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Research Article

We investigated the effect of the acute promyelocytic leukemia (APL) specific PML/RARalpha fusion protein on the sensitivity to TNF-alpha-mediated apoptosis. The U937 leukemia cell line was transduced with PML/RARalpha cDNA. PML/RARalpha expression caused a markedly reduced sensitivity to TNF-alpha, even if apoptosis was triggered by agonistic antibodies to TNF-alpha receptors I and II (TNF-alphaRI, II). PML/RARalpha induced a 10-20-fold decrease of the TNF-alpha-binding capacity via downmodulation of both TNF-alphaRI and TNF-alphaRII: this may mediate at least in part the reduced sensitivity to TNF-alpha. Furthermore, the fusion protein did not modify Fas expression (CD95) or sensitivity to Fas-mediated apoptosis. The pathophysiological significance of these findings is supported by two series of observations. (a) Fresh APL blasts exhibit no TNF-alpha binding and are resistant to TNF-alpha-mediated apoptosis. Conversely, normal myeloblasts-promyelocytes show marked TNF-alphaR expression and are moderately sensitive to TNF-alpha-mediated cytotoxicity. Similarly, blasts from other types of acute myeloid leukemia (AML M1, M2, and M4 FAB types) show an elevated TNF-alpha binding. (b) The NB4 APL cell line, which is PML/RARalpha+, shows low TNF-alphaR expression capacity and is resistant to TNF-alpha-triggered apoptosis; conversely a PML/RARalpha- NB4 subclone (NB4.306) exhibits detectable TNF-alpha-binding capacity and is sensitive to TNF-alpha-mediated cytotoxicity. These studies indicate that the PML/RARalpha fusion protein protects against TNF-alpha-induced apoptosis, at least in part via downmodulation of TNF-alphaRI/II: this phenomenon may play [...]

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The PML/RAR α Fusion Protein Inhibits Tumor Necrosis Factor- α -induced Apoptosis in U937 Cells and Acute Promyelocytic Leukemia Blasts

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Abstract

We investigated the effect of the acute promyelocytic leukemia (APL) specific PML/RAR α fusion protein on the sensitivity to TNF- α -mediated apoptosis. The U937 leukemia cell line was transduced with PML/RAR α cDNA. PML/RAR α expression caused a markedly reduced sensitivity to TNF- α , even if apoptosis was triggered by agonistic antibodies to TNF- α receptors I and II (TNF- α RI, II). PML/RAR α induced a 10–20-fold decrease of the TNF- α -binding capacity via downmodulation of both TNF- α RI and TNF- α RII: this may mediate at least in part the reduced sensitivity to TNF- α . Furthermore, the fusion protein did not modify Fas expression (CD95) or sensitivity to Fas-mediated apoptosis.

The pathophysiological significance of these findings is supported by two series of observations. (a) Fresh APL blasts exhibit no TNF- α binding and are resistant to TNF- α -mediated apoptosis. Conversely, normal myeloblasts-promyelocytes show marked TNF- α R expression and are moderately sensitive to TNF- α -mediated cytotoxicity. Similarly, blasts from other types of acute myeloid leukemia (AML M1, M2, and M4 FAB types) show an elevated TNF- α binding. (b) The NB4 APL cell line, which is PML/RAR α ⁺, shows low TNF- α R expression capacity and is resistant to TNF- α -triggered apoptosis; conversely a PML/RAR α ⁻ NB4 subclone (NB4.306) exhibits detectable TNF- α -binding capacity and is sensitive to TNF- α -mediated cytotoxicity.

These studies indicate that the PML/RAR α fusion protein protects against TNF- α -induced apoptosis, at least in part via downmodulation of TNF- α RI/II: this phenomenon may play a significant role in APL, which is characterized by prolonged survival of leukemic blasts. (*J. Clin. Invest.* 1998. 101:2278–2289.) Key words: leukemia • tumor necrosis factor • apoptosis • fusion protein

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Introduction

Acute promyelocytic leukemia (APL)¹ is characterized by the accumulation of blasts blocked at the promyelocytic stage of differentiation. The differentiation block is a major determinant in the maintenance of leukemic phenotype, as induction of terminal differentiation with retinoic acid (RA) therapy leads to clinical remission (for reviews see references 1 and 2). The blast proliferation rate in APL is lower than that in the other acute myeloid leukemias (AMLs), thus indicating that prolonged cell survival significantly contributes to accumulation of APL blasts (3, 4). In most APL cases, the molecular hallmark is the PML/RAR α fusion gene, generated by the t(15;17) translocation (5–8). RAR α encodes one of the RAR (9, 10); PML encodes a protein of unknown function localized in nuclear structures called nuclear bodies (NB) or PML oncogenic domains (11–13). The PML/RAR α fusion protein, expressed in the vast majority of APL cases (14), has been implicated in both leukemogenesis and response to RA. Thus, ectopic expression of PML/RAR α in human leukemic cell lines blocks differentiation by physiological stimuli, i.e., vitamin D3, D3 + TGF- β 1, or hemin (15–18), and increases susceptibility to the differentiative RA stimulus (15–18), whereas at low serum concentration it enhances cell proliferation due to diminished cell death (15). Furthermore, mice transgenic for PML/RAR α show myeloid differentiative alterations similar to those of promyelocytic leukemia (19). In the chicken model, PML/RAR α transforms early hematopoietic progenitor cells (HPCs) in vitro and induces acute leukemia in vivo (20). In spite of these advances, the cellular and molecular mechanisms mediating the PML/RAR α biological activity are poorly understood. Recent studies have shown that APL cells secrete several cytokines, including TNF- α (21, 22). This pleiotropic cytokine, primarily produced by macrophages, plays a major role in inflammatory, immunological, and pathophysiological reactions (reviewed in 23), but also exerts in vitro a direct cytotoxic activity on a variety of normal and tumor cells (24, 25). TNF- α induces its cellular responses through interaction with two specific membrane receptors of different molecular masses, 55–60 kD (TNF- α RI) and 75–80 kD (TNF- α RII) (reviewed in 26). The cytotoxic effects of TNF- α are triggered by signals activated through interaction with p60TNF- α R (27); in other cellular systems p80TNF- α R either cooperates with p60TNF- α R (28) or by itself mediates cytotoxic signals (29, 30). Accordingly, in the myeloid precursor leukemic cell line

1. *Abbreviations used in this paper:* AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; HPC, hematopoietic progenitor cell; NB, nuclear bodies; RA, retinoic acid; wt, wild type.

U937, which expresses both TNF receptors, cytotoxicity of TNF- α is mainly mediated by p60TNF- α R (27, 31), although an involvement of p80TNF- α R has also been proposed (28, 32, 33).

In these studies we have investigated the relationship between the antiapoptotic effect of PML/RAR α and TNF- α cytotoxicity. Particularly, we have evaluated (a) the effect of PML/RAR α expression on TNF- α -mediated cell apoptosis using the U937 target cell line, which is highly sensitive to TNF- α cytotoxicity (27, 31). Further studies were carried out on (b) a PML/RAR α^+ APL cell line (NB4) and a PML/RAR α^- subclone (NB4.306), and (c) fresh blasts from APL and other AML type cases.

Methods

Cells. The wild-type (wt) U937 myeloid cell line was maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) and 10% FCS. Detailed cloning methodology to obtain PML/RAR α^+ U937 cells has been reported previously (15).

The wt PML/RAR α^+ NB4 promyelocytic cell line (34) as well as

the NB4.306 subclone were grown in RPMI medium containing 10% FCS. The NB4.306 clone, derived from the NB4 cell line (35), is characterized by the loss of PML/RAR α protein synthesis, in spite of the presence of the t(15;17) translocation.

Fresh leukemic blasts from 11 AML patients (one pertaining to M1, two to M2, five to M3, and three to M4 FAB subtypes), obtained after informed consent, were isolated from bone marrow by Ficoll Hypaque density centrifugation. All M3 cases were PML/RAR α^+ .

Peripheral blood HPCs were obtained from normal adult donors after informed consent. The HPCs, purified as reported (36, 37), were grown in liquid suspension cultures under conditions allowing selective growth of granulocytopoietic-neutrophilic cells (i.e., in the presence of 1 U/ml IL-3, 0.1 ng/ml GM-CSF, and 500 U/ml of G-CSF) (37).

Analysis of cell growth, cell cycle distribution, and apoptosis. Cell growth was determined by counting the number of viable cells after trypan blue exclusion test. Apoptosis was determined by four different methods: (a) flow cytometric analysis of cells labeled by the TdT method (TUNEL assay, 38) using a kit from Boehringer Mannheim (Mannheim, Germany); (b) determination of hypodiploid cells by flow cytometric analysis of PI-stained nuclei (39); (c) detection of histone-associated DNA fragments in the cytoplasm of dying cells using an ELISA kit (Boehringer Mannheim); and (d) annexin-V-binding assay using a kit from R & D Systems (Minneapolis, MN).

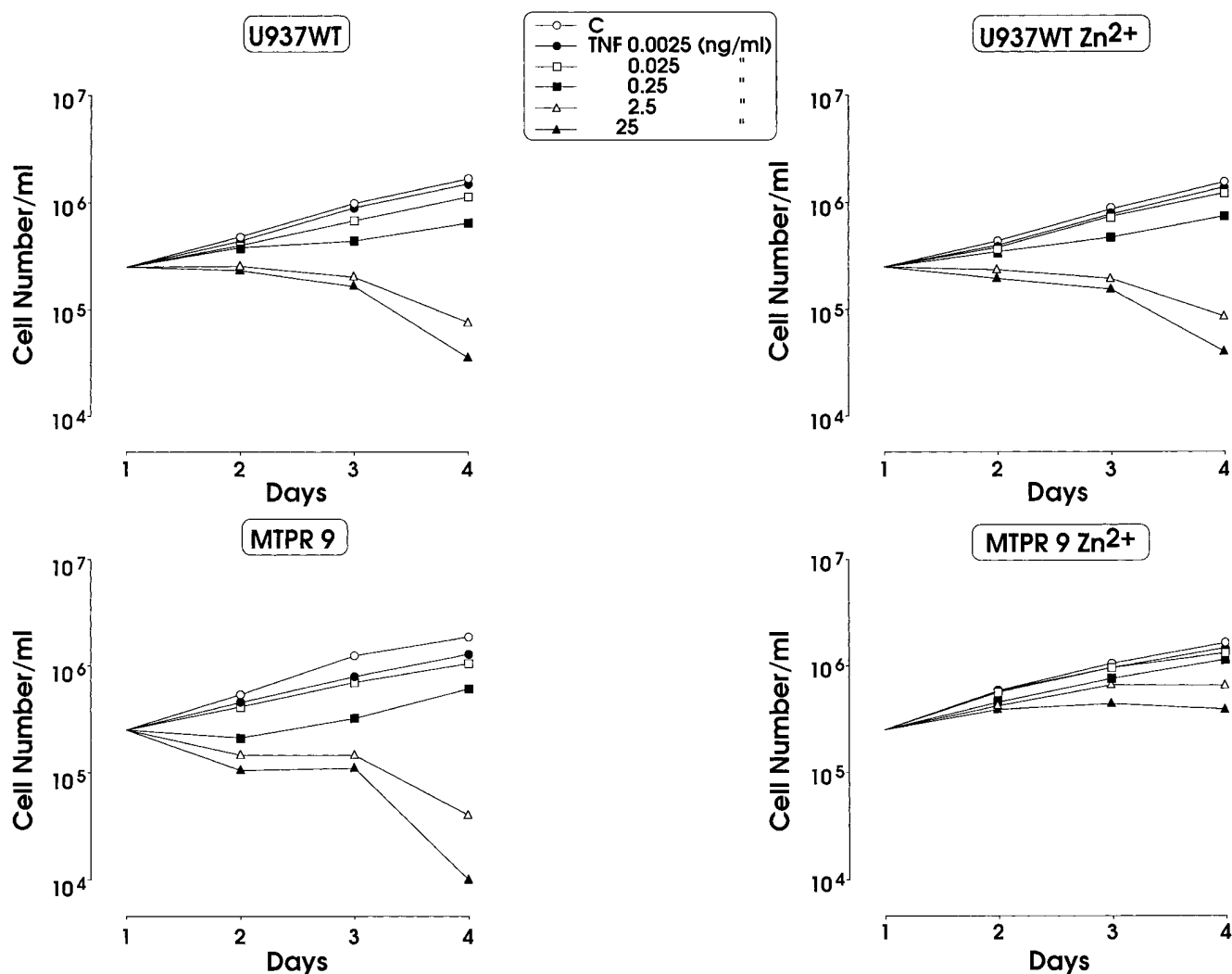


Figure 1. Growth curve of wt and MTPR9 U937 cells grown in absence or presence of Zn²⁺ with increasing doses of TNF- α . At each day of culture the number of living cells was determined by trypan blue staining. Representative results are shown.

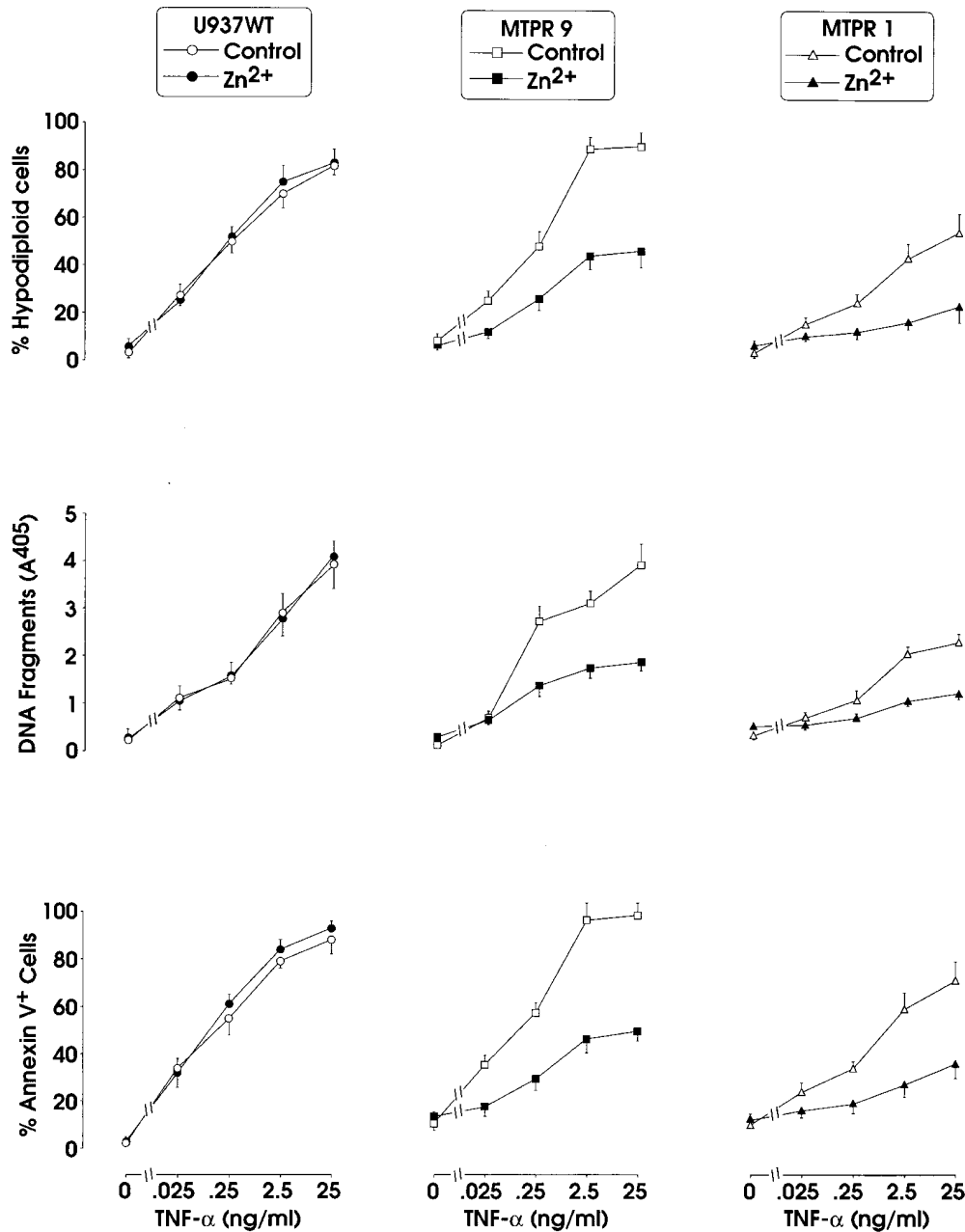


Figure 2. Effect of PML/RAR α expression in U937 cells on TNF- α -mediated cytotoxicity. Wt, MTPR9, and MTPR1 U937 cells were treated with serial dilutions of TNF- α and evaluated after a 40-h incubation for apoptosis according to three different assays: (i) flow cytometric analysis of DNA degradation in PI-stained nuclei (top); (b) ELISA detection of histones associated with DNA fragments (middle); (c) annexin V-binding assay (bottom). Mean \pm SEM values from three separate experiments are presented.

To trigger apoptotic damage, cells were incubated for 24–48 h in the presence of either recombinant human TNF- α (specific activity 2×10^7 U/mg, R & D Systems) or agonistic rabbit anti-human TNF- α RII serum M80 (40) or the TNF- α RI-specific mAb H398, which has been shown to possess agonistic activity in some (41, 42) but not all cellular systems (27).

In some experiments, the cells were first incubated for 60 min with antagonistic anti-TNF- α RI mAb directed against soluble TNF- α R (R & D Systems) or anti-TNF- α RII mAb directed against the extracellular domain of the receptor (Genzyme, Boston, MA) before TNF- α addition.

Receptor-binding assay and TNF- α R characterization. Receptor-binding assay was carried out using biotin-labeled rh-TNF- α (R & D Systems).

For analysis of TNF- α RI and TNF- α RII expression, the cells were first incubated with 5 μ g/ml of either TNF- α RI-specific mAb H398 (27) or TNF- α RII-specific mAb 80M2 (40), washed with cold PBS, and

then incubated with an appropriate dilution of phycoerythrin-labeled goat anti-mouse Igs (DAKOPATTS, Copenhagen, Denmark).

Fas antigen expression and Fas agonist-mediated apoptosis. Fas antigen (CD95) expression was evaluated by flow cytometry using a specific mAb (DX2) directly conjugated with phycoerythrin (PharMingen, San Diego, CA).

For evaluation of Fas-mediated cytotoxicity MTPR9 cells grown either in absence or presence of Zn²⁺ were incubated in 24-well cell culture plates (Costar, Cambridge, MA) at 2×10^5 cells/ml in complete medium in the presence of a cytotoxic anti-Fas mAb (clone CH-11, Upstate Biotechnology Inc., Lake Placid, NY) added at different concentrations (from 30 to 500 ng/ml). After 20 h of incubation, cells were recovered, washed in PBS, and processed for apoptotic cell detection (see above).

TNFR Western blotting. U937 cells grown in the absence or in the presence of Zn²⁺ were washed, and then lysed in lysis buffer (137 mmol/liter NaCl, 10 mmol/liter Tris-HCl, pH = 7.40, 10% glycerol,

1% Triton X-100, and protease inhibitors [10 $\mu\text{g/ml}$ leupeptin, 2 mmol/liter phenylmethylsulphonyl fluoride, 10 $\mu\text{g/ml}$ aprotinin]) for 30 min at 4°C followed by centrifugation of the lysate for 15 min at 4°C. Samples were boiled in running buffer for 2 min in the absence of 2-mercaptoethanol before separation of proteins by SDS-PAGE on 10% polyacrylamide gels.

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electroblotting and immunoreactive proteins were detected by chemiluminescence using the enhanced chemiluminescence kit (Amersham, Little Chalfont, UK) following the manufacturer's instructions.

Northern blot analysis. Total RNA was isolated by the guanidium-cesium chloride method (43). RNA samples (10 μg), after electrophoresis through denaturing agarose gels (1.2%) containing formaldehyde, were transferred onto a Hybond-*N* nylon membrane (Amersham, Buckinghamshire, UK) and hybridized with random-primed ^{32}P -labeled human TNF- α RI and TNF- α RII cDNA probes.

Results

PML/RAR α expression protects U937 cells from TNF- α , but not from anti-Fas. In a first set of experiments, the effect of TNF- α was evaluated on the growth of both wild-type and PML/RAR α -expressing U937 cells. Low concentrations (0.0025–0.25 ng/ml) of TNF- α progressively reduced the growth rate; high concentrations of TNF- α induced a marked inhibition of cell growth, associated with massive cell death (Fig. 1). MTPR9 cells grown in the absence of Zn^{2+} behave like wt U937 cells. However, in MTPR9 induced to express PML/RAR α by Zn^{2+} addition, low TNF- α concentrations (0.0025–0.25 ng/ml) only slightly reduced the rate of cell growth, whereas high TNF- α concentrations induced inhibition of cell growth to an extent markedly lower than that observed in wt U937 cells (Fig. 1).

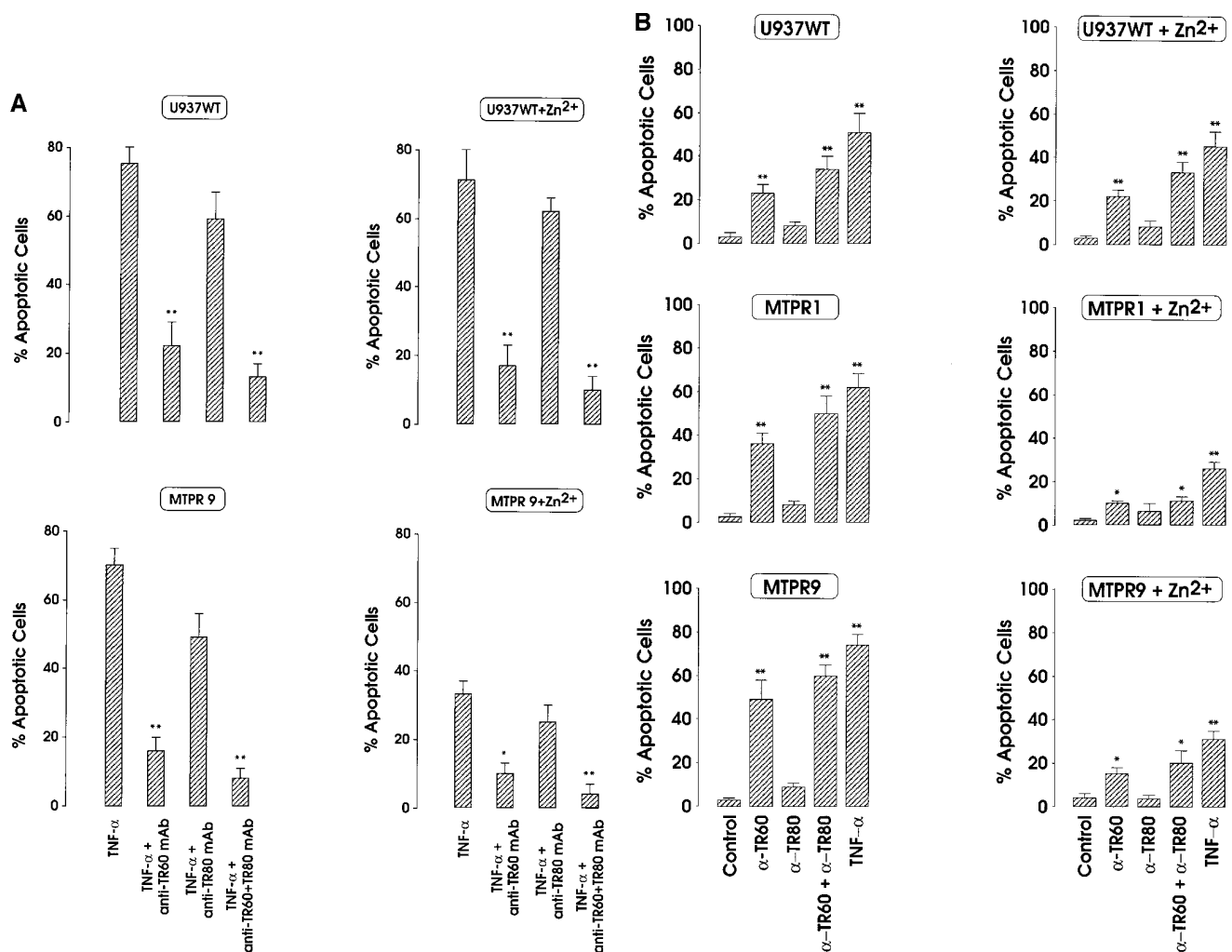


Figure 3. (A) Effect of antagonistic anti-TR60 and -TR80 mAbs on TNF- α -mediated cytotoxicity in wt and MTPR9 U937 cells. U937 and MTPR9 cells were grown for 24 h either in absence or presence of Zn^{2+} , incubated for 1 h with no additives or with antagonistic anti-TR60 or -TR80 mAb or both mAbs and then for an additional 24 h with TNF- α (25 ng/ml). The percentage of apoptotic cells was determined using the TUNEL reaction. Mean \pm SEM values from three separate experiments. ** $P < 0.01$, * $P < 0.05$, when compared with TNF- α -treated group. (B) Role of TR60 and TR80 in induction of wt U937 and MTPR9 clone cytotoxicity. U937 and MTPR9 cells were grown for 24 h in absence or presence of Zn^{2+} and then for an additional 24 h in presence of medium alone (Control), or TNF- α (25 ng/ml), or H398 agonistic anti-TR60 mAb (10 $\mu\text{g/ml}$) + 10 $\mu\text{g/ml}$ mouse Ig-specific cross-linking antibody, or anti-TR80 rabbit serum Ab or both anti-TR60 and -TR80 Abs. Mean \pm SEM values from three separate experiments are presented. ** $P < 0.01$, * $P < 0.05$, when compared with TNF- α -treated group.

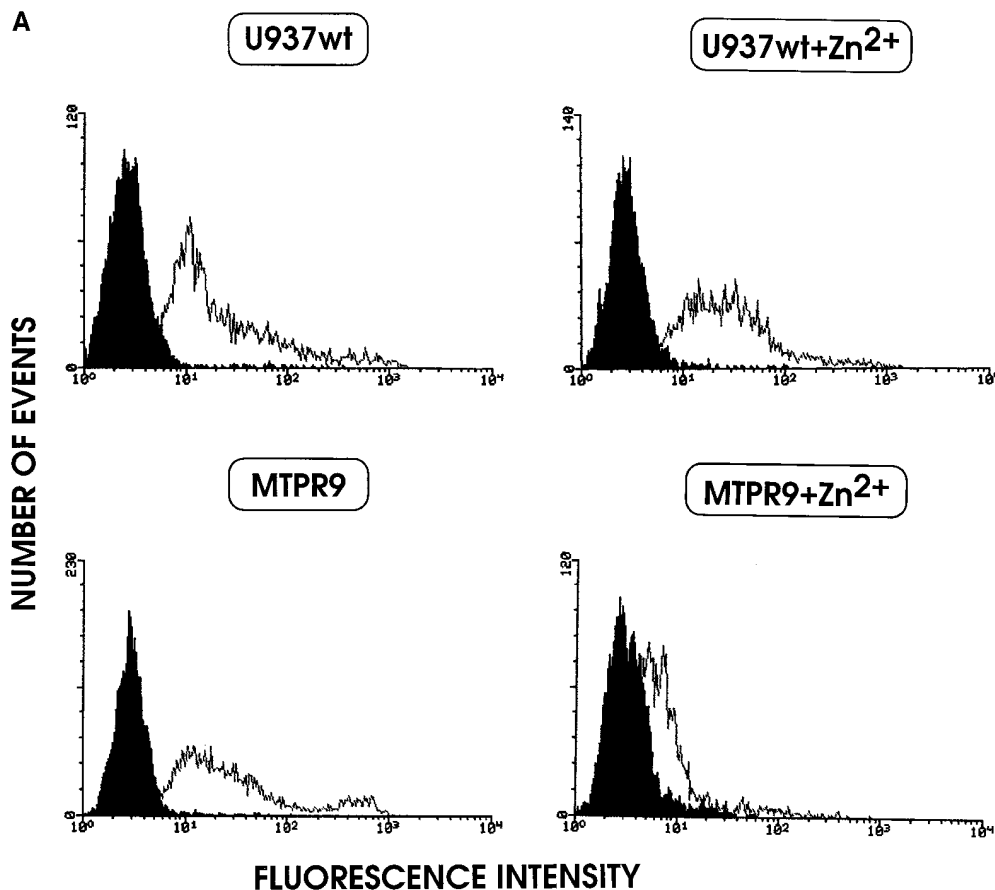


Figure 4. (A) Expression of TNF- α R in wt U937 and MTPR9 cells grown in absence or presence of Zn²⁺. Cells were incubated with biotinylated TNF- α and then stained with fluorescein-conjugated avidin. Fluorescence analysis was performed using Lysis II software with FACScan[®]. (B) Expression of TNF- α RI and TNF- α RII in MTPR1 and MTPR9 cells grown in absence or presence of Zn²⁺. Cells were incubated with specific anti-human TNF- α RI and TNF- α RII mAbs and stained with phycoerythrin-conjugated anti-mouse IgG second antibody. Fluorescence analysis was carried out as above. (C) Expression of Fas antigen (CD95) in wt U937, MTPR1, and MTPR9 cells grown in the absence or presence of Zn²⁺.

In a second set of experiments, we determined the sensitivity of wt U937 cells and the MTPR9 and MTPR1 clones expressing PML/RAR α to the apoptotic effects mediated by TNF- α . Different apoptosis assays provided evidence that PML/RAR α expression in U937 cells elicited a significant level of protection against TNF- α -induced apoptosis. (Fig. 2 and data not shown). This effect is specifically related to the induction of PML/RAR α expression. This protective effect requires an incubation with Zn²⁺ for at least 6 h before TNF- α triggering, thus suggesting that it requires optimal PML/RAR α expression and gene regulation. Growth of wt U937 cells in the presence of Zn²⁺ for 24 h before TNF- α addition did not modify the sensitivity to TNF- α -induced apoptosis (Fig. 2).

In parallel, the effect of PML/RAR α expression on the

Table I. Sensitivity of U937 wt and MTPR9 to Fas Agonist-mediated Cytotoxicity

Cell type	Treatment	Apoptotic cells
		%
U937 wt	None	5
U937 wt + Zn ²⁺	None	7
U937 wt	Fas agonist mAb (25 ng/ml)	75
U937 wt + Zn ²⁺	Fas agonist mAb (25 ng/ml)	77
MTPR9	None	4
MTPR9 + Zn ²⁺	None	7
MTPR9	Fas agonist mAb (25 ng/ml)	77
MTPR9 + Zn ²⁺	Fas agonist mAb (25 ng/ml)	86

sensitivity of U937 cells to anti-Fas-mediated cytotoxicity was also evaluated (Table I). Anti-Fas mAb (CH-11) at 250 ng/ml induced DNA fragmentation after 20 h. The extent of apoptosis induced by anti-Fas triggering was similar both in control U937 cells and in MTPR9 cells induced to express PML/RAR α by Zn²⁺ addition (Table I), indicating that PML/RAR α specifically acts on the TNF- α pathway.

Role of TNF- α RI and TNF- α RII in mediating apoptosis of PML/RAR⁺ U937 cells. Antagonistic antibodies inhibited

Table II. Sensitivity of Normal Myeloblasts/Promyelocytes to TNF- α -mediated Cytotoxicity (Mean \pm SEM Values from Three Separate Experiments)

Cell type	Treatment	Apoptotic cells
		%
Day 7 granulopoietic cells (+HGFs)	None	4 \pm 2.1
	(-HGFs)	None
	(+HGFs) TNF- α (25 ng/ml)	12 \pm 2.9*
	(-HGFs) TNF- α (25 ng/ml)	17 \pm 2.5*

Normal myeloblasts/promyelocytes (85–90% pure, see Methods) were derived from unilineage granulopoietic culture at day 7. One aliquot of cells was maintained in the original culture medium (+HGFs), while another aliquot was washed twice and incubated in culture medium without (-HGFs) or with HGFs (+HGFs). The cells were then grown for 24 h in either absence or presence of TNF- α ; thereafter, the percentage of apoptotic cells was determined using the annexin V-binding assay. * $P \leq 0.02$ when compared to control group.

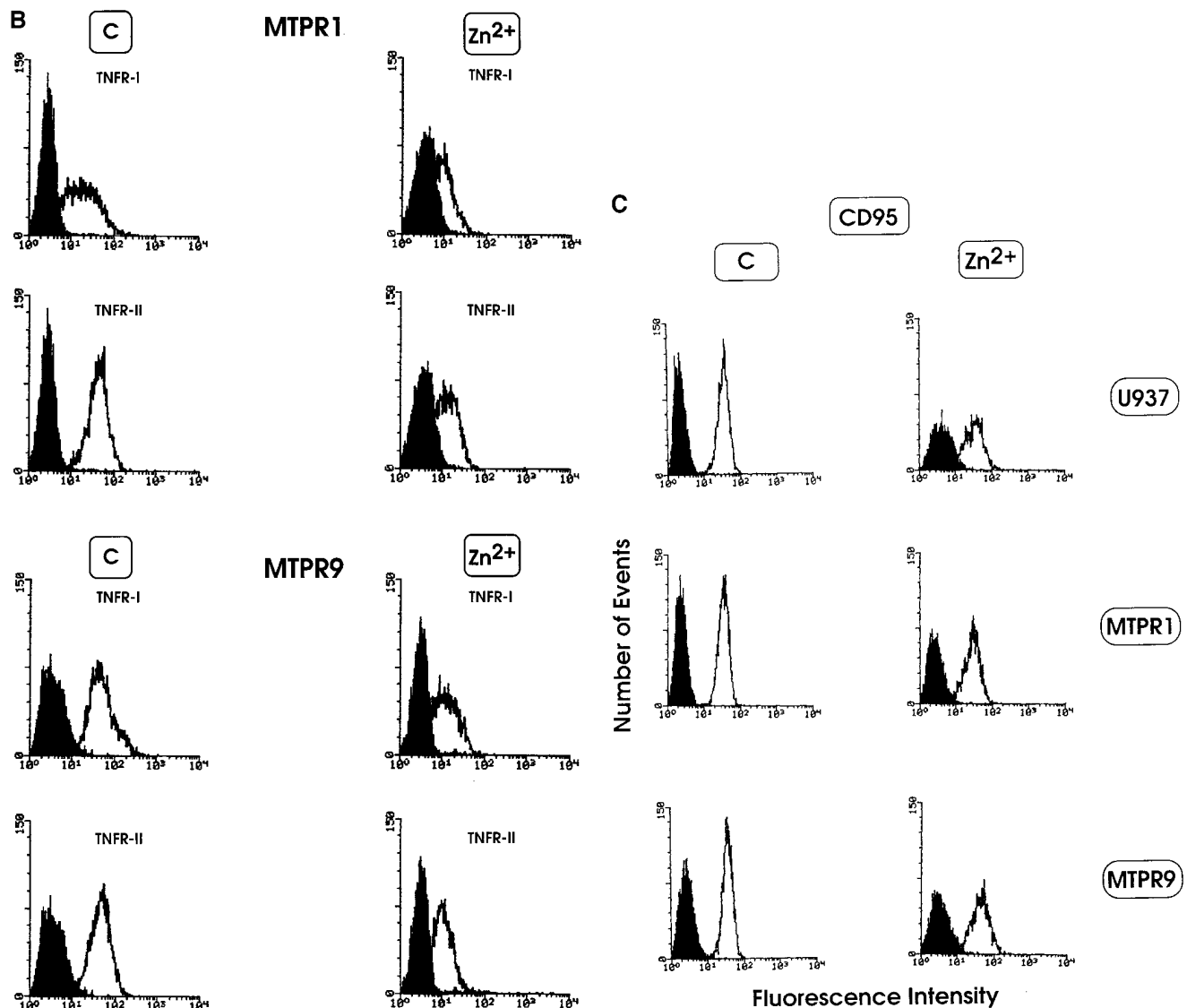


Figure 4 (Continued)

TNF- α binding to p60 and p80TNF- α R, respectively. In the presence of antagonistic anti-p80TNF- α R mAb, TNF- α still induced extensive apoptosis, but to a lesser extent in U937 clones expressing PML/RAR⁺ as compared with cells expressing little or no PML/RAR α (Fig. 3 A); in contrast, addition of antagonistic anti-p60TNF- α R mAb significantly decreased TNF- α -induced apoptosis, both in U937 cells expressing or not expressing PML/RAR α (Fig. 3 A). The addition of both antagonistic mAbs together produced an inhibition of TNF- α -mediated cytotoxicity greater than that elicited by anti-TNF- α R mAb (Fig. 3 A).

We further examined the effect PML/RAR had on cytotoxicity induced by agonistic anti-p60TNF- α R and anti-p80TNF- α R Abs. Agonistic M80 rabbit anti-TNF- α RII Ab did not induce significant cytotoxicity in wt or PML/RAR⁺ U937 clone (Fig. 3 B). The anti-TNF- α RI mAb H398 induced a significant level of toxicity, whose extent was distinctly lower in PML/RAR⁺ U937 cells induced to synthesize PML/RAR α protein, as compared with levels observed in cells where PML/RAR α expression was absent or low (Fig. 3 B). The addition of both

agonistic Abs elicited a small, but not significant, enhancement of cytotoxicity over the values observed with anti-TNF- α RI mAb alone (Fig. 3 B); also in this condition U937 cells expressing PML/RAR α showed a level of Ab-triggered apoptosis markedly lower than that observed in cells where PML/RAR α was absent or low.

Expression of apoptosis-related molecules in U937 PML/RAR α clones. Because expression of PML/RAR in U937 induced partial resistance to apoptosis triggered by TNF- α and anti-p60TNF- α R Ab, we compared the expression level of TNF- α R in U937 wt and MTPR1-MTPR9 clones expressing PML/RAR α . Flow cytometric analysis carried out using biotinylated TNF- α revealed that the induction of PML/RAR α expression in MTPR9 cells elicited a marked downmodulation of TNF- α R (Fig. 4 A), whereas Zn²⁺ addition to wt U937 cells did not modify TNF- α R expression.

Flow cytometric experiments carried out using specific Ab against p60 and p80TNF- α R showed that both receptors were downmodulated in U937 cells induced to express PML/RAR α protein (Fig. 4 B).

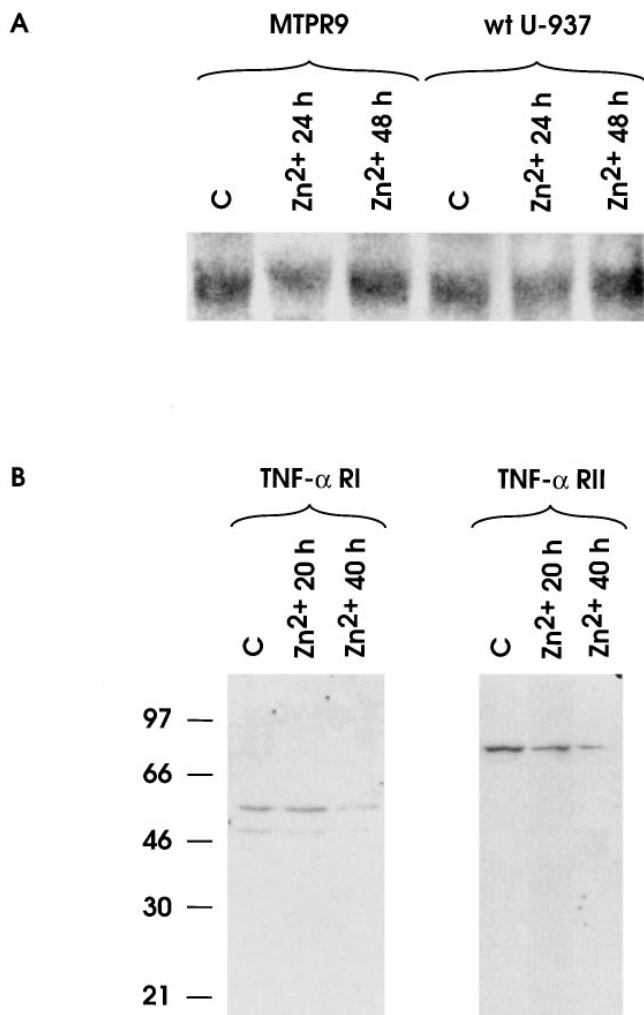


Figure 5. Northern blot analysis of TNF- α RI mRNA (A) and Western blot analysis of TNF- α RI and TNF- α RII (B) in wt U937 and MTPR9 cells grown in the absence or in the presence of Zn²⁺.

In contrast, other membrane molecules involved in different apoptotic pathways such as CD95 (Fas antigen) are not modulated by induction of PML/RAR α expression (Fig. 4 C).

Northern blot analysis showed that induction of PML/RAR α expression in MTPR9 cells did not significantly modify the level of TNF- α RI (Fig. 5) or TNF- α RII (data not shown) mRNAs as compared to the level observed in controls. Western blot analysis showed that in MTPR9 clone the induction of PML/RAR α expression was associated with a decline of protein level of both p60 and p80TNF- α Rs (Fig. 5).

Fresh APL blasts are resistant to TNF- α -mediated apoptosis and do not bind TNF- α . Five cases of newly diagnosed PML/RAR α ⁺ APL were analyzed for sensitivity to TNF- α , capacity to bind TNF- α , and expression of CD95. In all cases, the blasts were resistant to TNF- α -mediated cytotoxicity and barely bound TNF- α : this indicates that they possess a very low number of TNF- α Rs (Fig. 6 A and data not shown) (Fig. 4 A). Conversely, a relatively elevated expression of CD95 was observed in all APL cases (Fig. 6 A).

Furthermore, clearly detectable TNF- α binding and CD95 expression were observed in other AML patients, including

one M1, two M2, and three M4 cases (representative results in Fig. 6 B).

Normal myeloblasts/promyelocytes displayed detectable levels of TNF- α R (Fig. 6 C) CD95 expression comparable to those observed in U937 cells and moderate sensitivity to TNF- α -mediated cytotoxicity (Table II).

Expression of TNF- α R and sensitivity of APL cell line NB4 to TNF- α -mediated cytotoxicity depends on the synthesis of PML/RAR α protein. NB4 displayed low TNF- α -binding capacity, whereas NB4.306 exhibited a clearly detectable level of TNF- α binding (Fig. 7 A). Furthermore, NB4 cells displayed a slight sensitivity to TNF- α -mediated cytotoxicity, whereas NB4.306 cells were highly sensitive to TNF- α -induced apoptosis (Fig. 7 B).

Discussion

The proliferative rate of APL blasts is lower than that of other AMLs, thus suggesting that prolonged survival may contribute to the accumulation of the leukemic blasts (3, 4). As previously mentioned, expression of PML/RAR α protein in U937 inhibits cell differentiation, while increasing cell proliferation via diminished apoptotic cell death under low serum conditions (15). Furthermore, in the GM-CSF-dependent erythroleukemic TF-1 cell line, PML/RAR α expression improves cell survival upon growth factor starvation (44). However, the cellular and molecular mechanisms underlying these phenomena are poorly understood.

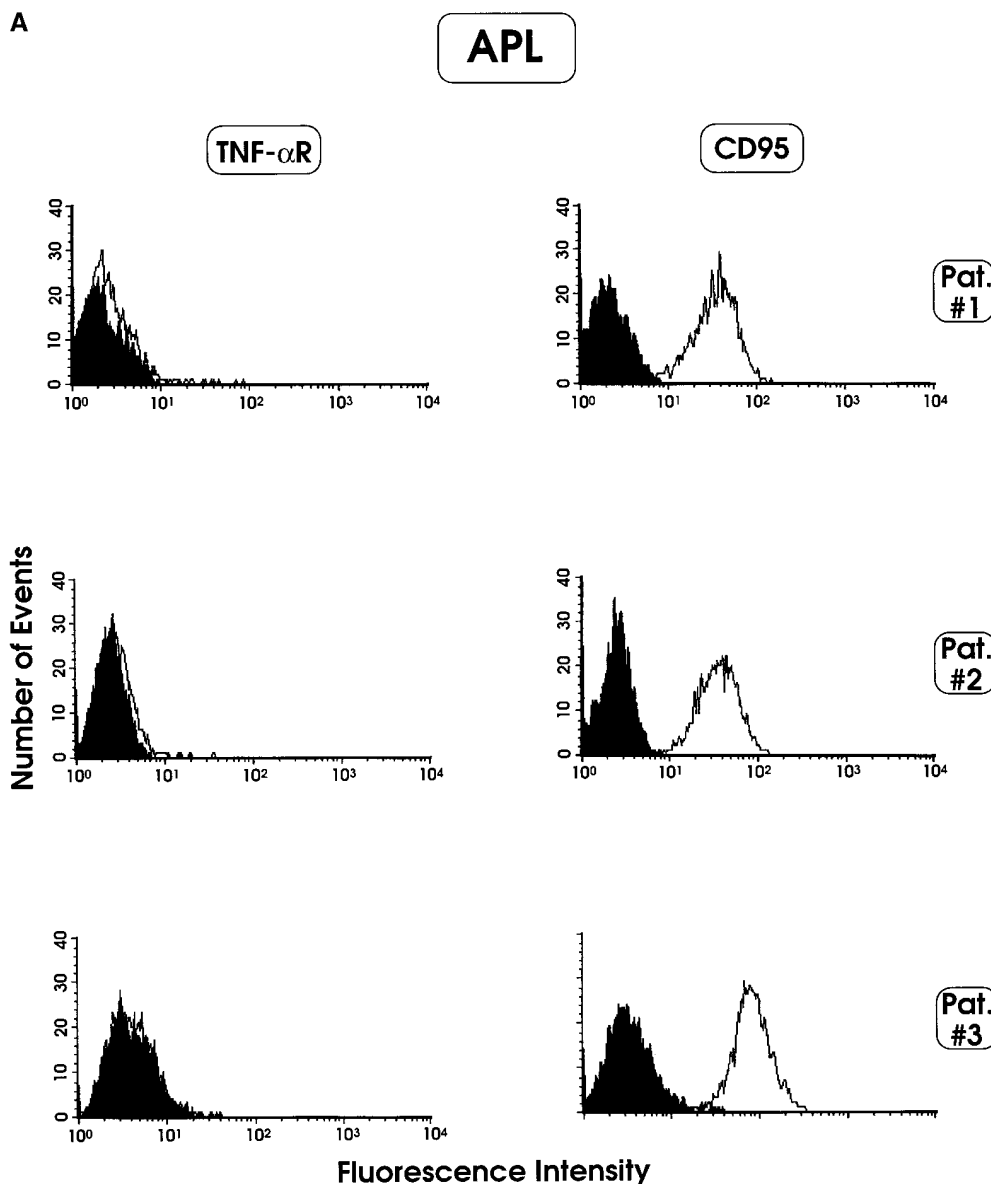
The U937 cell line expressing a high number of both TNF- α Rs (TNF- α RI and TNF- α RII) is TNF- α -sensitive (27, 28). Here we investigated the effect of PML/RAR α expression on TNF- α -mediated cytotoxicity in these cells.

The MTPR1 and MTPR9 U937 clones display a high sensitivity to TNF- α -mediated cytotoxicity; however, when grown in the presence of Zn²⁺ these clones are only scarcely sensitive to TNF- α -triggered cytotoxicity. This phenomenon is specifically related to the induction of PML/RAR α protein expression, in that: (a) the clones grown in the absence of Zn²⁺ displayed a sensitivity to TNF- α cytotoxicity comparable to that of wt U937 cells; (b) wt cells grown in absence or presence of Zn²⁺ showed comparable levels of sensitivity to TNF- α -triggered cytotoxicity.

A specific role for PML/RAR α protein in the protection against apoptosis is further supported by the scarce sensitivity to TNF- α of the APL cell line NB4 and by the marked sensitivity of the NB4-derived NB4.306 cell line that had lost the expression of PML/RAR α .

More importantly, fresh APL blasts exhibit a virtually absent TNF- α R expression and no sensitivity to TNF- α -mediated cytotoxicity, whereas corresponding normal cells (myeloblasts/promyelocytes) show clearly detectable TNF- α -binding capacity and moderate but significant sensitivity to TNF- α -mediated apoptosis. Particularly, the binding level of APL blasts was even lower than that observed in PML/RAR α ⁺ U937 cells induced by Zn²⁺. Furthermore, preliminary observations in other AML FAB types (including M1, M2, and M4) indicate that the blasts exhibit detectable TNF- α -binding capacity. Altogether, it is apparent that TNF- α R downmodulation is specifically restricted to APL blasts.

APL cells are scarcely sensitive to the apoptotic mechanism in both their undifferentiated and differentiated states (45, 46). Thus, although all-*trans* retinoic acid induces apopto-



*Figure 6. (A) Expression of TNF- α R and CD95 in leukemic blasts derived from three representative APL patients. TNF- α R expression was measured by analysis of TNF- α biotinylated binding. (B) Expression of TNF- α R and CD95 in leukemic blasts derived from three representative non-APL AML patients (*M1*, *M2*, and *M4* FAB types). (C) Expression of TNF- α R and CD95 in normal myeloblasts/promyelocytes grown in unilineage granulopoietic cultures from purified HPCs.*

sis in a significant proportion of HL-60 cells (47), it fails to induce apoptosis in fresh APL cells *in vitro* (48).

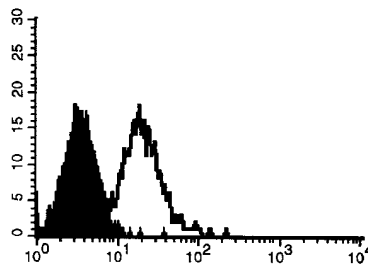
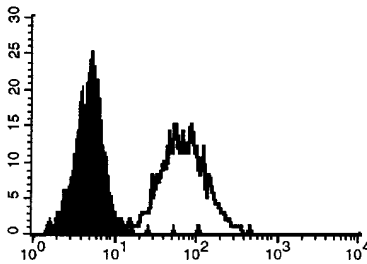
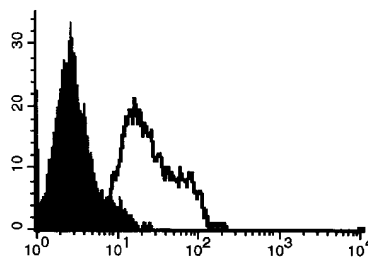
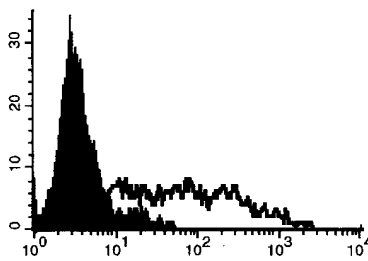
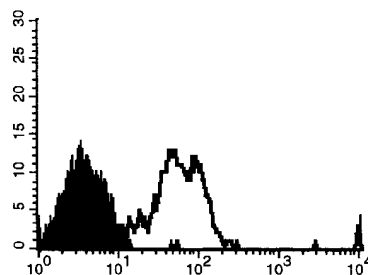
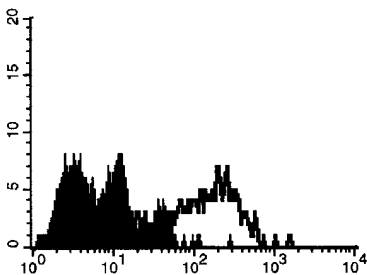
The specificity of the effect of PML/RAR α on TNF- α R in APL is further supported by the observation that this fusion protein does not affect the expression of FAS antigen (another member of the TNF- α R superfamily [26]) or the sensitivity to Fas-mediated apoptosis, as compared to both normal myeloblasts/promyelocytes and other AML FAB types.

The downmodulation of TNF- α RI may explain the reduced sensitivity to TNF- α -mediated cytotoxicity, in that U937 cells are particularly sensitive to apoptotic mechanisms triggered through TNF- α RI (28, 32, 33 and the present study). Our results, as well as previous studies (28, 32), further indicate that TNF- α RII cooperates with TNF- α RI in inducing TNF- α -mediated cytotoxicity. It may be hence assumed that the reduced expression of TNF- α RII induced by PML/RAR α also contributes to the decreased sensitivity to the cytotoxic action of TNF- α . Altogether, we suggest that the reduced cyto-

toxic action of TNF- α on APL blasts is at least in part mediated via downmodulation of TNFR. We cannot exclude, however, an additional mechanism(s) at the level of the TNFR signal transduction pathway.

The downmodulation of TNF- α R induced by PML/RAR α expression is seemingly mediated through posttranscriptional mechanisms: indeed, PML/RAR α ⁺ cells show an unmodified TNF- α RI and TNF- α RII mRNA level, whereas the total amount of receptor protein is significantly decreased. In other cellular systems, a posttranscriptional mechanism for TNFR modulation was similarly described (49).

Previous studies have shown that the cell surface expression of TNF- α Rs is upregulated by interferons, dibutyryl cAMP, butyrate, TNF- α , thyroid-stimulating hormone, and lectins (50), whereas it is downregulated by IL-1, granulocyte-monocyte colony-stimulating factor, phorbol esters, glucocorticoids, lipopolysaccharides, and okadaic acid (51, 52). However, this is the first report showing that an oncoprotein, i.e.,

B**AML****TNF- α R****CD95****M1****Number of events****M2****M4****Fluorescence Intensity***Figure 6 (Continued)*

the fusion PML/RAR α protein in APL, may specifically modulate the number of TNF- α R.

Our observations on TNF- α sensitivity of APL blasts relate to the physiological function of TNF- α in normal hematopoiesis. In this regard, growing evidence indicates an inhibitory role for TNF- α in normal myelopoiesis via a specific cytotoxic effect. Thus, TNF- α is produced by normal myeloid cells (53) and exerts an inhibitory action on myelopoiesis at the level of the progenitors/early precursors (54) and stimulates apoptosis of granulocytes (55). More important, TNFR-I^{-/-} mice showed a marked increase in the number of hematopoietic stem/progenitor cells, as compared with TNFR-I^{+/+} animals (56), thus suggesting that TNF- α is a physiological regulator of the early

hematopoietic cell compartment. Finally, TNF- α is produced by both myelodysplastic (57) and AML cells (58), and inhibits the clonogenic capacity of leukemic blasts, more than that of normal CFU-GM (59). In view of the cytotoxic TNF- α effect on normal myelopoietic cells, our studies suggest that in APL patients the loss of TNF- α sensitivity of leukemic blasts may contribute to their particularly prolonged survival and expansion (see 3, 4).

Other fusion genes specifically involved in the genesis of other leukemias, such as the E2A-HLF chimeric gene, protect, like the PML/RAR gene, leukemic cells from apoptosis (60). These observations suggest that protection from apoptosis, rather than stimulation of proliferation, may represent a com-

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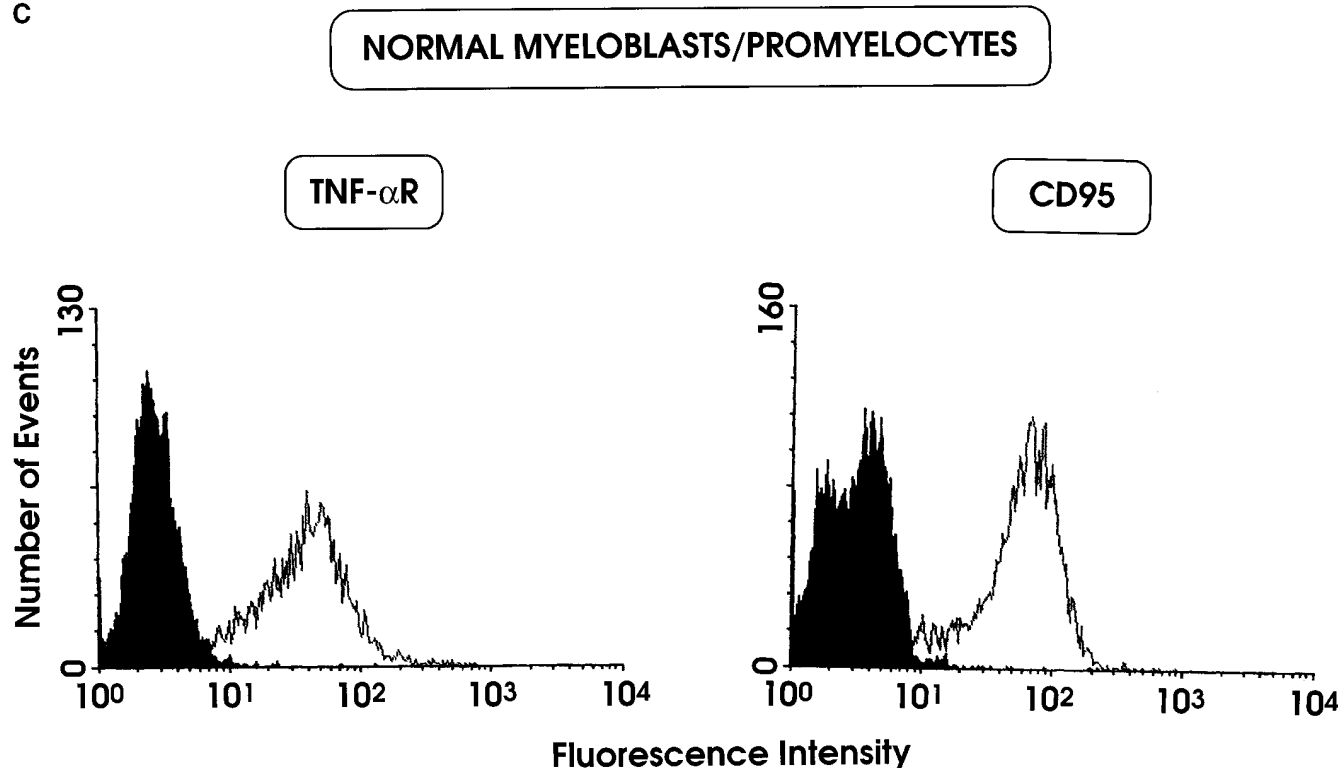


Figure 6 (Continued)

mon feature of the leukemogenic action of leukemia-specific chimeric genes.

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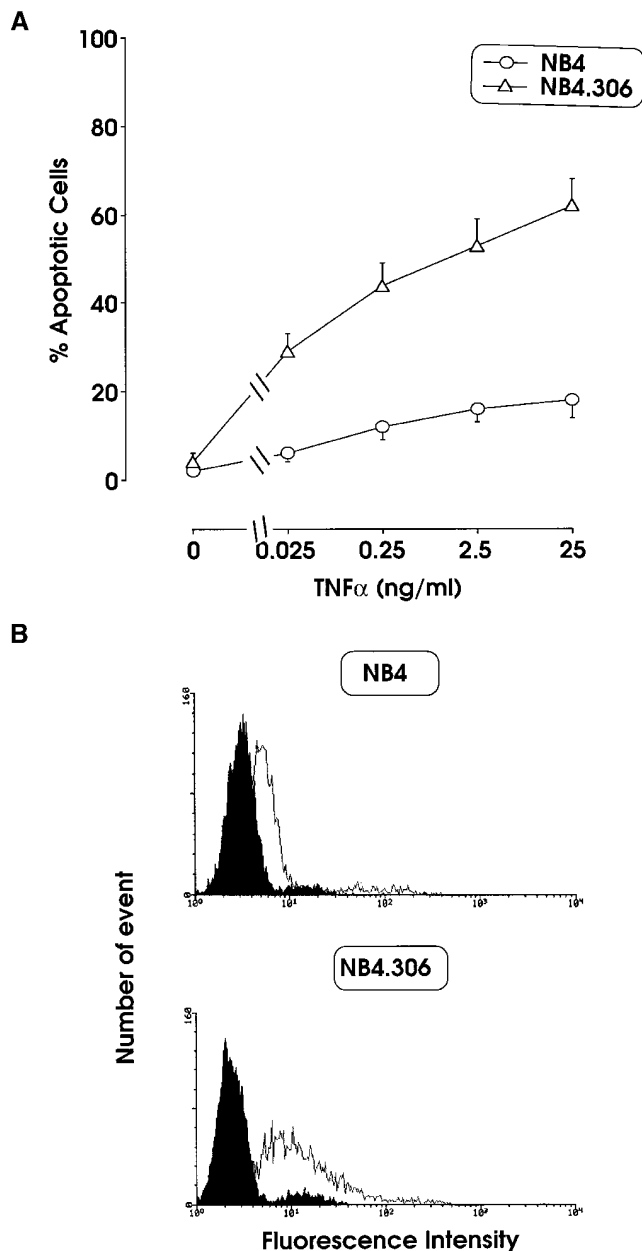


Figure 7. TNF- α -binding capacity and sensitivity of wt NB4 and NB4.306 cells to TNF- α -mediated cell apoptosis. (A) Cells were incubated with biotinylated TNF- α and then stained with FITC-Avidin. (B) NB4 and NB4.306 cells were grown for 24 h in absence or presence of increasing TNF- α concentrations and the percentage of apoptotic cells was then determined using the TUNEL reaction.

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