

# HIV type 1 Tat protein enhances activation- but not Fas (CD95)-induced peripheral blood T cell apoptosis in healthy individuals

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**Keywords:** activation-induced cell death, apoptosis, Fas, ICE, HIV, reactive oxygen intermediate, redox, Tat, T cells

## Abstract

T cell apoptosis may play an important role in the depletion and functional defects of T cells in HIV disease. A number of investigators have shown that peripheral blood T cells in HIV disease undergo spontaneous and activation-induced apoptosis. We found recently that peripheral blood T cells from HIV<sup>+</sup> individuals undergo apoptosis when stimulated through Fas. Also, a number of investigators have shown that Tat protein from HIV-1 can increase spontaneous and activation-induced apoptosis. In the present study we examined the effect of HIV type 1 Tat protein on spontaneous, activation-induced and Fas-induced apoptosis of peripheral blood T cells from HIV<sup>-</sup> individuals. We find that Tat protein has no effect on spontaneous apoptosis but does enhance activation-induced apoptosis of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Tat, however, failed to enhance Fas-induced apoptosis of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Examining the mechanisms by which Tat induces apoptosis, we found that inhibitors of reactive oxygen intermediate (ROI) generation or neutralizers of ROI, such as rotenone, a potent inhibitor of mitochondrial complex I of the respiratory chain, and 3,3,5,5-tetramethylpyrrolidine *N*-oxide (TMPO), an electron spin trap, could both enhance the spontaneous apoptosis induced by Tat. This enhancement of Tat-induced apoptosis by rotenone and TMPO was independent of ICE activation as it could not be inhibited by the tripeptide z-VAD-fmk, an irreversible inhibitor of ICE/ced-3 protease homologs. These findings suggest that Tat induced enhancement of activation-induced cell death may involve complex mechanisms, some of which are ROI independent. These results indicate that a HIV-specific mechanism other than Tat is responsible for the previously observed increased susceptibility of peripheral blood T cells from HIV-infected individuals to undergo apoptosis in response to Fas stimulation.

## Introduction

T cell apoptosis has been suggested to play an important role in the pathogenesis of HIV disease by contributing to the loss of T cell numbers and function (1). We recently showed that peripheral blood T cells in HIV<sup>+</sup> individuals are especially prone to undergo apoptosis in response to Fas antigen stimulation (2). Activation-induced cell death (AICD) of peripheral blood T cells, which has also been described in HIV<sup>+</sup> individuals (3-5), we found is Fas independent (6), suggesting that these two mechanisms of apoptosis may play different pathogenic roles.

The mechanism, however, of such T cell priming to undergo

activation or Fas-induced apoptosis has not yet been elucidated. A number of studies have suggested that cross-linking of CD4 on T cells by gp120 protein results in T cells undergoing AICD when this cross-linking is followed with stimulation through the TCR (5,7). Recent reports have shown that Tat primes normal peripheral blood T cells to undergo spontaneous apoptosis or AICD in response to TCR or anti-CD3 stimulation (8-10); however, the role of Tat in priming T cells to undergo Fas-induced apoptosis is not clear. Tat may also play an important role in the defective proliferative T cell responses observed in HIV infection (1), as Tat has been

shown to inhibit antigen-specific proliferative responses of human peripheral blood T cells (10,11). This immunosuppressive effect has been attributed to the inhibition of IL-2 production and IL-2 receptor  $\alpha$  chain expression which is observed in Tat-transfected Jurkat T cells (12). However, in light of recent reports (8,9), Tat may also inhibit proliferative responses via its enhancing effect on activation-induced T cell apoptosis.

In this report we show that Tat enhances activation-induced apoptosis of peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells but does not prime T cells to undergo Fas-induced apoptosis. These findings suggest that Tat may contribute to some, but not all, aspects of T cell apoptosis in HIV disease. Investigating the mechanism by which Tat may exert its effect, we found that Tat induces marked apoptosis when ROI generation is inhibited or ROI are neutralized. Elucidating the mechanisms of T cell priming to undergo activation and Fas-induced apoptosis may prove useful in designing novel strategies to prevent T cell depletion and restore the functional defects observed in HIV disease.

## Methods

### *Samples and materials*

Buffy coats from HIV seronegative healthy volunteers were provided by the Stanford Medical School Blood Center. Mouse mAb to Fas antigen (M3, IgG1 isotype) was a generous gift of Dr C. A. Smith (Immunex, Seattle, WA). Anti-CD3 mAb antibody OKT3 and the isotype control antibody DS-1 (anti-mouse IgM of the a allotype) were kindly provided by Drs M. Feldmann (London, UK) and A. B. Kantor (Stanford, CA) respectively. Recombinant Tat and p51 were kind gifts from Dr Michael Lederman (Chase Western Reserve University, Cleveland, OH). Recombinant Tat was produced and purified from *Escherichia coli* transfected with plasmid pTat6xHis as previously described (13,14). The control p51 protein, recombinant polyhistidinylated p51 subunit of HIV-1 reverse transcriptase, was similarly prepared as above. Rotenone, an inhibitor of complex I of the respiratory chain (15,16), and 3,3,5,5-tetramethylpyrroline *N*-oxide (TMPO), a free radical spin trap (17), were both commercially purchased (Sigma, St Louis, MO). The tripeptide ICE inhibitor z-VAD-fmk (fluoromethylketone) and the control dipeptide z-FA-fmk were commercially purchased (Enzyme Systems Products, Dublin, CA).

### *Tat priming experiments and anti-Fas- and anti-CD3-induced apoptosis.*

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by density centrifugation on Ficol-Hypaque and were cultured for 24 h at  $1 \times 10^6$  cells/ml in RPMI 1640/10% FCS in 24-well plates coated with or without 1  $\mu$ M Tat or p51 protein in the presence or absence of immobilized monoclonal anti-Fas antibody M3, anti-CD3 antibody OKT3 or isotype control IgG1 mAb DS-1. Plates were coated with 500  $\mu$ l/well of 10  $\mu$ g/ml of antibodies in RPMI 1640 for 2 h at 37°C, plates were then washed twice briefly with 500  $\mu$ l/well RPMI 1640/10% FCS and then washed one more time as above for 30 min at 37°C. In the Fas priming experiments PBMC were first cultured with or without 1  $\mu$ M Tat/p51 and immobilized anti-CD3 or isotype control for 24 h, and then

transferred to plates coated with immobilized anti-Fas or isotype control antibody. Specific anti-Fas-induced apoptosis was calculated by subtracting the isotype control-induced apoptosis from the anti-Fas-induced apoptosis under identical conditions, i.e. specific anti-Fas CD4<sup>+</sup> or CD8<sup>+</sup> T cell apoptosis of Tat/anti-CD3 combination pretreated PBMC = (anti-Fas-induced CD4<sup>+</sup> or CD8<sup>+</sup> apoptosis of Tat/anti-CD3 pretreated PBMC) - (isotype control-induced CD4<sup>+</sup> or CD8<sup>+</sup> apoptosis of Tat/anti-CD3 pretreated PBMC). In all the above experiments cells were cultured for 24 h at  $1 \times 10^6$  cells/ml/well in RPMI 1640/10% FCS in 24-well plates.

### *Effect of rotenone or TMPO on Tat-induced apoptosis*

Freshly isolated PBMC were cultured with rotenone or TMPO at 500 nM and 40 mM respectively in the presence or absence of Tat protein for 24 h. For the ICE inhibitor experiments PBMC were first preincubated for 3 h with 50  $\mu$ M z-VAD-fmk or z-FA-fmk at 37°C and then cells were treated with rotenone or TMPO and Tat. PBMC were cultured at  $10^6$  cells/ml at 37°C in a 5% CO<sub>2</sub> incubator. Following 24 h of culture, cells were harvested and stained as above.

### *Apoptosis measurement and DNA fragmentation*

Apoptosis was determined by FACS analysis, as previously described (2), by staining cells with 1  $\mu$ g/ml Hoechst 33342 (HO342; Molecular Probes, Eugene, OR) for 7 min on ice. Cells were co-stained with anti-CD8-phycoerythrin (PE) (Becton Dickinson, Mountain View, CA), anti-CD4-FITC (PharMingen, San Diego, CA) and anti-CD5-CyC5 (PharMingen) for 15 min on ice. Following washes and fixing in paraformaldehyde,  $3 \times 10^4$  cells were analyzed by flow cytometry using a FACStar (Becton Dickinson) and Desk software (18). Apoptosis was determined by measuring the percentage of HO342 staining cells, in cells gated on forward and side scatter for lymphocytes. Live CD4<sup>+</sup> and CD8<sup>+</sup> T cell absolute numbers were determined by multiplying the fraction of live non-apoptotic CD4<sup>+</sup> and CD8<sup>+</sup> T cells in culture (determined by flow cytometry) times the total number of PBMC in culture measured by a Coulter Counter model ZM (Coulter, Hialeah, FL).

DNA fragmentation was examined by gel electrophoresis. PBMC ( $2 \times 10^6$  cells) were treated in culture as described above for 5 h, cells were centrifuged and DNA was extracted by resuspending cells in 20  $\mu$ l of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% sodium lauryl sarcosylate, 0.5 mg/ml proteinase K and incubated for 1 h at 50°C. RNase was then added at 100  $\mu$ g/ml and the extract was further digested for 1 h at 50°C. DNA was run on a 2% agarose gel in 45 mM Tris borate, 1 mM EDTA, pH 8.0, stained with 0.5  $\mu$ g/ml ethidium bromide and visualized under UV light.

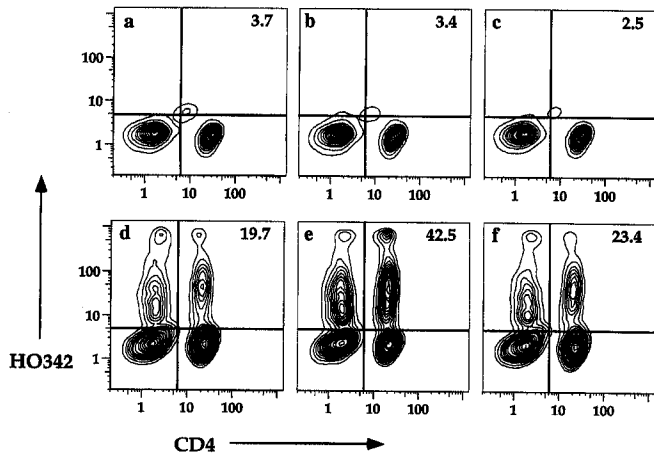
### *Statistical analysis*

Data were analyzed by Mann-Whitney *U*-test and Wilcoxon's signed-rank test for paired data, using JMP Statistics Guide (SAS Institute, Cary, NC).

## Results

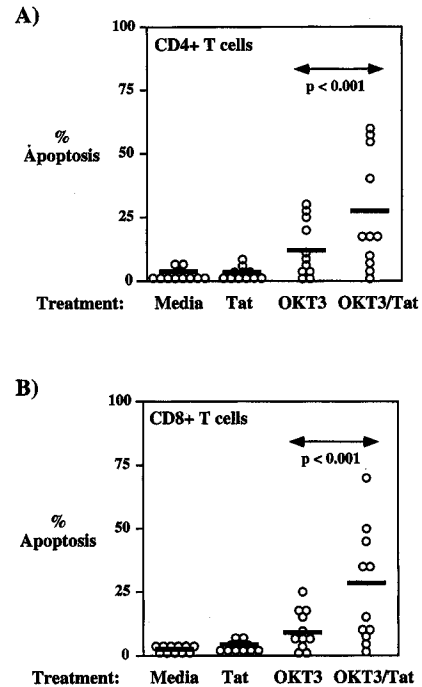
### *Tat enhances AICD of normal T cells*

Using multiparameter flow cytometry to measure apoptosis, we found that Tat protein enhances AICD of normal peripheral



**Fig. 1.** Tat protein enhances activation-induced death of peripheral blood CD4<sup>+</sup> T cell from healthy individuals. Tat does not enhance spontaneous apoptosis. The control protein HIV p51 affects neither spontaneous nor activation-induced cell death. Similar results were obtained when CD8<sup>+</sup> T cells were examined. Apoptosis measured by Hoechst 33342 in multiparameter flow cytometry. Cells treated for 24 h with: (a) control antibody alone, (b) Tat and control antibody, (c) p51 and control antibody, (d) anti-CD3 alone, (e) Tat and anti-CD3, and (f) p51 and anti-CD3. Percent apoptosis shown in upper right corner of plots. Representative experiment of 11 performed shown.

blood T cells. In concordance with previous reports (8,9), we found that anti-CD3-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cell apoptosis in normal PBMC was enhanced by Tat protein in 24 h cultures. Anti-CD3-induced apoptosis was  $11.5 \pm 3.5$  and  $8.2 \pm 2.4\%$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively, Tat enhanced anti-CD3-induced apoptosis to  $26.3 \pm 6.9$  and  $27.3 \pm 6.5\%$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively ( $n = 11$ , mean  $\pm$  SE shown) (Figs 1 and 2, and Table 1). The control protein p51 had no significant effect on AICD of T cells (data not shown and Figs 1 and 2). Tat also enhanced DNA fragmentation and reduced significantly the absolute numbers of cells recovered in the above anti-CD3-treated cultures (data not shown). Apoptosis was observed at 1  $\mu$ M Tat protein, concentrations below 1  $\mu$ M failing to have any effect on apoptosis. Tat did not increase the spontaneous (no Tat treatment:  $1.8 \pm 0.5$  and  $1.8 \pm 0.3\%$ , Tat treatment:  $2.3 \pm 0.6$  and  $1.9 \pm 0.4\%$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively,  $n = 11$ ) or isotype control-induced T cell apoptosis in these PBMC cultures (data not shown and Figs 1 and 2, and Table 1), indicating that Tat affects only activation-induced T cell apoptosis and does not directly induce apoptosis in resting T cells. The control HIV protein p51 had no effect on neither spontaneous nor anti-CD3 or isotype control antibody-induced apoptosis or cell counts. Similarly with PBMC T cells, Jurkat T cell line anti-CD3-induced apoptosis was also enhanced significantly by Tat but not p51 protein (data not shown) and this is in agreement with previously published work (9,13). Spontaneous or isotype control-induced apoptosis of the Jurkat T cell line was not enhanced by Tat or p51 control protein (data not shown).



**Fig. 2.** Anti-CD3-induced apoptosis of CD4<sup>+</sup> and CD8<sup>+</sup> T cells is enhanced by Tat protein. (A) CD4<sup>+</sup> T cell apoptosis and (B) CD8<sup>+</sup> T cell apoptosis shown. Anti-CD3<sup>+</sup> Tat-induced apoptosis is significantly increased compared to anti-CD3 treatment alone ( $n = 11$ , pooled data from five independent experiments,  $P < 0.001$  by Wilcoxon's signed rank test). Horizontal bars indicate the mean.

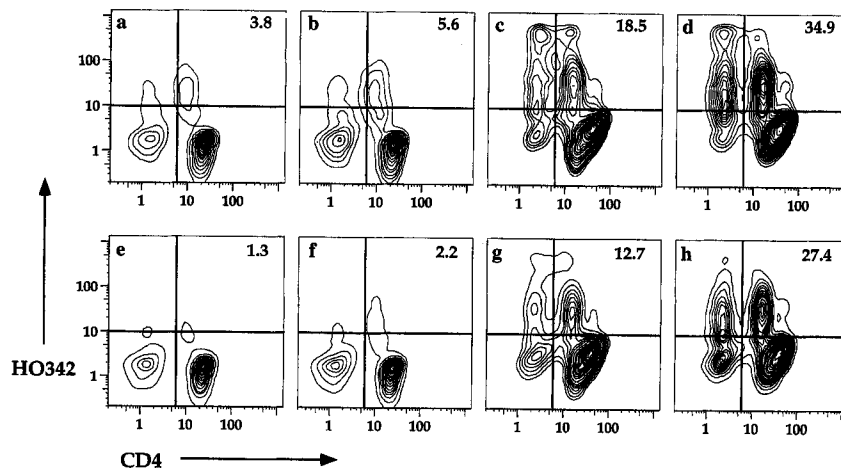
**Table 1.** Effect of Tat on Fas-induced apoptosis of peripheral blood T cells from healthy individuals ( $n = 7$ )

Pretreatment	Apoptosis (%)			
	Anti-Fas-induced		Isotype control	
	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>
Media	$9 \pm 1.9^a$	$6.2 \pm 1.8$	$1.8 \pm 0.2$	$1.4 \pm 0.4$
Tat	$10.9 \pm 2.1$	$8.8 \pm 1.3$	$2.2 \pm 0.3$	$1.6 \pm 0.3$
Anti-CD3	$16.1 \pm 2.8$	$12 \pm 2.1$	$7.4 \pm 2.7$	$6.2 \pm 1.3$
Anti-CD3 + Tat	$24.8 \pm 4.9$	$21.4 \pm 3.5$	$16.3 \pm 2.4$	$12.6 \pm 2.7$

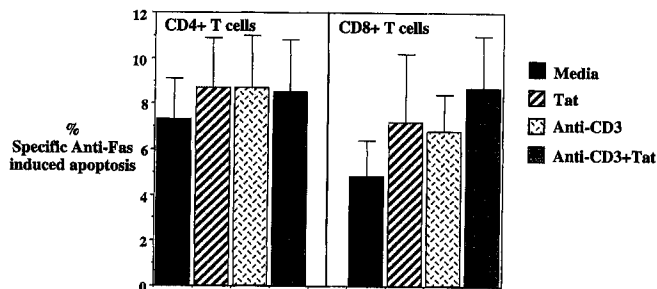
<sup>a</sup>Mean  $\pm$  SE.

#### Tat does not enhance Fas-induced T cell apoptosis

Pretreatment of PBMC with 1  $\mu$ M Tat alone for 24 h did not enhance specific anti-Fas-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cell apoptosis in 24 h cultures (Figs 3 and 4, and Table 1). Although Tat appeared to enhance anti-Fas-induced apoptosis when cells are treated with Tat and anti-CD3 (Fas-induced apoptosis of anti-CD3 pretreated cultures:  $16.1 \pm 2.8$  and  $12 \pm 2.1$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively; Fas-induced apoptosis of anti-CD3<sup>+</sup> Tat pretreated cultures:  $24.8 \pm 4.9$  and  $21.4 \pm 3.5\%$  for CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively,  $n = 7$ ), this enhancement is all due to the effect Tat has on anti-CD3-induced apoptosis, since a similar increase was also observed in apoptosis-induced by the isotype control antibody (Fig. 3



**Fig. 3.** Effect of Tat protein on anti-Fas-induced apoptosis of peripheral blood T cells from healthy individuals. Cells were pretreated for 24 h with: (a and e) nothing, (b and f) Tat protein, (c and g) anti-CD3 alone or (d and h) Tat and anti-CD3. Cultures were then treated with: (a–d) anti-Fas antibody or (e–h) isotype control antibody. Tat alone had no effect on anti-Fas-induced apoptosis. Anti-CD3 with or without Tat failed to enhance anti-Fas-induced apoptosis. The difference in apoptosis between anti-CD3 alone and Tat/anti-CD3 pretreated cultures is the same in the anti-Fas or control antibody treated cultures (16.4 and 14.7% respectively). Apoptosis was measured at 24 h by Hoechst 33342 stain using multiparameter flow cytometry. CD4<sup>+</sup> T cells shown, similar results were obtained for CD8<sup>+</sup> T cells. Percent apoptosis shown in upper right corner.



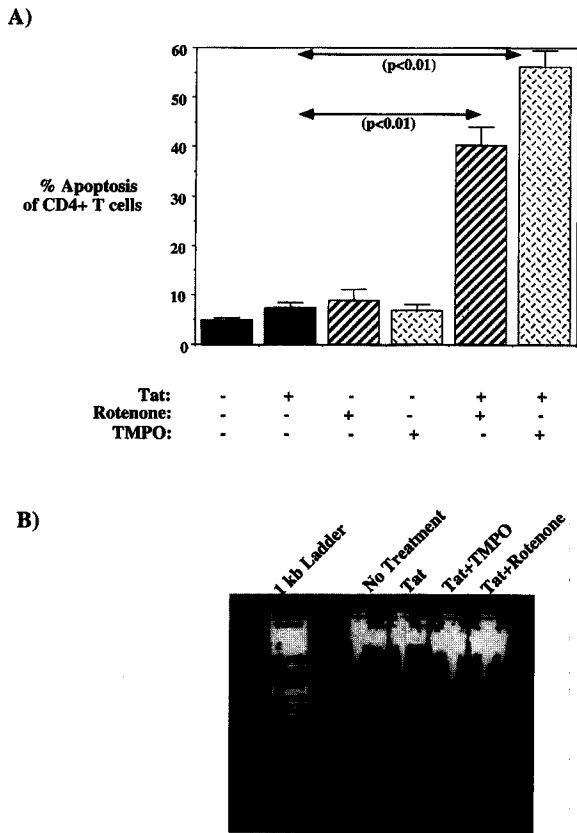
**Fig. 4.** Tat does not enhance specific Fas-induced apoptosis of peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Specific anti-Fas-induced apoptosis of Tat + anti-CD3 pretreated cells = (anti-Fas-induced apoptosis of Tat + anti-CD3 pretreated cells) – (isotype control-induced apoptosis of Tat + anti-CD3 pretreated cells). Pooled data ( $n = 7$ ) of specific Fas-induced apoptosis shown. Mean and SE shown.

and Table 1). When specific anti-Fas apoptosis was calculated, Tat did not significantly enhance specific anti-Fas-induced T cell apoptosis (Fig. 4). Tat treatment also did not induce any significant effect on DNA fragmentation and cell numbers in the anti-Fas treated cultures above those induced by anti-CD3 alone. Tat protein added simultaneously with anti-Fas treatment also had no effect on Fas-induced apoptosis and cell counts in these cultures (data not shown). No effect on anti-Fas antibody-induced apoptosis was observed either when cultures were preincubated for 3 days with Tat alone or in combination with anti-CD3 (data not shown). These data taken collectively show that Tat enhances anti-CD3-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cell apoptosis but fails to have any effect on anti-Fas-induced apoptosis.

#### *Tat induces apoptosis when $O_2^-$ superoxide anion generation is inhibited*

Since it has been suggested that Tat may affect apoptosis by down-regulating Mn superoxide dismutase (MnSOD), thus altering the redox state of cells and lowering their reducing capacity (19,20), we investigated the effect of inhibiting the generation of ROI on Tat-induced apoptosis. Reagents that block the generation of ROI or neutralize ROI, such as rotenone, an inhibitor of complex I of the mitochondrial respiratory chain (15,16), and TMPO (17), an electron spin trap, significantly enhanced AICD in the presence of Tat for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Data not shown). This apparent enhancement of AICD was due to increased spontaneous T cell apoptosis induced by Tat in the presence of rotenone and TMPO (Fig. 5A). Twenty-four hour apoptosis of CD4<sup>+</sup> T cells was  $4.8 \pm 0.45\%$  in untreated cultures,  $7.4 \pm 1.1\%$  for 1  $\mu\text{M}$  Tat alone treatment,  $8.8 \pm 2.3\%$  for 500 nM rotenone treatment,  $6.8 \pm 1.2\%$  for 40 mM TMPO treatment,  $40.3 \pm 3.7\%$  for 1  $\mu\text{M}$  Tat plus 500 nM rotenone treatment and  $56.1 \pm 3.4\%$  1  $\mu\text{M}$  for Tat plus 40 mM TMPO treatment ( $n = 7$ ). CD8<sup>+</sup> T cell apoptosis was similar to the CD4<sup>+</sup> T cell results (data not shown). Rotenone and TMPO also enhanced DNA fragmentation in Tat-treated PBMC cultures (Fig. 5B). Rotenone or TMPO alone did not induce any DNA fragmentation above background (data not shown). This effect of rotenone and TMPO on Tat-induced apoptosis was shown to be dose dependent (Fig. 6A and data not shown).

In order to further elucidate the above, we also performed the rotenone/Tat and TMPO/Tat experiments in the presence of an irreversible inhibitor of the ICE protease family. The tripeptide inhibitor z-VAD-fmk inactivates ICE and is expected to inhibit other ICE-like proteases (21). z-VAD-fmk can inhibit IL-1 $\beta$  secretion by lipopolysaccharide-stimulated monocytes

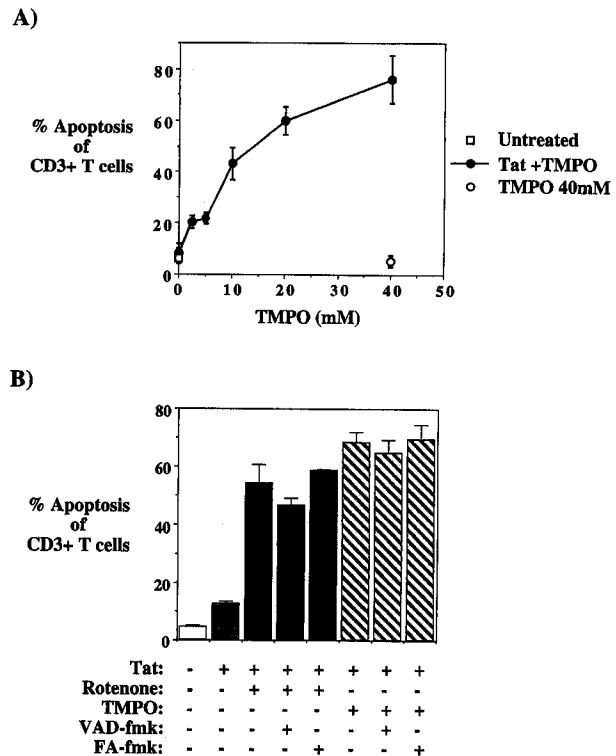


**Fig. 5.** Inhibitors of  $O_2^-$  superoxide anion generation rotenone and TMPO enhance Tat-induced apoptosis of  $CD4^+$  T cell. (A) Apoptosis of  $CD4^+$  T cells measured by flow cytometry shown, pooled data ( $n = 7$ ,  $P < 0.01$  by Wilcoxon's signed rank test). (B) DNA fragmentation is enhanced when Tat treatment is combined with rotenone or TMPO (representative experiment shown). Rotenone or TMPO alone did not induce any DNA fragmentation above background (data not shown).

(P. Katsikis, unpublished observation), and can inhibit apoptosis-induced by Fas (P. Katsikis, unpublished observation) (22), overexpression of REAPER (23) and FLICE/MACH (24) and a variety of other stimuli (25,26). The ICE family homolog inhibitor z-VAD-fmk (50  $\mu$ M) could not inhibit the enhanced Tat-induced apoptosis in neither rotenone- nor TMPO-treated cultures (Fig. 6B). At this concentration z-VAD-fmk inhibits 80% of anti-Fas-induced apoptosis of the Jurkat T cell line (data not shown). The control dipeptide z-FA-fmk had no effect in any of the above cultures (data not shown). These findings clearly show that Tat can induce apoptosis by an ROI independent mechanism which is also ICE protease independent. This, however, does not exclude that other cysteine proteases, such as CPP32 which are not inactivated by z-VAD-fmk (22), are involved in rotenone or TMPO enhancement of Tat-induced apoptosis.

**Discussion**

The mechanism of priming of T cells for apoptosis in HIV disease has not yet been fully elucidated. Recent reports have suggested that Tat may be involved by priming normal



**Fig. 6.** (A) Dose response of TMPO on Tat-induced  $CD3^+$  T cell apoptosis. Representative experiment shown (mean and SE of triplicates shown). (B) The ICE protease homolog inhibitor z-VAD-fmk (50  $\mu$ M) cannot abrogate the rotenone (500 nM) and TMPO (40 mM) enhancement of Tat-induced  $CD3^+$  T cell apoptosis (mean and SE of triplicates shown).

peripheral blood T cells to undergo AICD (8,9). We have recently shown that peripheral blood T cells from HIV+ individuals undergo apoptosis in response to stimulation of Fas antigen (CD95) (2), this Fas-induced apoptosis preferentially kills  $CD4^+$  compared to  $CD8^+$  T cells, whereas anti-CD3 induces apoptosis equally in both  $CD4^+$  and  $CD8^+$  T cells (6), and is Fas independent (6). Fas-mediated apoptosis does not require T cells to engage with their respective antigen since activated cytotoxic  $CD8^+$  T cells with up-regulated FasL could kill bystander T cells. AICD, on the other hand, requires T cells to be activated following binding to their antigen. These two mechanisms of apoptosis, Fas-mediated apoptosis of T cells and T cell AICD, may play different roles in the T cell depletion and dysfunction in HIV disease.

The above findings suggest that activation and Fas-induced apoptosis may play different pathogenic roles in HIV disease (6). If these two modes of apoptosis are independent one may expect that different apoptosis priming mechanisms may be responsible for T cell apoptosis in HIV disease. Tat protein has been suggested as one such priming mechanism, enhancing spontaneous or activation-induced T cell apoptosis (8-10,13). In our study we show that Tat enhances anti-CD3-induced  $CD4^+$  and  $CD8^+$  T cell apoptosis of normal peripheral blood T cells but does not affect spontaneous apoptosis. In superantigen-stimulated T cells the apoptosis enhancing effect of Tat has been attributed to enhanced Fas ligand up-

regulation (9). This, however, may not be universally true for activation-induced T cell death as differences between superantigen and anti-CD3-induced apoptosis of T cells may exist. Superantigen-induced T cell apoptosis has been shown to require Fas-Fas ligand interactions whereas anti-CD3-induced T cell death can proceed in the absence of Fas (27). We show here that Tat does not increase the number of T cells primed (sensitized) to undergo Fas-induced apoptosis. Therefore some other factor/mechanism is responsible for the increased Fas sensitivity/priming which is observed in peripheral blood T cells from HIV-infected individuals.

Inappropriate CD4 cross-linking on T cells by gp120 and antibodies to gp120 has been suggested as another mechanism that enhances activation-induced apoptosis in HIV disease (5,7). In one report the combination of CD4 cross-linking and Tat resulted in enhanced AICD, without increasing sensitivity to Fas-induced apoptosis (9). In our hands, CD4 cross-linking by the Leu3a monoclonal anti-CD4 antibody or recombinant gp120  $\pm$  polyclonal anti-gp120 antibodies failed to induce spontaneous or enhance anti-CD3- or anti-Fas-induced T cell apoptosis of normal PBMC from healthy individuals (P. Katsikis unpublished observations). The reason for such discrepancies remain unclear at the moment.

A number of studies have suggested that Tat can inhibit apoptosis both when added in solution and when transfected in a number of cell lines including the Jurkat T cell line (28,29). These studies have been performed with Tat-transfected cells cultured under low or no serum conditions. Tat may be acting via different mechanisms in this experimental set up, since Tat transfected Jurkats cultured in normal serum conditions show enhanced apoptosis (13). In our study Tat added in solution to Jurkat cell resulted in enhanced AICD of these cells. These discrepancies are likely to be due to differences in cell cycling which result from culturing cells under different conditions, since it has been suggested that Tat mediates its effect on apoptosis by activating cyclin-dependent kinases (8).

Another possible mechanism by which Tat may induce apoptosis is by modulating the redox status of cells. Recently, it has been shown that Tat down-regulates MnSOD, decreases the total amount of glutathione (GSH) and lowers the ratio of reduced:oxidized glutathione (GSH:GSSG), thus shifting the cellular redox state to a pro-oxidative condition (19,20). This pro-oxidative shift decreases the antioxidant defense capabilities of cells and has been proposed as the possible mechanism by which Tat induces apoptosis with ROI being important mediators of such apoptosis (19,20). Our data indicate that the mechanism by which Tat induces apoptosis is ROI independent. Here we clearly show that the generation of  $O_2^-$  superoxide anion is not required for Tat to induce apoptosis. On the contrary, inhibition of  $O_2^-$  superoxide anion generation or neutralization of ROI enhances apoptosis induced by Tat. This Tat-induced apoptosis was not mediated by ICE protease activity as the ICE inhibitor z-VAD-fmk had no effect on this apoptosis. The above, however, does not exclude that other members of the ICE protease family may be involved in this apoptosis.

Oxidation of Tat results in its inactivation (11), therefore it is possible that when  $O_2^-$  superoxide anion generation is inhibited the activity of Tat is potentiated due to its decreased rate of oxidation and inactivation. Under such a perspective

the Tat-induced down-regulation of MnSOD could be viewed as a strategy by which HIV virus secures maximal Tat transcriptional activity in infected cells via NF- $\kappa$ B activation (19) whilst minimizing cell death induced by Tat. Although the above data do not directly address the enhancement of AICD by Tat, they do raise the possibility that Tat can induce apoptosis by a ROI independent mechanism.

Although HIV-infected cells are prone to undergo apoptosis in response to stimulation of Fas (30), direct infection as a mechanism of priming for Fas-mediated apoptosis or AICD for peripheral blood T cells in HIV disease is excluded by the fact that the frequency of HIV-infected T cells in peripheral blood (31,32) is much lower than the levels of Fas-induced apoptosis or AICD (2,6). In addition a number of reports have shown that cells undergoing apoptosis in lymph nodes of HIV-infected individuals or the thymus of SCID mice reconstituted with human fetal thymus and liver are not infected with HIV virus (33,34). Therefore an indirect mechanism for priming must be responsible for the enhanced T cell apoptosis in response to Fas stimulation or activation in HIV. Our results here show that Tat is not involved in the priming of T cells to undergo Fas-induced apoptosis but does enhance T cell AICD. Thus the mechanism of priming of T cells in HIV infection to undergo Fas-mediated apoptosis remains elusive.

Our study shows that Tat does not enhance the spontaneous apoptosis of normal T cells and the Jurkat T cell line. Although Tat can enhance AICD of peripheral blood T cells from healthy individuals and the Jurkat T cell line it can neither prime cells to undergo Fas-mediated apoptosis nor enhance Fas-induced apoptosis. Tat induced peripheral blood T cell apoptosis when  $O_2^-$  superoxide anion generation was inhibited or ROI were neutralized, raising the possibility that Tat under certain conditions may induce apoptosis by ROI independent mechanisms. Determining the mechanisms responsible for priming T cells to undergo Fas-mediated apoptosis in HIV may prove valuable in understanding the pathogenesis of HIV infection.

### Acknowledgements

The authors would like to thank Mr E. S. Wunderlich for excellent technical assistance, and Drs Abhey Patki and Michael Lederman (Division of Infectious Diseases, Chase Western Reserve University, Cleveland, OH) for reagents and suggestions. M. E. G. O. was supported by a National Science Foundation fellowship. J. F. T. R was supported by Stanford Immunology Training Grant AI-07290. This work was supported by NIH grants CA 42509, LM 04836 and AI 31770, and a grant from the Unicorn Foundation.

### Abbreviations

AICD	activation-induced cell death
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
ROI	reactive oxygen intermediate
SOD	superoxide dismutase
TMPO	3,3,5,5-tetramethylpyrrolone <i>N</i> -oxide

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