

# Membrane Tumor Necrosis Factor (TNF) Induced Cooperative Signaling of TNFR60 and TNFR80 Favors Induction of Cell Death Rather Than Virus Production in HIV-infected T Cells

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## Summary

Tumor necrosis factor (TNF) and lymphotoxin (LT) are highly pleiotropic cytokines that play a central role in regulating HIV-1 replication. These cytokines express their activities through two membrane receptors, TNFR60 (p55-60) and TNFR80 (p75-80). In the present study we have demonstrated by means of antagonistic and agonistic receptor-specific antibodies that in latently infected lymphocytic (ACH-2) cells the TNFR60 plays a dominant role in signaling HIV production, although selective activation of TNFR80 by receptor-specific antibodies can also induce HIV production. Unexpectedly, when both TNFRs were activated simultaneously by agonistic antibodies or coculture with cells expressing a noncleavable membrane form of TNF, HIV production was downregulated and induction of cell death was enhanced in ACH-2 cells. More relevant, *in vitro* HIV-infected peripheral blood lymphocytes cocultured with cells expressing membrane TNF underwent rapid induction of apoptosis with a subsequent reduced HIV production of these lymphocytes cultures. This was not observed with HIV-infected lymphocytes treated with soluble TNF. These data provide evidence for the differential trigger potential of membrane versus soluble TNF and show that TNFR80 is an important modulator of TNF responsiveness of HIV-infected T cells via cooperative signaling with TNFR60.

TNF is suspected to play an important role in HIV infection and progression of AIDS. This reasoning is based on the finding that TNF enhances or induces HIV replication *in vitro* in chronically infected, established cell lines and in freshly isolated peripheral blood mononuclear cells from HIV-infected individuals (1-4). On the other hand, it has been reported that, *in vitro*, HIV infection stimulates TNF gene expression and protein production (5). This finding is in accordance with a clinical correlation of enhanced TNF serum levels and disease state (6). It is conceivable that an autocrine-positive feedback loop exists between HIV infection and TNF production, in which TNF would act as a progression factor of virus replication. Aside from this direct influence of TNF on the HIV replication cycle, it is apparent that several of the AIDS associated pathophysiological changes observed during late stages of the disease (e.g., cachexia and neurodegeneration) are correlated with and could be due to chronically elevated TNF levels (reviewed in reference 7).

Lymphotoxin (LT)<sup>1</sup>, which is structurally and functionally similar to TNF, has also been shown to activate HIV

replication *in vitro* (3, 8). Both cytokines, TNF and LT, share the same membrane receptors for initiation of their cellular responses, the 55-60-kD TNF receptor 1 (TNFR60) and the 75-80 kD TNF receptor 2 (TNFR80) (9, 10). Both TNFRs are coexpressed in many tissues including hematopoietic cells, although membrane expression is independently regulated and may differ considerably, depending on the cell type (11-13). The individual contribution of the two TNFRs to TNF responses is not yet fully understood. *In vitro* models indicate that both receptors activate distinct signal pathways and can be functional on their own (14-16), but may also cooperate at the level of receptor-ligand interaction (17) and at the level of signal transduction (16).

With respect to TNF-mediated enhancement of virus production or induction of latent HIV, the critical role of NF- $\kappa$ B in this process has been shown for T lymphocytes and monocytes/macrophages as well as for neuronal cells (18-22). As TNF activation of NF- $\kappa$ B appears predominantly mediated via TNFR60-linked pathways (23-25), a role of this TNFR subtype in TNF-mediated HIV replication can be assumed and has been shown for a monocytic cell line (26), whereas the role of TNFR80 remained unclear. This is of particular interest, as TNFR80 is the prevailing TNFR sub-

<sup>1</sup>Abbreviations used in this paper: FDA, fluorescein diacetate; LT, lymphotoxin; RT, reverse transcriptase.

type in normal T cells, whereas cells of the myelomonocytic lineage usually express equal levels of both TNFRs (12, 27).

To understand whether both TNFRs are capable to transmit signals relevant to modulation of HIV replication, we have employed the natural ligand TNF in a soluble and in a stably membrane integrated form as well as LT $\alpha$ . Further, agonistic and antagonistic, receptor subtype-specific antibodies were used to mimic and block, respectively, TNF/LT $\alpha$  action. For these studies the T cell line ACH-2 was used as a model of postintegration HIV latency (reviewed in 28). This cell line has a very low basal level of HIV production, which is enhanced dramatically by external stimuli, in particular TNF or inducers of endogenous TNF (29), and has previously been used to study inhibition of TNF-mediated HIV replication by soluble TNFR constructs (30). In a second model, we have used in vitro activated and HIV-infected peripheral blood T cells to study the response to the 26-kD membrane expressed form of TNF, which has been recently shown to differ from soluble TNF in its receptor binding and cellular activation capacity (31).

## Materials and Methods

**Cell Lines.** The ACH-2 cell line (HIV-1 latent T-cell clone; 32) and the parental cell line CEM-SS were obtained from Dr. Thomas M. Folks, through the AIDS Research and Reference Reagent Program (Rockville, MD). The cells were propagated in RPMI-1640 (Gibco, Paisley, Scotland), 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin (Amimed, Basel, CH), 2 mM L-glutamine, and 10 mM Hepes buffer (Gibco) containing 10% FCS (Gibco) (complete tissue culture medium). CHO and NIH 3T3 cells were obtained from American Type Culture Collection (Rockville, MD). The generation and characteristics of the cell line B2.20, a stable CHO transfectant and 3T3mTNF, both expressing a membrane-bound, noncleavable, but biologically active deletion mutant of human TNF (huTNF $\Delta$ 1-12), have been described (31). 3T3mTNF( $\Delta$ 1-12) cells were a kind gift of Chiron Corp. (Emeryville, CA).

**Reagents and Antibodies.** Recombinant human TNF, LT $\alpha$ , rabbit anti-human TNF polyclonal antibody, and rabbit anti-human LT $\alpha$  polyclonal antibody were purchased from Genzyme (Cambridge, MA). The generation and specificity of the TNFR60-specific antagonistic mAb H398 (11), the TNFR60-specific agonistic mAb htr-1 (13), and the TNFR80-specific mAb MR2-1 (33) have all been described. The generation of the rabbit anti-human TNFR80 polyclonal agonistic serum, purification of IgG-fraction thereof and subsequent Fab fragment preparation has also been described previously (34). As control antibodies, a rabbit immune serum and a mouse mAb (IR $\gamma$ 2), each specific for the human IFN $\gamma$  receptor, as well as UPC10 (mouse IgG2a) and normal rabbit IgG (Sigma Chemical Co., St. Louis, MO) were used.

**Detection of Cell Surface Expression of TNF Receptors by Indirect Immunofluorescence.** ACH-2 and CEM-SS cells ( $0.5 \times 10^6$ /well) were dispensed in V-bottom 96-well plates (Nunc, Wiesbaden, Germany) and washed with ice cold PBS containing 0.1% NaN $_3$ . Cells were incubated in a blocking buffer designed to prevent nonspecific binding of antibodies containing 10% heat-inactivated human AB serum and 500  $\mu$ g/ml human IgG (Sigma) in PBS plus 0.1% NaN $_3$  for 30 min on ice. Specific anti-TNF receptor antibodies (H398, M80 IgG) were added to the cells at a final concentration of 50  $\mu$ g/ml and incubated for an additional 30 min on ice. Cells were washed twice with cold PBS and resuspended in

100  $\mu$ l of blocking buffer containing secondary antibody (20  $\mu$ g/ml) conjugated to a fluorochrome (goat F(ab') $_2$  anti-mouse IgG-R-phycoerythrin (Tago Inc., Burlingame, CA) or goat F(ab') $_2$  anti-rabbit IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) were mixed with the cells and incubated on ice in the dark for 30 min. Cells were washed twice with cold PBS plus NaN $_3$  and fixed in 2% formalin. UPC10 (mouse IgG2a) and normal rabbit IgG were used as isotype controls, as well as the addition of the secondary antibody alone to monitor nonspecific binding. Samples were placed in FACScan<sup>®</sup> cytofluorimeter and 10,000 events were acquired and analyzed with the *LYSIS II TM* software (Lysis, Mountainview, CA) and WinMDI software (provided by J. Trotter, Salk Institute, La Jolla, CA).

**Induction of HIV in ACH-2 Cells and Cell Quantification Assay.** ACH-2 cells, in log phase growth, were seeded in 250  $\mu$ l (30,000 cells) in 96-well flat-bottom plates (Nunc, Roskilde, Denmark). 24 h later, inducing agents were added to a final volume of 300  $\mu$ l. Cultures were incubated at 37°C in 5% CO $_2$ . Cell free supernatants were removed at 24, 48, and 72 h after induction and stored at -20°C until tested for HIV-1 production. At the end of the experiment (72 h) 1/3 of the cells were removed from the 96-well plates, placed in U-bottom 96-well plates, washed two times with PBS to remove serum, and resuspended in 100  $\mu$ l of PBS and 100  $\mu$ l of PBS containing 0.05 g/ml fluorescein diacetate (FDA) (Serva Fein Biochemica, Heidelberg, Germany). Stock FDA was prepared at 5 mg/ml in acetone and stored at -20°C. Cells were incubated in the dark for 30 min at room temperature and fluorescence was measured with a Titertek Fluoroscan II (Flow Laboratories, McLean, VA), programmed on the absolute scale, using a 485-nm excitation filter and a 538-nm emission filter. The fluorescence values obtained are expressed in arbitrary units (35).

**Preparation and HIV-1 Infection of Primary Human Lymphocytes.** Peripheral blood mononuclear cells were obtained from the blood of healthy HIV seronegative donors by a combination of leukapheresis and counter-current centrifugal elutriation as previously described (36). Lymphocytes were cultured in the presence of 0.25  $\mu$ g/ml phytohemagglutinin (Wellcome Diagnostics, Temple Hill, Dartford, UK) for 2 d in complete tissue culture medium (see ACH-2). Activation was confirmed by the increase in cell size (scattergram, flow cytometric analysis). These cells were washed, resuspended at  $8 \times 10^6$  cells/0.9 ml of medium and infected for 6 h with 0.1 ml of HIV-1/LAV containing cell-free supernatant (multiplicity of infection 0.14). This virus (LAV.04/A3.01) was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) (37), and passaged in lymphocytes. After viral adsorption, cells were washed and resuspended in complete medium supplemented with 100 U/ml of human recombinant IL-2 (Genzyme, Cambridge, MA).  $9 \times 10^4$  cells/well (0.3 ml) were plated in flat-bottom 96-well plates and cultured for 3 d. Noninfected lymphocytes were treated under similar conditions. At this point the cells were collected, washed, and resuspended at  $10^6$ /ml in complete medium.

**Evaluation of Cell Membrane Expressed, Noncleavable TNF Action on ACH-2 or PBLs (Coculture System).** The transfectant cells 3T3mTNF and B2.20 or the control parental cell lines 3T3 and CHO were trypsinized and plated ( $1-1.2 \times 10^4$  cells/0.25 ml per well) in 96-well flat bottom microtiter plates in complete tissue culture medium. After 2-3 d, ~80% confluency was achieved. To these monolayers, ACH-2 cells ( $32 \times 10^3$ ) or HIV-1/LAV-infected or noninfected PBLs ( $20 \times 10^4$ ) were added. Plates were centrifuged at 1,200 rpm for 3 min to produce an evenly distributed layer of cells. In the case of PBLs, after 16-18 h of coculture (37°C in 5%

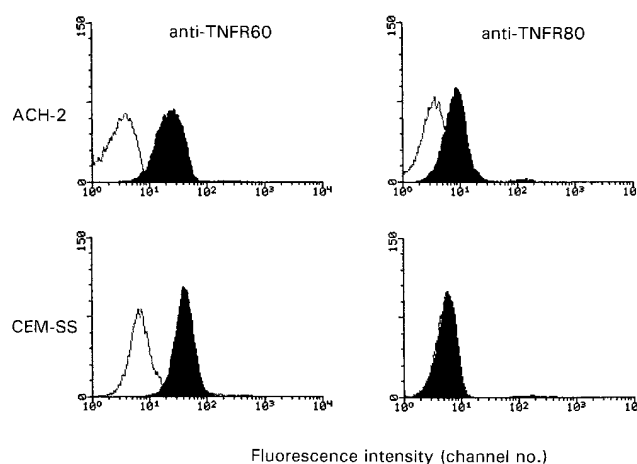
CO<sub>2</sub>), the nonadherent cells were removed from the 96-well culture plates by gently pipetting up and down four times with a multichannel pipettor. Replicate wells were pooled. Based on the different sizes, flow cytometric analysis (FACScan®; Becton Dickinson, Mountain View, CA) was used to distinguish PBLs from 3T3 or 3T3mTNF cells. Though there was an overlap in the forward scatter characteristics of the two cell populations, it was found that by using bivariate analysis of light scatter characteristics (i.e., forward scatter versus side scatter) the human PBLs could be easily distinguished from the 3T3 cells. This was confirmed by further cell analysis using direct fluorochrome conjugated monoclonal antibodies to CD4 and CD8 (Becton Dickinson). For each sample, the percentage of human PBLs was calculated and used to adjust the total cell recovery.

To quantify the effect of cell membrane bound TNF on viability of ACH-2 cells or lymphocytes, two different approaches were used. Due to the morphological features of the ACH-2 cells, direct quantification of dead cells (apoptotic) was performed using the propidium iodide/morphology method or the YO-PRO-1™ method. In the case of propidium iodide (38), ACH-2 cells were centrifuged, resuspended in 1 ml PBS ( $1-2 \times 10^6$ ), and fixed in 10 ml pre-cooled ( $-20^{\circ}\text{C}$ ) 70% ethanol. For labeling of cellular DNA (apoptosis analysis), stored samples ( $-20^{\circ}\text{C}$ ) were washed twice with PBS to remove the ethanol and rehydrate the cells. Centrifuged cell pellets were resuspended in PBS containing 20  $\mu\text{g/ml}$  of propidium iodide (Molecular Probes, Inc., Eugene, Oregon) and 100  $\mu\text{g/ml}$  RNase A (Sigma) and incubated overnight at  $4^{\circ}\text{C}$ . Fragmented nuclei in each sample were evaluated morphologically using a Leitz Diaplan fluorescence microscope (Wild Leitz AG, Zürich, Switzerland) equipped with a long pass filter (580 nm). A total of 200 cells per sample were scored for nuclear morphology. ACH-2 cells were also examined using labeling with YO-PRO-1™ (39) (Molecular Probes, Inc.). Equal volumes of cells and dye (final concentration of 10  $\mu\text{M}$ ) were mixed. After 10 min incubation at room temperature, samples were examined under a fluorescent light microscope (long pass filter, 520 nm), and fluorescent and nonfluorescent cells were counted. On the other hand, for PBLs we found that more consistent results within the study period were obtained when the total number of viable cells at the end of the coculture was determined instead of scoring dead or damaged cells, which appeared to be short lasting. For these cells, the YO-PRO-1™ method allowed to distinguish viable (nonfluorescent) from apoptotic or dead cells with accuracy.

**Quantification of HIV Production: Cell-free Supernatant Reverse Transcriptase Determination.** Virus production was determined as virion-associated reverse transcriptase (RT) activity in the supernatants of infected cells. RT was measured as described previously (40) with the following modifications: the reaction mixture was adjusted to 0.1% Nonidet P-40, 0.8 mM EDTA, and 10  $\mu\text{g/ml}$  of poly(A) with 0.16  $\mu\text{g/ml}$  oligo(T) as the template primer. The assay was performed in a 96-well format.

## Results

**Inhibition of TNF and LT $\alpha$  Induced HIV Production by TNFR Selective Antagonistic Antibodies.** To assess the role of the two TNFRs in TNF-mediated activation of HIV production in ACH-2 cells, membrane expression of both TNFR60 and TNFR80 was verified by immunofluorescence flow cytometry (Fig. 1). In contrast, the non HIV-infected parental cell line CEM only expressed TNFR60,

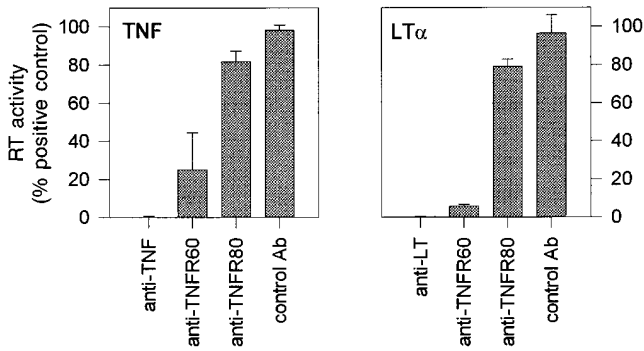


**Figure 1.** Coexpression of TNFR60 and TNFR80 in ACH-2 cells. TNFR expression was determined by indirect immunofluorescence flow cytometry analyses using TNFR60-specific monoclonal (H398) and TNFR80-specific polyclonal antibodies (M80) and FITC-conjugated secondary reagents as described in Materials and Methods. The open histogram represents staining by control antibody, fluorescence intensity obtained by staining with receptor-specific antibodies is shown in black.

suggesting an induction of TNFR80 in ACH-2 cells during establishment of latent HIV infection.

Next, HIV replication was induced by treatment of cultures with 10 ng/ml TNF or 100 ng/ml of LT $\alpha$ , concentrations typically evoking 70–90% of maximum HIV production (data not shown). TNF and LT $\alpha$  dependence of RT induction was affirmed by addition of anti TNF and anti LT $\alpha$  mAbs, respectively, resulting in a full suppression of supernatant RT activity in each case (Fig. 2, column 1). To determine the involvement of the two TNFRs in this response, neutralizing Fab fragments of receptor-specific antibodies (27, 34) were used for receptor blockade. Treatment of ACH-2 with TNFR60-specific H398 Fab provided, on average, 75% reduction (Fig. 2, column 2). By contrast, TNFR80-specific antagonists (M80 Fabs) caused only a minor ( $\sim 20-25\%$ ) inhibition of TNF-induced supernatant RT activity. The same inhibition profile was obtained, when cells were stimulated with LT instead of TNF (Fig. 2), indicating that both cytokines predominantly employ the TNFR60 for signaling HIV replication.

**Induction of HIV Replication by TNFR-specific Agonistic Antibodies.** To further scrutinize the individual role of the two TNFRs in induction of HIV, we used agonistic monoclonal (htr-1, MR2-1) and polyclonal (M80) antibodies against TNFR60 and TNFR80 to substitute for TNF in this response. Stimulation with the TNFR60 agonist htr-1 induced a strong response similar to saturating concentrations of TNF (Fig. 3), indicating that selective TNFR60 triggering is sufficient to induce maximum TNF responses with respect to RT activation. However, selective TNFR80 triggering by either polyclonal (M80) (Figs. 3 A and 4 A) or monoclonal (MR2-1; Fig. 3 B) receptor-specific antibodies induced significant RT activity in ACH-2 cells, too, reaching 35–50% of the maximum response induced by TNF or htr-1 stimulation (Figs. 3 and 4 A). Specificity was



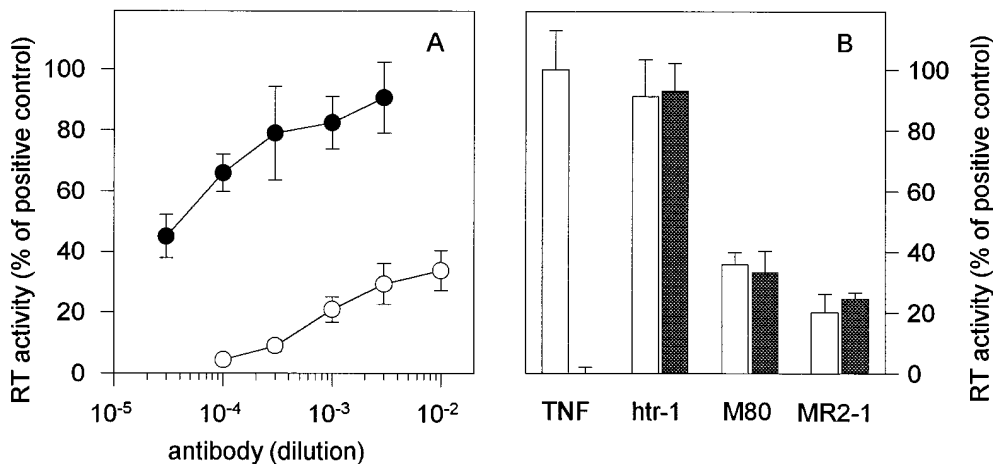
**Figure 2.** Inhibition of TNF and LT induced HIV production by TNFR-specific monovalent antibody fragments. ACH-2 cells were left untreated or were preincubated for 1 h at 4°C with 10 µg/ml of the respective monoclonal or polyclonal reagent before culture for 3 d in the presence of TNF or LT as indicated. Lanes 1, anti TNF/anti LT, respectively; lanes 2, anti TNFR60 (H398Fab); lanes 3, anti TNFR80 (M80Fab); lanes 4, control Fab. Data presented are the mean values SEM from three independent experiments (triplicate cultures) in the case of TNF and mean values SD from one experiment in the case of LT. Data are expressed as relative supernatant RT activity in % of the positive control (TNF- and LTα-treated cells, 10 and 100 ng/ml, respectively). The mean induced supernatant RT activity for individual experiments was between 525 and 1,100 cpm/µl).

controlled by the use of mono- and polyclonal antibodies directed against the huIFNγ receptor, which exerted no HIV-inducing activity (56 and 59 cpm, respectively, versus a background value of 66 cpm). Accordingly, TNFR80 is also linked to signal pathways leading to HIV replication.

The induction of autocrine TNF has been described as a mechanism for stimulation of HIV production in the monocytic cell line U1 (29). Whether endogenous TNF production is involved in TNFR-selective activation of HIV replication in ACH-2 cells was assessed by the use of neutralizing anti-TNF antibodies. The data obtained show

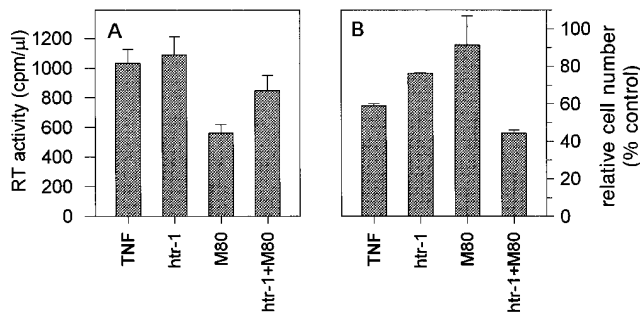
that neither TNFR60- nor TNFR80-mediated induction of RT activity can be inhibited by anti-TNF antibodies (Fig. 3 B) suggesting a direct TNFR mediated induction of HIV production.

Because each of the TNFR-specific agonistic antibodies independently elicited HIV production in ACH-2, it was of interest to investigate whether the combined action of the two receptor-specific antibodies induces a further increase in RT activity. Unexpectedly, when the receptor selective agonists were used in combination, each at its optimum activating concentration, the cellular response of ACH-2 was less than that expected from addition of the individual responses and even less than the response obtained by htr-1 stimulation only (Fig. 4 A). When the cell numbers were determined it became apparent that, although TNF is a potent inducer of virus replication, it simultaneously has an effect on ACH-2 growth or viability during the three day culture (Fig. 4, A and B, column 1). This could be a direct consequence of cell damage due to production and release of virions or alternatively reflect an independent action of TNF on this particular cell line. Microscopical analyses of ACH-2 confirmed that TNF treated cultures not only contain a reduced total cell number, but also a significant proportion of trypan blue positive cells (25 vs. 5% in untreated cultures; data not shown), indicating that TNF-induced cell death is at least in part responsible for the observed reduction in cell numbers. Interestingly, each of the agonistic antibodies studied here, also when used at a maximum RT inducing concentration, showed less effect on cell numbers when compared to TNF treated ACH-2 (Fig. 4 B). A combination of both receptor-specific agonists, however, induced an enhanced cytotoxic effect, even exceeding that of saturating TNF concentrations (Fig. 4 B). These data suggest that a major reason for the lack of an additive RT enhancing effect of the two receptor-specific agonistic antibodies is due to a predominant induction of cell death in



**Figure 3.** Induction of HIV production by agonistic TNFR-specific antibodies. (A) ACH-2 were cultured in the presence of the indicated dilutions of TNFR60-specific mab htr-1 (hybridoma supernatant, ●) or TNFR80-specific antiserum M80 (○) and supernatant RT activity was determined after 72 h. For comparison, control cultures were set up in parallel with unstimulated cells and TNF (10 ng/ml)-treated cells. Shown are the mean values SD of triplicates of a typical experiment ( $n = 3$ ). Basal supernatant RT activity of untreated cells (60 cpm/µl) has been subtracted and data are expressed in percentage of the positive

control (10 ng/ml TNF: 925 ± 55 cpm/µl = 100%). (B) ACH-2 cells were stimulated for 72 h with htr-1 (hybridoma supernatant 1/100), M80 IgG (4 µg/ml) or MR2-1 (2 µg/ml) in the absence (open bars) or presence (filled bars) of 10 µg/ml of a neutralizing anti-human TNF antibody. Data shown are the mean values SD of six replicate cultures expressed in percentage of the RT activity induced by 10 ng/ml TNF (100% = 934 ± 126 cpm, the background of 66 cpm has been subtracted).

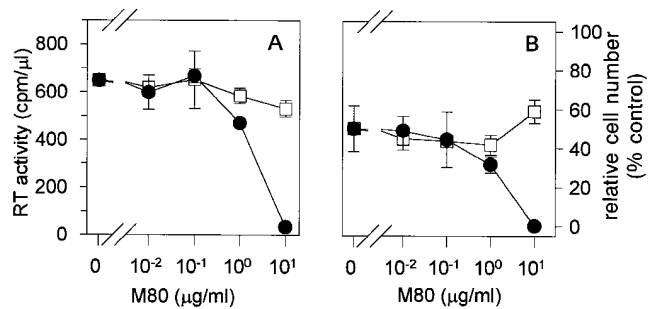


**Figure 4.** Coactivation of TNFR60 and TNFR80 downregulates HIV production and enhances cell death. ACH-2 cells were cultured for 3 d in the presence of TNF (100 ng/ml) and TNFR-specific antibodies (htr-1, 1/50; M80, 40 μg/ml) as indicated. (A) RT activity (mean values SD of five replicate cultures) was determined in the supernatants as described in material and methods. (B) Total cell number of ACH-2 was determined by the FDA method from the groups shown in A in parallel. Data are expressed as relative cell numbers (100% = untreated cells).

ACH-2. This effect was not seen when either of the TNFR-specific antibodies was combined with an irrelevant control antibody (data not shown).

**Enhancement of TNF-induced Cell Death by TNFR80-specific Agonistic Antibodies.** The previous set of experiments had indicated that TNFR-specific agonistic antibodies in combination can induce an ACH-2 cell response pattern distinct from that inducible by TNF. To determine which of the two TNFRs is triggered differentially by natural ligand versus agonistic antibody, we titrated the antibodies into ACH-2 cultures stimulated by a constant concentration of TNF. In all experiments the TNFR60 agonist htr-1 displayed no significant additional effects on either TNF-induced RT induction or growth inhibition (data not shown), whereas the TNFR80 agonist M80 strongly diminished the TNF-induced RT induction and enhanced the TNF-induced cytotoxicity in parallel. Although the quantity of these M80 effects varied considerably within the four experiments performed, they were significant in all cases and could even lead to a total inhibition of RT induction, paralleled by killing of all ACH-2 cells at the end of a three-day culture (Fig. 5). To achieve this activation of TNFR80, complete antibodies were required, as Fab fragments of the agonist M80 showed no effect (Fig. 5). In the presence of the TNFR60 antagonistic antibody H398, not only virus replication (Fig. 2) but also the TNF dependent cell death was fully reverted (data not shown). Together, these data suggest that efficient induction of cell death in ACH-2 requires cooperative signaling via both TNFRs.

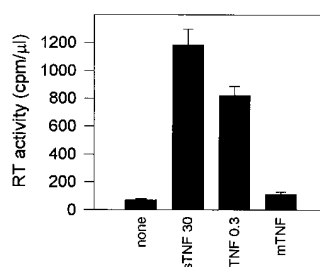
**Induction of Apoptosis but not HIV Replication by the Membrane form of TNF.** The enhancement of cell death, upon combined activation of both TNFRs, either by agonistic antibodies or by TNF plus agonistic antibodies specific for TNFR80, pointed to a differential triggering of TNFR80 by the soluble form of the natural ligand versus receptor-specific antibodies. Because we have recently identified membrane TNF (mTNF) to be the physiological equivalent to antibody-mediated TNFR80 activation (31), we in-



**Figure 5.** TNFR80 enhances TNF mediated cytotoxicity in ACH-2 cells. ACH-2 cells were cultured in triplicates with TNF (10 ng/ml) in the presence of the indicated concentrations of the TNFR80-specific agonist M80 (filled circle) or Fab produced from purified M80 IgG (open square). Shown is one experiment out of a total of four with similar results. (A) Supernatant RT activity (mean values SD) was determined after 3 d of culture. (B) The total cell number of ACH-2 was determined by the FDA method. Data are expressed as the relative cell numbers (100% = control cells in the absence of TNF, mean values ± SD).

vestigated the role of mTNF in induction of HIV replication and apoptosis in HIV-infected cells. In a first set of experiments, a stable transfectant cell line, 3T3mTNF, expressing an exclusively membrane bound, nonsecretable mutant form of TNF has been used in coculture experiments with ACH-2 cells and compared to the RT activity induced by recombinant, soluble TNF (sTNF). Different mTNF-expressing cell lines have been recently shown to be superior to optimum concentrations of sTNF in the induction of a variety of cellular responses (31, 41, 42). As predicted from the data obtained upon combined TNFR antibody stimulation (Figs. 4 and 5), mTNF was found to be a rather poor activator of HIV replication in ACH-2 cells. A representative experiment is shown in Fig. 6, where coculture of ACH-2 with 3T3mTNF resulted in <10% of the RT activity induced with low concentrations of sTNF (0.3 ng/ml).

Control of cell numbers at the end of a three-day culture revealed massive cell death in the HIV-infected cell line cocultured with mTNF-expressing transfectants. This was readily detectable by microscopical analysis, although a reliable quantification of cell numbers by the FDA method was not possible because of the high background values from the NIH 3T3 cells. To quantify this effect, we used the cell line B2.20, a CHO clone transfected with the non-



**Figure 6.** Membrane TNF expressing transfectants fail to induce HIV replication. ACH-2 cells were cultured alone in the absence or presence of TNF (30, 0.3 ng/ml) or were cocultured with transfectants expressing mTNF. Supernatant RT activity was determined after 3 d of coculture. Shown are the mean values ± SD of six replicate cultures from a representative experiment ( $n = 3$ ) performed with 3T3mTNF cells.

**Table 1.** Membrane TNF Expressing B2.20 Cells Induce Apoptosis but Not HIV Replication in ACH-2 Cells

Treatment	Cell No.*	Apoptotic Cells <sup>‡</sup>	RT activity <sup>§</sup>
	$\times 10^3$	%	cpm/ $\mu$ l
None	111	4	38 $\pm$ 8
sTNF	93	18	212 $\pm$ 20
CHO	125	6	27 $\pm$ 5
B2.20	68	28	54 $\pm$ 15

ACH-2 cells were seeded at  $125 \times 10^3$  cells/well, in the absence or presence of sTNF (30 ng/ml) or in wells containing monolayers of CHO or B2.20 cells as described in Materials and Methods. 22 h after onset of cultures,  $10 \mu$ l of supernatant was removed and RT activity determined. Thereafter, the nonadherent ACH-2 cells were removed from the wells (60 wells for each experimental condition), pooled, and counted.

\*Average calculated number of cells recovered per well.

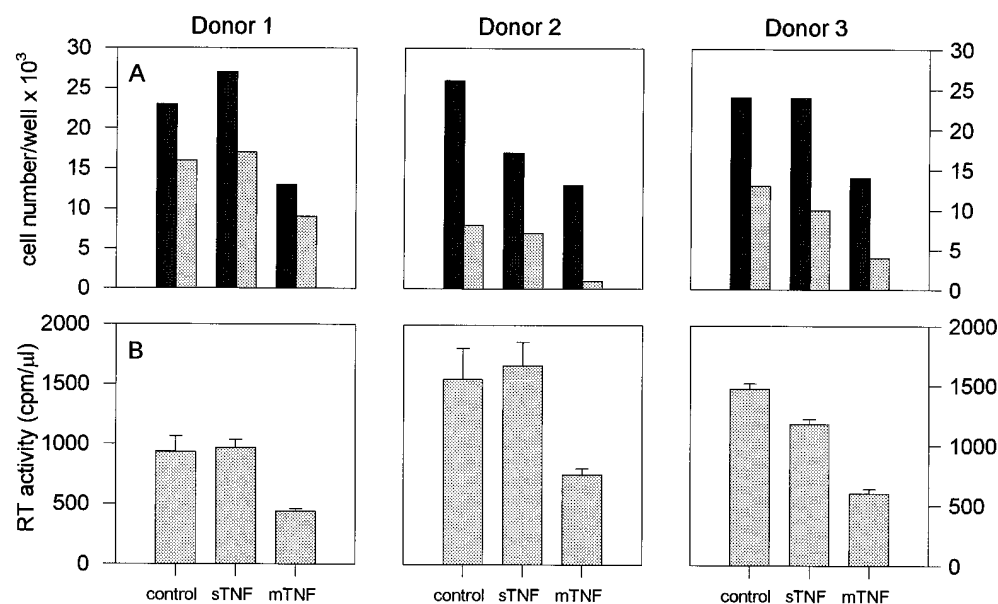
<sup>‡</sup>The cells were further stained with YO-PRO-1<sup>™</sup>, 200 cells for each group were counted and cells displaying fragmented or condensed nuclei were scored as apoptotic.

<sup>§</sup>Mean values  $\pm$  SD of six replicates.

cleavable form of human TNF (31). These cells are easily distinguishable by size and morphology from ACH-2 cells. As shown in Table 1, the ACH-2 cell recovery after 22 h of coculture with B2.20 cells was significantly reduced compared to ACH-2 cocultured with control CHO and even less compared to cultures in the presence of high concentrations of soluble TNF. This clearly correlated with a higher number of apoptotic cells present in the ACH-2/

B2.20 cocultures at this time point, indicating that induction of cell death is more efficient or follows a faster kinetics when mTNF instead of sTNF was the activating ligand. Furthermore, in accordance with the results obtained with 3T3mTNF cocultures (Fig. 6), it was also clear from this experiment that sTNF induced strong HIV (RT) production, while this was not the case in the ACH-2/B2.20 coculture (Table 1). According to these observations, the failure of mTNF to promote virus replication may thus be directly related to a preferential activation of apoptotic processes in HIV-infected cells preceding the production of infectious virus particles.

To confirm the above described observations in a cellular system that reflects more closely the in vivo situation, we studied the role of mTNF in induction of apoptosis and virus production in HIV-infected, PHA-activated peripheral blood lymphocytes. The effect of mTNF was compared to that of sTNF in HIV-infected and noninfected PBLs. Fig. 7 shows results obtained in independent experiments with three different blood donors. 16–18 h after onset of cocultures, a significantly higher reduction in viable cell numbers was observed in all cultures containing mTNF expressing 3T3 cells as compared to cultures with control 3T3 cells plus high concentrations of sTNF. Although mTNF was found to induce apoptosis in both, the infected and the noninfected, PHA-activated T cells, in two out of the three donors examined here (numbers 2 and 3), an apparent preferential kill of the HIV-infected population (number of viable cells 13 and 30% of control) as compared to noninfected cultures (number of viable cells 50 and 58% of control) was observed (Fig. 7A). More revealing, the T cells surviving a 16 h 3T3mTNF coculture were signifi-



**Figure 7.** Effect of 3T3mTNF cells on in vitro HIV-1-infected and noninfected human lymphocytes. PBLs prepared from three different healthy blood donors were in vitro activated with PHA (0.25  $\mu$ g/ml) for 2 d, left noninfected or were infected with HIV-1/LAV. 3 d later, activated lymphocytes were plated on a near confluent monolayer of mTNF expressing 3T3mTNF transfectants (mTNF) or 3T3 control cells without (control) or with sTNF (10 ng/ml) (sTNF) (six replicate cultures). At the onset of cocultures, the RT activity of HIV-infected cells was  $2219 \pm 247$  (donor 1),  $585 \pm 88$  (donor 2) and  $332 \pm 62$  (donor 3) cpm/ $\mu$ l. (A) 16 h after coculture, replicate cultures were pooled, the YO-PRO negative number of cells analysed and the average number of viable cells per well

was determined. Black bars indicate noninfected cells and grey bars indicate infected cells. (B) HIV-1 production (cell-free supernatant RT activity) of the infected lymphocytes from the three donors upon separation from the 3T3 coculture after 16 h and replating in wells without 3T3 for an additional 2 d. The carry over of 3T3 cells was minimal as assessed by the absence of adherent cells after the 2-d culture. Each group represents the mean  $\pm$  SD from six replicate cultures.

cantly impaired in their virus production capacity, as revealed upon transfer and reculture in the absence of mTNF expressing cells (Fig. 7 B), suggesting that the number of infected, virus producing cells has been reduced. In contrast, upon replating HIV-infected T cells pretreated for 16 h with sTNF RT activity was similar to control cultures. These data indicate a higher sensitivity of HIV-infected, primary lymphocytes towards apoptosis induced by mTNF.

## Discussion

The aim of this study was to delineate the specific contribution of the two TNF receptors to TNF responses of HIV-infected T cells. Our data not only show a differential function of the two TNFRs in regard to activation of HIV replication, with TNFR60 as the major mediator of HIV activation, but also provide evidence for TNFR80 as an important modulator of the cellular response. We show that TNFR80 is capable of converting, upon appropriate activation, the response phenotype from promoting virus replication to induction of cell death.

Based on the existing knowledge of the critical involvement of NF- $\kappa$ B in promoting HIV replication (18–22) and its activation via TNFR60 signal pathways (23–25) as well as from observations with the OM-10.1 cell line (26) it was anticipated to find a dominant role of TNFR60 in HIV induction in the T cell line ACH-2 as well. This holds true for both TNF and LT $\alpha$ . With regard to TNFR80, we show by use of two distinct receptor-specific, agonistic reagents that this receptor is also capable to convey activation signals for virus production in this T cell line. Our data further show that the TNFR80-mediated activation of HIV replication in ACH-2 cells is likely to be a direct effect and not an indirect action via endogenously produced TNF, the latter potentially activating, in an autocrine feedback loop, HIV replication via TNFR60. A direct TNFR80-mediated signal pathway to HIV replication is in full accordance with the recent demonstration that, in T cells, TNFR80 can trigger NF- $\kappa$ B activation (43). As both TNFRs employ a common receptor associated signal transducer, TRAF 2, for NF- $\kappa$ B signaling (44, 45), it appears likely that shared signal pathways are used by each of the receptor subtypes to induce HIV replication in ACH-2 cells.

Quite unexpected, however, was our observation with the T cell line ACH-2 and acutely HIV-infected PBLs that a simultaneous triggering of both TNFRs with agonistic, receptor-specific antibodies (ACH-2) or membrane TNF-expressing cells (ACH-2, PBL) was not equivalent to a treatment with maximum activating concentrations of soluble TNF. Rather, this resulted in a response counteracting virus replication, namely rapid induction of apoptosis. Although, at present, we cannot rule out that upon combined activation of both TNFRs, unknown signal pathways negatively controlling HIV replication become effective independent of induction of apoptosis, the available *in vitro* data suggest a correlation between reduction in viable cell number and inhibition of virus replication.

The differential signal capability of TNFR-specific agonistic antibodies versus sTNF could be mapped to TNFR80, yet the shift of the cellular response towards enhanced cell death was brought about by cooperative signaling via both receptors, in which mTNF or antibody mediated induction of TNFR80-elicited signal pathway(s) determine(s) the overall outcome of the cellular response. This interpretation is based on the following findings: (a) stimulation with a potent TNFR60 agonist, htr-1, led to the induction of a full response with respect to HIV induction in ACH-2 cells, making TNFR80 signaling dispensable for this action; (b) with respect to concomitantly induced apoptosis of HIV-infected cells, signaling via both receptors is evident from a limited (htr-1) or virtually no (M80) cytotoxic activity of each of the agonistic antibodies on their own as compared to TNF; (c) the enhanced apoptosis upon treatment with sTNF plus TNFR80-specific antibody, but not sTNF plus TNFR60-specific antibody, suggests that a distinct signal quality is transduced via TNFR80, which is not inducible by soluble TNF.

The superior capability of agonistic antibodies over sTNF to induce cellular responses via TNFR80 is not without precedence (31, 46), and evidence for cooperative signaling of both TNFRs, leading to quantitatively and/or qualitatively distinct responses, has been obtained by us in several other experimental systems (31, 41, 42, 47, 48). It is evident from these *in vitro* studies that mTNF, unlike sTNF, is an efficient inducer of TNF responses due to simultaneous activation of both TNFR subtypes. According to these studies, the underlying mechanism of mTNF versus sTNF action resides, at least in part, in a 10-fold higher off rate of sTNF at TNFR80 as compared to TNFR60, resulting in a low signal capacity of TNFR80 under these conditions. In contrast, binding of mTNF by its cognate receptors during cell–cell contacts is likely to persist for a time long enough to ensure the formation of active signaling complexes at TNFR80, too. Agonistic antibodies mimic this stable receptor ligand interaction. Consistent with this interpretation is the finding that a nonagonistic, receptor-specific antibody, which stabilizes sTNF binding to TNFR80, restores a high signal capacity of this receptor (31).

A possible *in vivo* relevance mTNF in conveying protective TNF responses during host defense against parasites and tumors had already been suggested previously (49–51), although, at that time, the underlying mechanisms for a superior action of mTNF were not understood. We now extend these findings by showing that in *in vitro* models of acute HIV infection and HIV latency, mTNF promotes apoptosis of infected T cells rather than virus replication and that the simultaneous application of TNFR80-specific agonistic antibodies can convert a response to sTNF into a mTNF like response.

What are the implications of the data presented here for understanding of HIV replication *in vivo*? Provided TNF plays a similar role for propagation of virus infection *in vivo*, our results point to a potentially important regulatory function of TNFR80 in HIV-infected cells because of a

differential response to soluble versus membrane bound TNF. Accordingly, both the TNFR status of infected cells and the relative concentrations of soluble versus membrane TNF in their close vicinity should influence the type of response. TNFR80 expression is highly regulated and, in particular in lymphoid cells, activation dependent (9, 10, 12). As efficient induction of apoptosis by mTNF in HIV-infected T cells requires cooperative signaling via both TNFRs, regulation of TNFR80 expression could ensure selectivity. The constitutive TNFR80 expression in the chronically infected ACH-2, but not the parental CEM cell line, could be a correlate of the situation during primary infection of lymphocytes. Further, for the OM-10.1 cell line, another in vitro model of chronically infected cells, it has been shown that TNFR60 activation not only caused HIV replication, but simultaneously a selective upregulation of TNFR80 expression (26), thus potentially rendering this HIV-infected cell line more sensitive to apoptosis. In this context, it is important to note that TNF-induced apoptosis appears, at least in vitro, not exclusively restricted to HIV-infected cells. Rather, it has already recently been shown that, upon repeated in vitro activation, T cells can undergo apoptosis via a TNF/TNFR80-dependent pathway, too (52). The particular role of membrane TNF in this process becomes apparent from the data presented here, showing a greater apoptotic response to mTNF as compared to sTNF also on noninfected, but PHA-activated T cells. Despite this principle sensitivity of in vitro polyclonally activated T cells to TNF mediated apoptosis, we have here obtained evidence for a higher sensitivity of HIV-infected versus noninfected peripheral blood lymphocytes towards apoptosis induced by mTNF. More revealing, only in the presence of mTNF expressing cells, but not sTNF, was a reduction of virus

production observed in acutely infected lymphocyte cultures. The intracellular mechanism(s) causing increased sensitivity of HIV-infected cells to mTNF-induced apoptosis are unknown at present.

Soluble TNF is proteolytically derived from its membrane precursor, and mTNF expression has been verified on activated monocytes, T cells and endothelial cells (42, 53, 54). In the context discussed here it is interesting to note that HIV-specific CD4<sup>+</sup> T cell clones express membrane TNF (54). Accordingly, in vivo, not only macrophages but also virus-specific T cells are a potential source of mTNF and could, independent of a T cell receptor mediated cytolytic effector function, signal cell death to TNFR60 and TNFR80 coexpressing, HIV-infected cells. However, mTNF-expressing cells could potentially also contribute to the bystander effect by signaling apoptosis to those non infected, yet activated lymphocytes that coexpress sufficient amounts of both TNF receptors. Independent on the in vivo frequency of the latter, the model predicts that all nonactivated and thus TNFR80 negative T cells (likely the vast majority) would be spared.

Based on the data presented, we hypothesize that an in vivo prevention of proteolytic processing of membrane TNF to its soluble form, e.g., by TNF-specific protease inhibitors (55–58), should not only downmodulate systemic toxicities associated with high serum TNF levels (55), but also could limit virus replication by enhanced elimination of HIV-infected cells. Further studies in appropriate animal models will be necessary to evaluate whether or not an inhibition of TNF processing or antibody mediated TNFR80 coactivation are effective means to reduce the number of HIV-infected T cells in vivo and could be employed together with other antiviral strategies.

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