTR/CAT reporter gene construct transfected



**Fig. 7.** Effect of full-length two-exon Tat (Tat$_{1-86}$) and truncated one-exon Tat (Tat$_{1-72}$) on Mn-SOD expression. Jurkat T cells were cultured for 20 h in the presence of 1 µg/ml Tat$_{1-86}$ or Tat$_{1-72}$, respectively. Total cell lysates ($10^6$ cells, equal amounts of protein) were dotted onto a nitrocellulose membrane and blots developed as described in the legend to Figure 5.

into HeLa cells was transactivated equally well by both proteins (data not shown). Taken together, our data demonstrate that the repression of Mn-SOD gene expression, like MHC class I expression, requires the C-terminal part of HIV-1 Tat. They further suggest a common mechanism of gene repression by Tat distinct from HIV-1 gene transactivation.
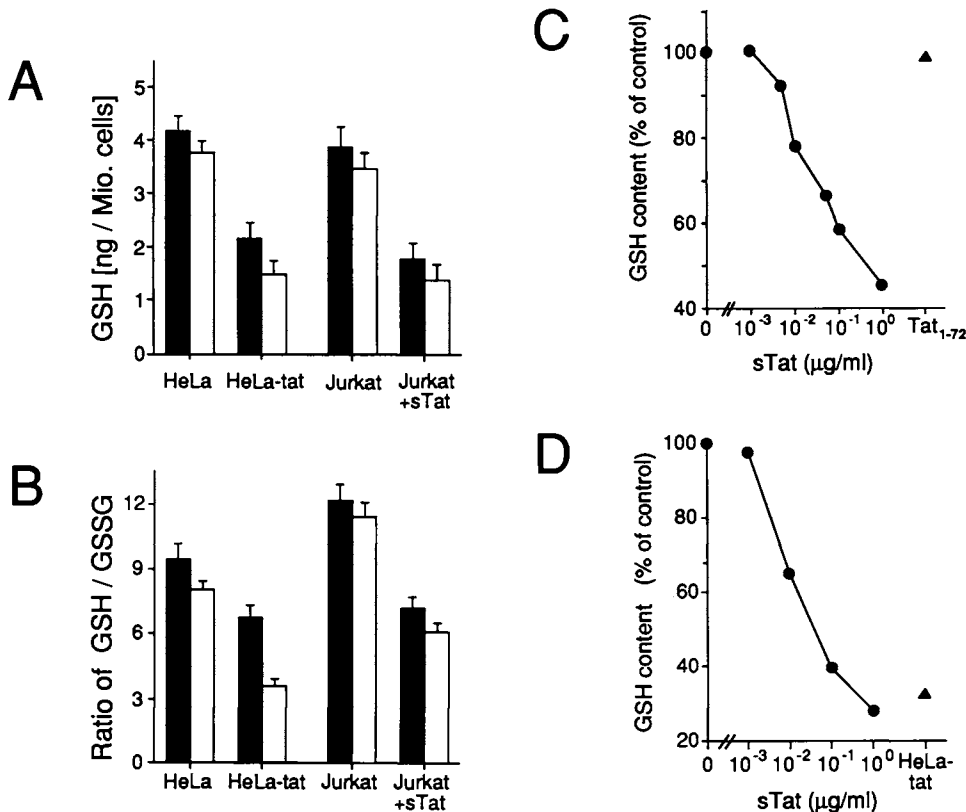
**Fig. 8.** Effect of HIV-1 Tat on total glutathione content and ratio of GSH:GSSG. (A) Glutathione in total cell lysates of Jurkat T cells and HeLa cells transfected with (HeLa-tat) or treated with Tat (+ sTat) was assayed by an enzymatic recycling procedure in which it is sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase. (B) For the determination of glutathione disulfide, GSH was derivatized with 2-vinylpyridine and then exposed to the DTNB assay. Values are given as the means of three independent determinations. (D and C) HeLa and HeLa-tat cells (D) or Jurkat T cells (C) ($5 \times 10^5$) pretreated for 20 h with the indicated amount of sTat were incubated with 20 μM monochlorobimane for 10 min at 37°C. $Tat_{1-72}$, truncated Tat protein lacking amino acids 73–86. The reaction was stopped by the addition of ice-cold culture medium. Then cells were washed three times with ice-cold PBS and the relative glutathione content was estimated by FACS analysis.

## Tat protein decreases the cellular glutathione content and lowers the GSH:GSSG ratio

A likely consequence of decreased SOD activity is increased oxidative stress reflected by a decrease in the total amount of glutathione or a decreased ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). GSH, an important antioxidant within cells, serves as a substrate in the reduction of peroxides catalyzed by the seleno-enzyme glutathione peroxidase (Meister and Anderson, 1983). In addition, GSH acts as a scavenger for hydroxyl radicals. We measured the influence of HIV-1 Tat on the total content of glutathione and the ratio of GSH:GSSG in both HeLa and Jurkat T cells. As shown in Figure 8A, the total GSH content present in HeLa-tat cells was decreased to ~50% of control cells. The ratio of GSH:GSSG was ~9.0 in the parental cells and 6.5 in HeLa-tat cells (Figure 8B). The addition of TNF slightly lowered glutathione content and GSH:GSSG ratios. The above results in HeLa-tat cells were confirmed in Jurkat T cells and HeLa cells treated with sTat (Figure 8A and B). As shown in Figure 8C, the treatment of Jurkat T cells with sTat protein decreased the glutathione content in a dose dependent manner from 93% at 5 ng/ml Tat to 45% at 1 μg/ml sTat, as measured by fluorescence intensity. The decrease of GSH levels in HeLa cells after treatment with sTat (Figure 8D) was even more pronounced than in Jurkat T cells. In contrast, the treatment of Jurkat T cells

with the truncated $Tat_{1-72}$ had no effect on GSH content (Figure 8C).

## Discussion

Several reports have demonstrated previously that TNF plays an important role in the progression of AIDS (for a review see Rosenberg and Fauci, 1990). High levels of TNF have been observed in sera and in supernatants of monocytes, peripheral blood mononuclear cells and alveolar macrophages from AIDS patients. TNF levels were shown to become progressively elevated with disease progression towards AIDS (Laehdevirta et al., 1988). Moreover, TNF is known to stimulate HIV-1 gene expression via activation of the transcription factor NF-κB (Duh et al., 1989; Osborn et al., 1989), which is modulated by the cellular redox state (Schreck et al., 1991; Schulze-Osthoff et al., 1993). Since virus replication is increased by TNF, we investigated whether HIV-1 gene products could also control TNF activity. Therefore, we examined the ability of HIV-1 Tat to modulate TNF-induced activation of NF-κB. We used HeLa cells stably transfected with the tat gene and found that the expression of Tat protein enhanced NF-κB activation in response to TNF. Similarly, synthetic Tat protein, when added to Jurkat T cells or HeLa cells, potentiated TNF-induced NF-κB activation and TNF-mediated cell death.

Tat-modulated activation of NF-κB by TNF was inhibited by antioxidants such as BHA. These data suggested that the activity of Tat is linked to cellular redox state. Therefore, we examined the influence of Tat on Mn-SOD, an enzyme which serves as the primary defense of cells against oxygen-derived free radicals. Two human genes encode intracellular SOD proteins: the gene for the Mn-dependent SOD and that for the Cu,Zn-dependent SOD. Only the former is known to be inducible and regulated by diverse oxidative stress-inducing agents (McCord *et al.*, 1977; Wong *et al.*, 1991). We observed that HIV-1 Tat, produced intracellularly or added exogenously, strongly reduced Mn-SOD protein expression. Furthermore, Mn-SOD RNA levels were strongly decreased in cells transfected with *tat* (see also Wong *et al.*, 1991; Flores *et al.*, 1993). Unlike Mn-SOD, Cu,Zn-SOD was not affected significantly. Similarly, sTat added exogenously to Jurkat T cells or HeLa cells decreased Mn-SOD transcription. Therefore, HIV-1 Tat produced and released by HIV-1-infected cells can potentially influence the cellular redox state of surrounding non-infected cells. To shed light on the mechanism of Mn-SOD repression by HIV-1 Tat, we employed a mutant lacking the C-terminus of Tat (amino acid positions 73–86) which is encoded by the second exon of the *tat* gene. This truncated protein (Tat$_{1-72}$) contains all elements required for HIV-1 gene transactivation (Kuppuswamy *et al.*, 1989). However, our experiments clearly demonstrate that Tat$_{1-72}$ does not repress Mn-SOD gene expression, suggesting that the C-terminal sequence encoded by the second exon of the *tat* gene is required for this Tat activity. Similar observations have been reported previously for the repression of MHC class I gene expression by Tat (Howcroft *et al.*, 1993). Taken together, we suggest that the repression of gene expression by HIV-1 Tat is based on a distinct mechanism which, unlike transactivation of HIV-1 gene expression, requires the C-terminal part of Tat.

Moreover, we found that Tat-induced down-regulation of Mn-SOD activity in Jurkat T cells and HeLa-tat cells was accompanied by a decreased glutathione content and by a decreased ratio of GSH:GSSG. Such an imbalance of the antioxidant defense system enhances the susceptibility of cells towards the cytotoxic action of TNF (Wong *et al.*, 1989; Schulze-Osthoff *et al.*, 1992, 1993). In accordance with this assumption, we found that HIV-1 Tat potentiated both the cytotoxic action of TNF and its inhibitory effect on T cell proliferation (Figures 2 and 4B). In line with the observed effect on Mn-SOD expression, Tat$_{1-72}$ had no effect on GSH content (Figure 8D) and TNF-mediated cytotoxicity (data not shown).

Based on these findings, we suggest that Tat modulates TNF activity by the following mechanisms. Inhibition of Mn-SOD synthesis by Tat leads to an increase in superoxide anion concentration. Superoxide is a poorly reactive radical and may diffuse in the cell before it encounters an appropriate reaction partner. Such partners may include metal ions such as $Fe^{3+}$ which becomes reduced to $Fe^{2+}$ ions. $Fe^{2+}$ catalyzes the production of the highly reactive hydroxyl radicals that give rise to secondary reactive products such as peroxides. Increasing amounts of peroxides are counteracted by glutathione peroxidase. This enzyme depletes reduced stores of glutathione. A decrease in reduced glutathione may result in a state of generalized
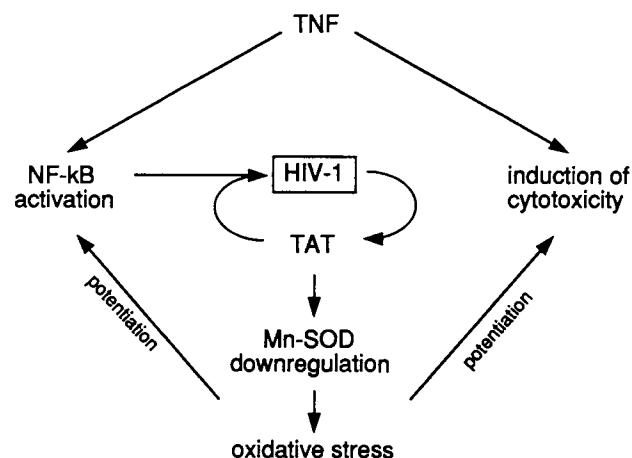


Fig. 9. Proposed interrelationship between Tat, TNF and oxidative stress in HIV-1 infection.

oxidative stress that amplifies TNF activity including TNF-induced activation of NF-κB and TNF-induced cytotoxicity. Furthermore, it is known that TNF production is progressively increased during HIV-1 infection. Therefore, we suspect that a positive feed-back loop may exist between Tat protein and TNF that stimulates rapid progression of the disease.

The interaction between Tat, the cellular redox system and TNF relevant to disease progression in AIDS is depicted in Figure 9. During the initial infection HIV-1 invades cells of the immune system, such as macrophages, and activates the expression of cellular genes including the TNF gene. TNF then stimulates HIV-1 replication and Tat production via activation of the transcription factor NF-κB. Tat induces oxidative stress which further enhances the activity of TNF. $CD4^+$ T cells incapable of repairing the ensuing oxidative stress are primed to undergo cell death (Fauci, 1993). A consequence of these events might be a decrease in the immunological effector functions. Tat is secreted by HIV-1-infected cells (Zauli *et al.*, 1992) and is also taken up by non-infected cells (Frankel and Pabo, 1988; Westendorp *et al.*, 1994). It has been speculated that these mechanisms also occur *in vivo*. Thus, Tat-producing and surrounding non-infected cells might be similarly affected, undergo a change in redox state and become more susceptible to infection or the cytotoxic action of cytokines. A vicious cycle may lead to a steady increase of TNF and Tat and to a decrease of antioxidants such as reduced glutathione. These events might finally cause a severe aggravation of the disease. The link between Tat and TNF action and sulfhydryl consumption may also account for the decreased glutathione levels observed in sera of HIV-1-infected patients (Gmünder *et al.*, 1990; Staal *et al.*, 1992).

Taken together, our data suggest that HIV-1 has found mechanisms that stimulate its own reproduction. These mechanisms involve the control of the cellular redox system and the generation of increased amounts of oxidants. The understanding of the interaction between TNF, Tat protein and the cellular redox system may open new ways for therapeutic intervention in AIDS. These may include drugs that interfere with Tat activity (Zapp *et al.*, 1993) or antioxidants, such as *N*-acetyl-cysteine,

which replenish depleted glutathione levels and restore a normal cellular redox state (Dröge *et al.*, 1992).

## Materials and methods

### Cell culture

Jurkat T cells and HeLa cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), HEPES (0.02 M) and 50 µg/ml gentamycin. HeLa cells stably transfected with an HIV-1 *tat* expression vector (Valerie *et al.*, 1988) were subcloned twice. The expression of HIV-1 *tat* was determined by transient transfection of the cells with a CAT reporter gene construct driven by the HIV LTR enhancer. CAT activity was determined as described (Gorman, 1985). Expression of the HIV-1 LTR CAT construct in HeLa cells transfected with the *tat* gene (HeLa-tat cells) was ~1200-fold, as compared with the parental HeLa cell line.

### Materials

rTNF with an activity of $5 \times 10^7$ U/mg protein was provided by BASF (Ludwigshafen, Germany). A mouse monoclonal antibody recognizing human Mn-SOD was obtained from Bender (Vienna, Austria). A CAT reporter gene construct carrying four copies of the NF-κB binding motif (4× NF-κB/CAT; Westendorp *et al.*, 1994) was used for the transient transfection of HeLa cells.

### Treatment of cells with sTat

The synthesis of full-length HIV-1 Tat (86 amino acids, HIV-1 strain BL 16) will be described elsewhere (Kraft *et al.*, 1994). A mutant of HIV-1 Tat comprising the first 72 amino acid positions (Tat$_{1-72}$) was synthesized accordingly. The biological activity of both sTat proteins was determined by treatment with sTat of HeLa cells transiently transfected with an HIV-1 LTR-driven CAT reporter gene construct, followed by assaying CAT activity. HeLa cells 'scrape-loaded' (Gentz *et al.*, 1989) with 1 µg/ml sTat showed an ~680-fold higher LTR activity as compared with control HeLa cells 'scrape-loaded' with 1 µg/ml bovine serum albumin (BSA). To examine the ability of sTat to influence TNF-mediated cytotoxicity and proliferation inhibition, cells were incubated with 100 ng/ml to 10 µg/ml sTat for 4–24 h as indicated in the text. To evaluate the modulation of TNF-induced activation of NF-κB by the protein, sTat was introduced into HeLa cells by 'scrape loading'. Briefly, $1 \times 10^5$ HeLa cells were grown in 35 mm Petri dishes, washed once with PBS and scraped off the plastic surface in 1 ml of cold PBS (4°C) containing 1 or 10 µg/ml sTat. Then 2 ml of culture medium were added to the cell suspension and incubation was continued at 37°C. In one experiment sTat was inactivated by exposing the protein solution to a flow of molecular oxygen.

### Proliferation and cytotoxicity assay

The cytotoxic activity of TNF was determined by a colorimetric assay (MTT test; Sladowski *et al.*, 1993). HeLa cells were seeded at a density of $1 \times 10^4$ cells per well in 96-well flat-bottomed microtiter plates and incubated for 16 h in 0.2 ml culture medium. The supernatant was then removed and replaced by fresh medium containing serial dilutions of TNF and actinomycin D (1 µg/ml). Incubation was continued for 16 h followed by the addition of 10 µl of an MTT solution (5 mg/ml PBS). After another 4 h incubation, supernatants were removed, followed by the addition of 150 µl of a solution containing 4% (v/v) HCl and 96% (v/v) isopropanol. The absorbance of each well was determined with an automated plate reader (SLT Easy Reader EAR 400 AT, Crailsheim, Germany) at 550 nm. Survival was calculated as the percentage of the staining value of untreated cultures. Percent cytotoxicity is the difference between control (100%) and percent survival.

Proliferation was measured by [$^3$H]thymidine incorporation (0.5 µCi, specific activity 185 GBq/mMol; Amersham, Braunschweig, Germany) for the last 6 h of a 20 h culture period. Cells were then lyzed with 1% SDS, 0.1 N HCl and radioactivity was counted.

### Determination of SOD activity

SOD was assayed by the cytochrome *c* method (McCord and Fridovich, 1969). The two forms of SOD (Cu,Zn-SOD and Mn-SOD) were distinguished by their differential sensitivity to cyanide.

### Intracellular GSH and GSSG measurement

GSH and GSSG were determined as described previously (Griffith, 1980). In some experiments GSH was estimated using FACS analysis

of monochlorobimane-stained cells (Staal *et al.*, 1990). Cells kept in culture medium were loaded with 20 µM monochlorobimane (Molecular Probes Inc., Eugene, OR, USA) for 10 min at 37°C. The reaction was stopped by the addition of ice-cold medium. Then cells were washed three times with cold PBS; FACS measurement was performed immediately after staining using the FACS Vantage (Becton Dickinson, Mountain View, CA) with the excitation set at 351–364 nm and the emission filter at 450 nm (Omega 450 DF-65). Dead cells were excluded by forward- and side-scatter gating; mean values from duplicate samples were considered for data analysis.

### Dot blot analysis

Total cell lysates ($1 \times 10^6$ cells, equal amounts of protein) were dotted onto a Hybond$^{TM}$ nitrocellulose membrane (Amersham) in log 2 dilutions. The membrane was washed six times with PBS, incubated with a mouse monoclonal antibody against human Mn-SOD, washed six times as described above, incubated for 90 min at room temperature with 1:4000 diluted peroxidase-labeled goat anti-mouse antibody (Dianova, Hamburg, Germany) and washed again as described above. Signals were developed using the enhanced chemoluminescence reaction as described by the manufacturer (Amersham); autoradiographs were quantified densitometrically.

### Northern blot analysis

Total cellular RNA was prepared according to Chirgwin *et al.* (1979). Cells were either left untreated or incubated for 18 h with HIV-1 Tat (1 µg/ml), followed by the addition of TNF (10 ng/ml) for another 4 h. RNA (20 µg) was subjected to electrophoresis employing 1% formaldehyde–agarose gels, transferred to a nylon membrane by capillary blotting and fixed by UV irradiation. Hybridization was carried out at 42°C in 50 mM Tris–HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, 0.2% BSA, 10% dextran sulfate and 50% formamide. The blots were washed to a stringency of 0.5× SSC (SSC: 15 mM sodium citrate, 150 mM NaCl) and 0.1% SDS at 62°C, and then exposed to an X-ray film at 70°C. A 650 bp fragment of human Mn-SOD cDNA and a 600 bp fragment of human Cu,Zn-SOD cDNA were labeled with $^{32}$P and used as hybridization probes (Amstad *et al.*, 1991). Autoradiographs were quantified densitometrically.

### Preparation of total cell extracts

Cells ($1 \times 10^6$ per sample) were incubated with TNF for 30 min as indicated, harvested, washed twice with ice-cold PBS and resuspended in 100 µl of high salt buffer containing 20 mM HEPES, pH 7.6, 400 mM NaCl, 1 mM MgCl$_2$, 0.5 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1% NP-40, 0.5 mM DDT, 10 µg/ml leupeptin, 1 mM PMSF and 0.001% aprotinin. After 15 min incubation on ice, lysates were centrifuged and supernatants were transferred to new vials and kept frozen at −70°C.

### EMSAs

EMSAs were performed as follows. Equal amounts of extracts (10 µg protein, determined by a protein assay kit; Bio-Rad, Munich, Germany) were incubated with an NF-κB-specific $^{32}$P-labeled oligonucleotide. The sequence of the double-stranded 30 bp probe comprising the NF-κB site and flanking sequences from the mouse kappa light chain enhancer is shown in Zabel *et al.* (1991). Binding reactions were performed in a 20 µl volume containing 2 µg poly(dI–dC), 2 µg BSA, 20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM DDT, 2.5 mM MgCl$_2$, 4% Ficoll and 3000–6000 c.p.m. of the random primer-labeled oligonucleotide. After 20 min at room temperature, samples were loaded onto a 4% non-denaturing polyacrylamide gel and run in 0.5× TBE buffer at pH 8.0. After electrophoresis, gels were dried and exposed overnight to a Kodak X-ray film with an intensifying screen.

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