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

Ugo Testa,* Francesco Grignani,*‡ Paola Samoggia,* Cristiana Zanetti,* Roberta Riccioni,* Francesco Lo Coco,§ Daniela Diverio,§ Nadia Felli,* Carlo Gambacorti Passerini,‖ Matthias Grell,¶ Pier Giuseppe Pelicci, and Cesare Peschle*‡‡**

**Department of Hematology-Oncology, Istituto Superiore di Sanità, 00161 Rome, Italy; ‡Istituto di Clinica Medica I, Policlinico Monteluce, Perugia University, 06100 Perugia, Italy; §Department of Cellular Biotechnology and Hematology, University La Sapienza, 00161 Rome, Italy; ‖Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milan, Italy; ¶Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany; **European Institute of Oncology, Department of Experimental Oncology, 20133 Milan, Italy; and ‡‡Thomas Jefferson University, Philadelphia, Pennsylvania 19107-5541*

Abstract

We investigated the effect of the acute promyelocytic leukemia (APL) specific PML/RARα fusion protein on the sensitivity to TNF-α–mediated apoptosis. The U937 leukemia cell line was transduced with PML/RARα cDNA. PML/RARα 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-αRI, II). PML/RARα induced a 10–20-fold decrease of the TNF-α–binding capacity via downmodulation of both TNF-αRI and TNF-αRII: 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-αR 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/RARα$^+$, shows low TNF-αR expression capacity and is resistant to TNF-α–triggered apoptosis; conversely a PML/RARα$^-$ NB4 subclone (NB4.306) exhibits detectable TNF-α–binding capacity and is sensitive to TNF-α–mediated cytotoxicity.

These studies indicate that the PML/RARα fusion protein protects against TNF-α–induced apoptosis, at least in part via downmodulation of TNF-αRI/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

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/RARα fusion gene, generated by the t(15;17) translocation (5–8). RARα 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/RARα 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/RARα in human leukemic cell lines blocks differentiation by physiological stimuli, i.e., vitamin D3, D3 + TGF-β1, 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/RARα show myeloid differentiative alterations similar to those of promyelocytic leukemia (19). In the chicken model, PML/RARα 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/RARα 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-αRI) and 75–80 kD (TNF-αRII) (reviewed in 26). The cytotoxic effects of TNF-α are triggered by signals activated through interaction with p60TNF-αR (27); in other cellular systems p80TNF-αR either cooperates with p60TNF-αR (28) or by itself mediates cytotoxic signals (29, 30). Accordingly, in the myeloid precursor leukemic cell line

Address correspondence to Cesare Peschle, Thomas Jefferson Cancer Institute, Thomas Jefferson University, BLSB, Room #528, 233 South 10th Street, Philadelphia, PA 19107-5541. Phone: 215-503-1763. FAX: 215-923-4153. E-mail: C_Peschle@lac.jci.tju.edu

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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-αR (27, 31), although an involvement of p80TNF-αR has also been proposed (28, 32, 33).

In these studies we have investigated the relationship between the antiapoptotic effect of PML/RARα and TNF-α cytotoxicity. Particularly, we have evaluated (*a*) the effect of PML/RARα 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/RARα$^+$ APL cell line (NB4) and a PML/RARα$^-$ 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/RARα$^+$ U937 cells has been reported previously (15).

The wt PML/RARα$^+$ 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/RARα 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/RARα$^+$.

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).

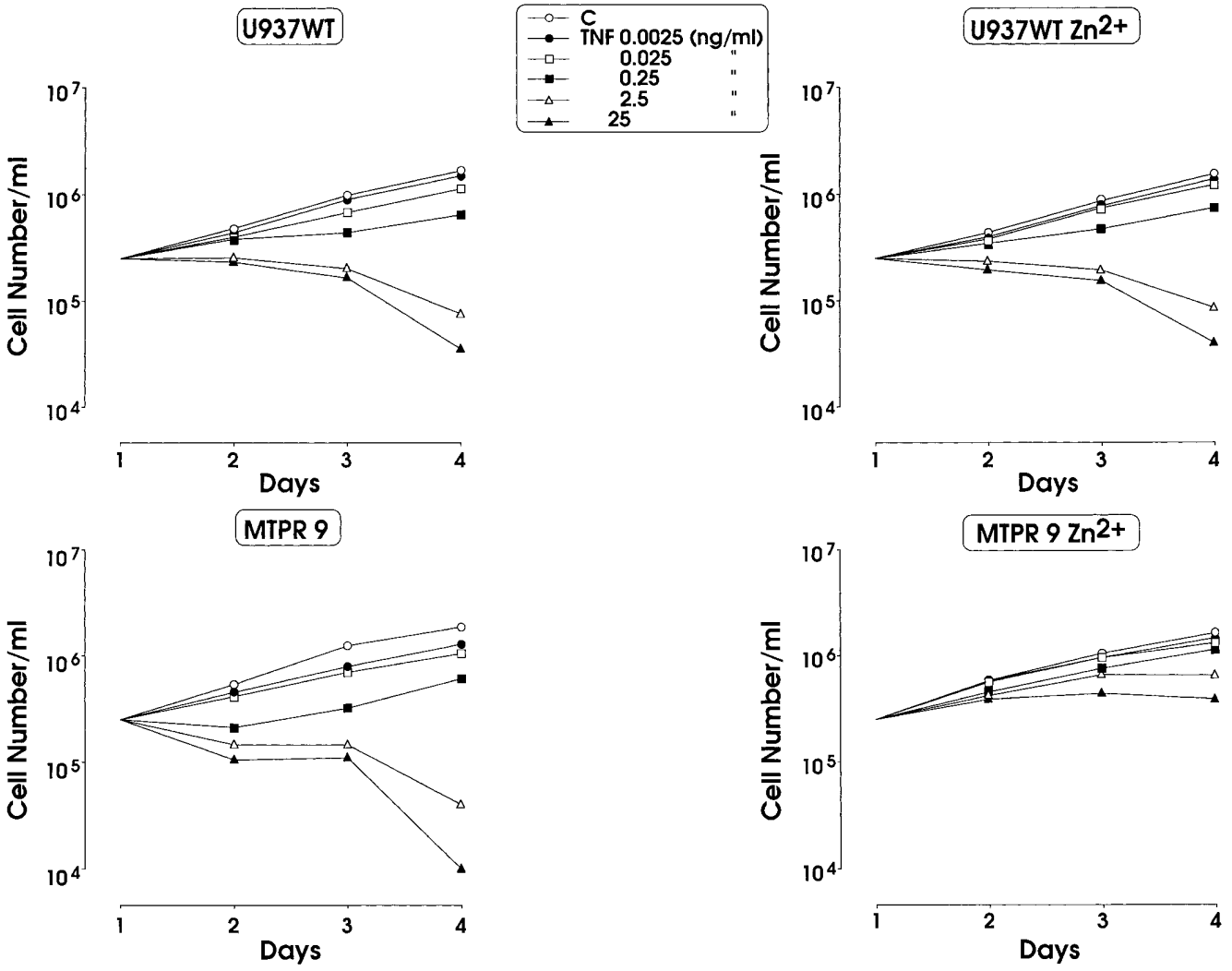

Figure 1. Growth curve of wt and MTPR9 U937 cells grown in absence or presence of Zn^{2+} with increasing doses of TNF-α. At each day of culture the number of living cells was determined by trypan blue staining. Representative results are shown.

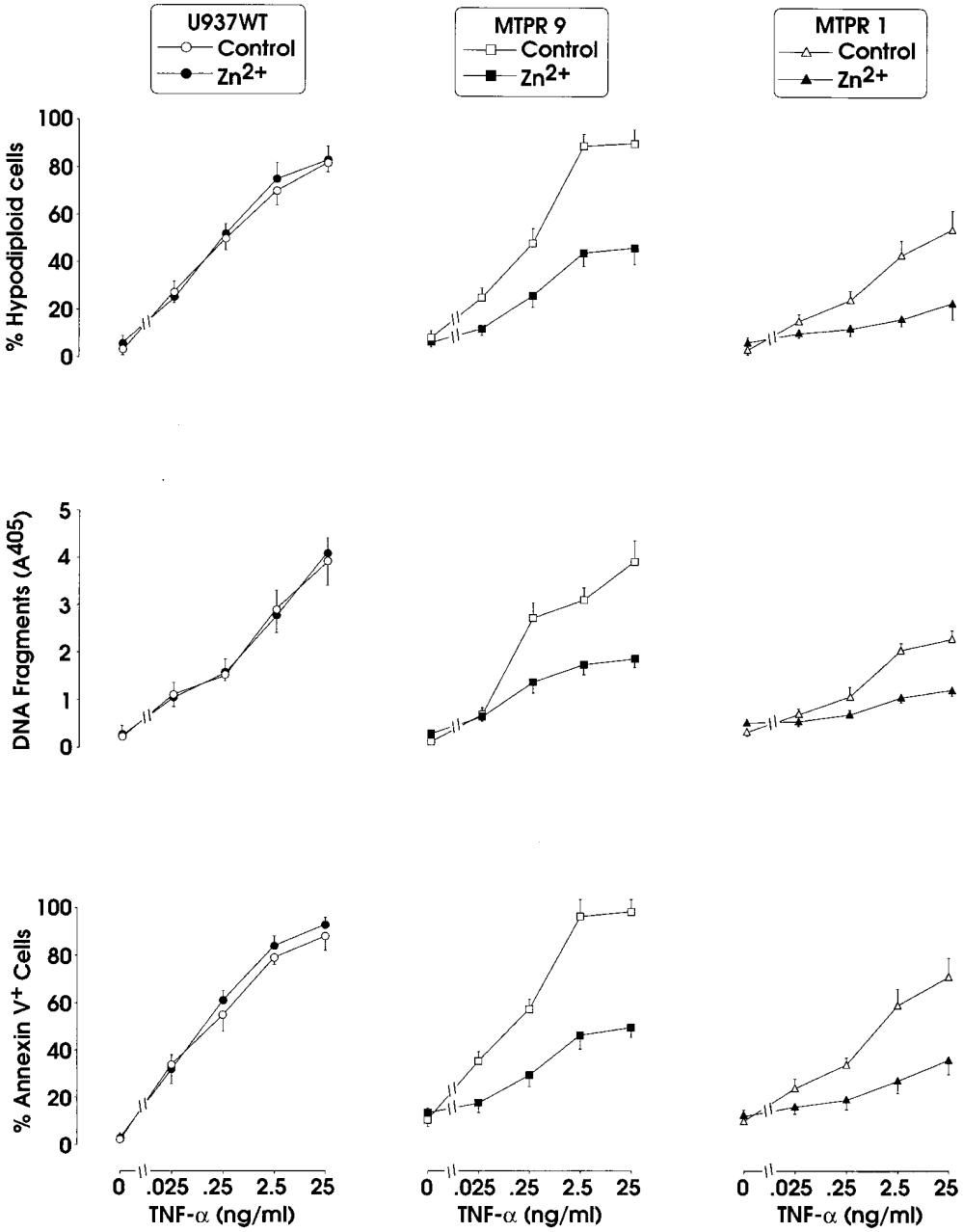

Figure 2. Effect of PML/RARα expression in U937 cells on TNF-α–mediated cytotoxicity. Wt, MTPR9, and MTPR1 U937 cells were treated with serial dilutions of TNF-α and evaluated after a 40-h incubation for apoptosis according to three different assays: (*i*) flow cytometric analysis of DNA degradation in PI-stained nuclei (*top*); (*b*) ELISA detection of histones associated with DNA fragments (*middle*); (*c*) annexin V–binding assay (*bottom*). Mean±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-αRII serum M80 (40) or the TNF-αRI–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-αRI mAb directed against soluble TNF-αR (R & D Systems) or anti–TNF-αRII mAb directed against the extracellular domain of the receptor (Genzyme, Boston, MA) before TNF-α αddition.

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

For analysis of TNF-αRI and TNF-αRII expression, the cells were first incubated with 5 μg/ml of either TNF-αRI–specific mAb H398 (27) or TNF-αRII–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 Zn^{2+} 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 Zn^{2+} 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 μg/ml leupeptin, 2 mmol/liter phenylmethysulphonyl fluoride, 10 μg/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-αRI and TNF-αRII cDNA probes.

Results

PML/RARα 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/RARα–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/RARα 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).

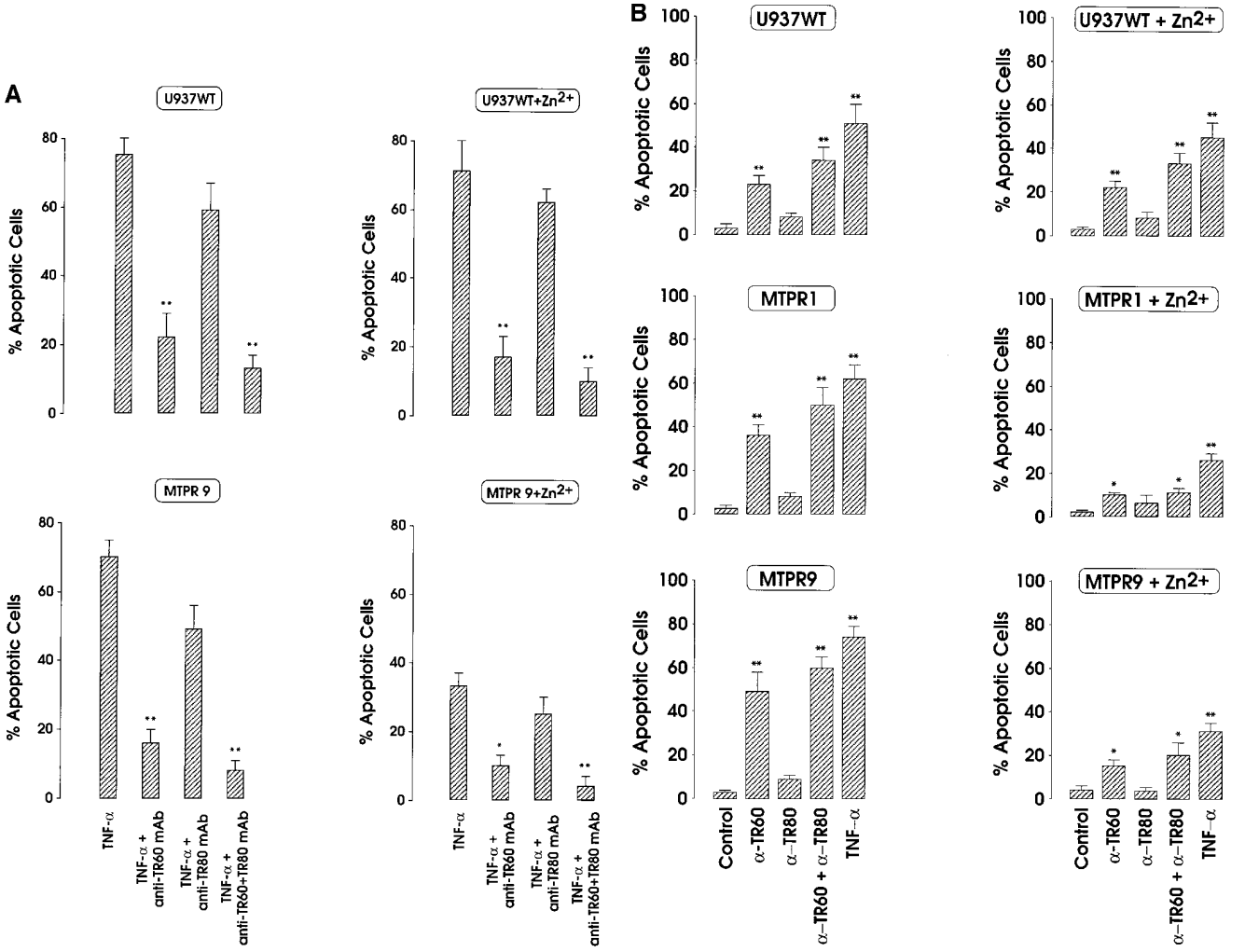

Figure 3. (*A*) Effect of antagonistic anti-TR60 and -TR80 mAbs on TNF-α–mediated cytotoxicity in wt and MTPR9 U937 cells. U937 and MTPR9 cells were grown for 24 h either in absence or presence of Zn^{2+}, incubated for 1 h with no additives or with antagonistic anti-TR60 or -TR80 mAb or both mAbs and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

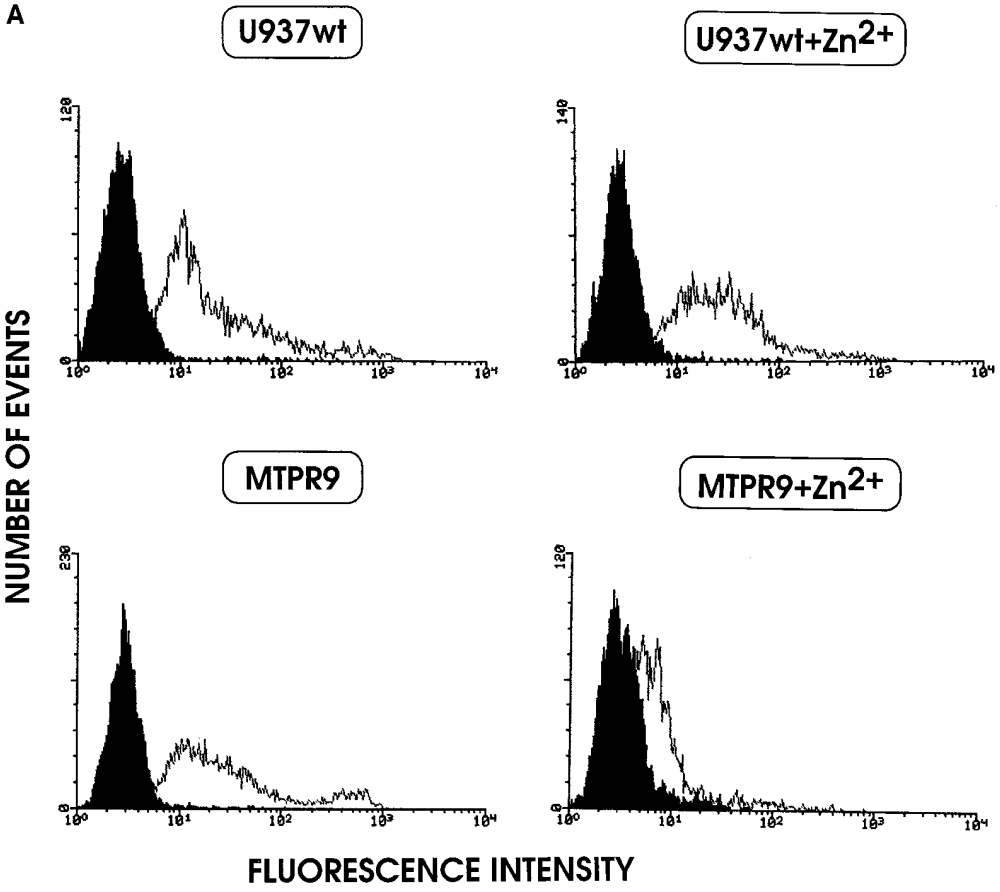

Figure 4. (*A*) Expression of TNF-αR in wt U937 and MTPR9 cells grown in absence or presence of Zn^{2+}. Cells were incubated with biotinylated TNF-α and then stained with fluorescein-conjugated avidin. Fluorescence analysis was performed using Lysis II software with FACScan®. (*B*) Expression of TNF-αRI and TNF-αRII in MTPR1 and MTPR9 cells grown in absence or presence of Zn^{2+}. Cells were incubated with specific anti–human TNF-αRI and TNF-αRII 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 Zn^{2+}.

In a second set of experiments, we determined the sensitivity of wt U937 cells and the MTPR9 and MTPR1 clones expressing PML/RARα to the apoptotic effects mediated by TNF-α. Different apoptosis assays provided evidence that PML/RARα 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/RARα expression. This protective effect requires an incubation with Zn^{2+} for at least 6 h before TNF-α triggering, thus suggesting that it requires optimal PML/RARα expression and gene regulation. Growth of wt U937 cells in the presence of Zn^{2+} 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/RARα expression on the 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/RARα by Zn^{2+} addition (Table I), indicating that PML/RARα specifically acts on the TNF-α pathway.

Role of TNF-αRI and TNF-αRII in mediating apoptosis of PML/RAR⁺ U937 cells. Antagonistic antibodies inhibited

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

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

Cell type	Treatment	Apoptotic cells
		%
Day 7 granulopoietic cells (+HGFs)	None	4±2.1
(−HGFs)	None	8±3.3
(+HGFs)	TNF-α(25 ng/ml)	12±2.9*
(−HGFs)	TNF-α(25 ng/ml)	17±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.

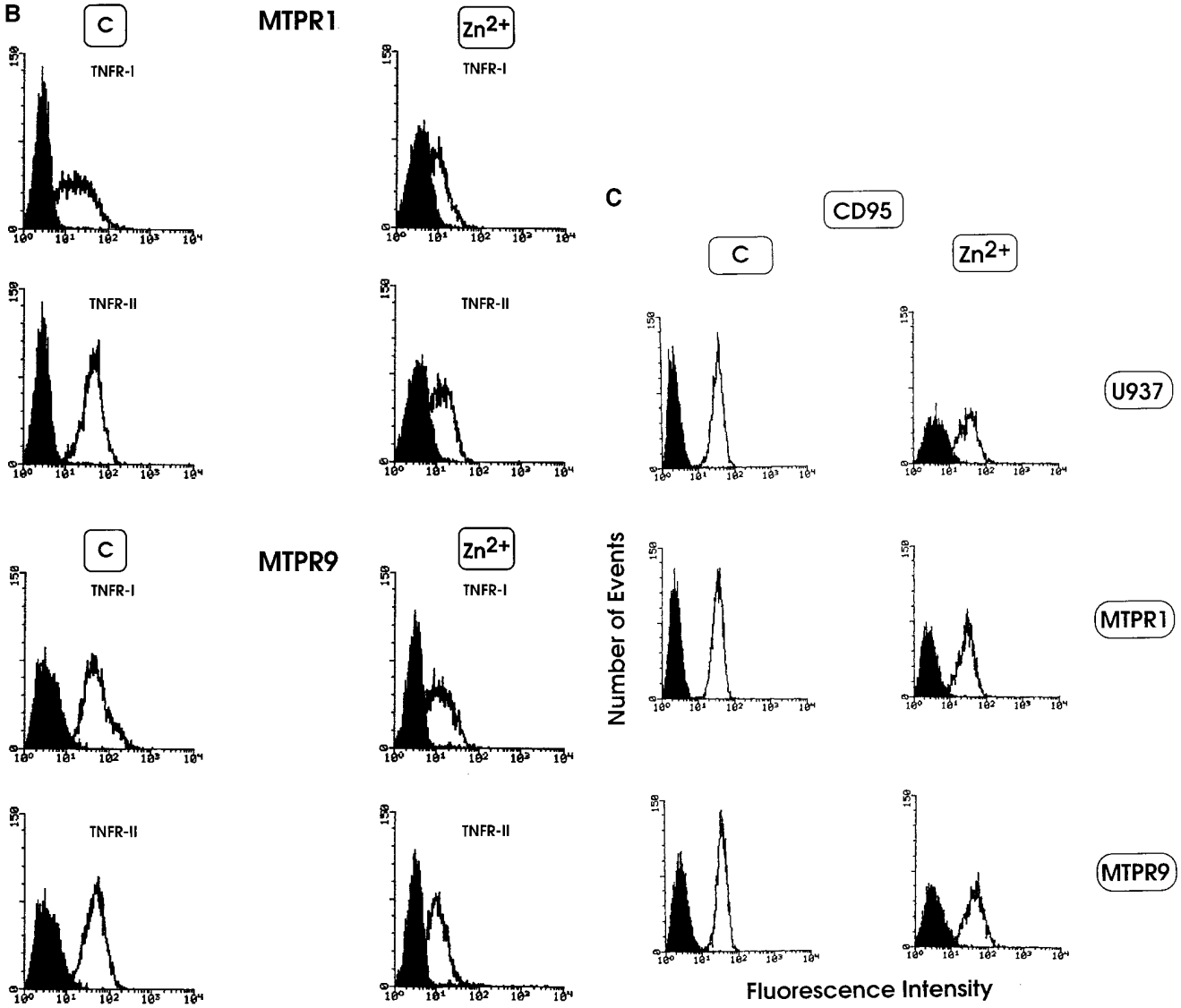

Figure 4 (Continued)

TNF-α binding to p60 and p80TNF-αR, respectively. In the presence of antagonistic anti–p80TNF-αR 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/RARα (Fig. 3 *A*); in contrast, addition of antagonistic anti–p60TNF-αR mAb significantly decreased TNF-α–induced apoptosis, both in U937 cells expressing or not expressing PML/RARα (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-αRI mAb (Fig. 3 *A*).

We further examined the effect PML/RAR had on cytotoxicity induced by agonistic anti–p60TNF-αR and anti–p80TNF-αR Abs. Agonistic M80 rabbit anti–TNF-αRII Ab did not induce significant cytotoxicity in wt or PML/RAR$^+$ U937 clone (Fig. 3 *B*). The anti–TNF-αRI mAb H398 induced a significant level of toxicity, whose extent was distinctly lower in PML/RAR$^+$ U937 cells induced to synthesize PML/RARα protein, as compared with levels observed in cells where PML/RARα 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-αRI mAb alone (Fig. 3 *B*): also in this condition U937 cells expressing PML/RARα showed a level of Ab-triggered apoptosis markedly lower than that observed in cells where PML/RARα was absent or low.

Expression of apoptosis-related molecules in U937 PML/RARα$^+$ clones. Because expression of PML/RAR in U937 induced partial resistance to apoptosis triggered by TNF-α and anti–p60TNF-αR Ab, we compared the expression level of TNF-αR in U937 wt and MTPRI-MTPR9 clones expressing PML/RARα. Flow cytometric analysis carried out using biotinylated TNF-α revealed that the induction of PML/RARα expression in MTPR9 cells elicited a marked downmodulation of TNF-αR (Fig. 4 *A*), whereas Zn^{2+} addition to wt U937 cells did not modify TNF-αR expression.

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

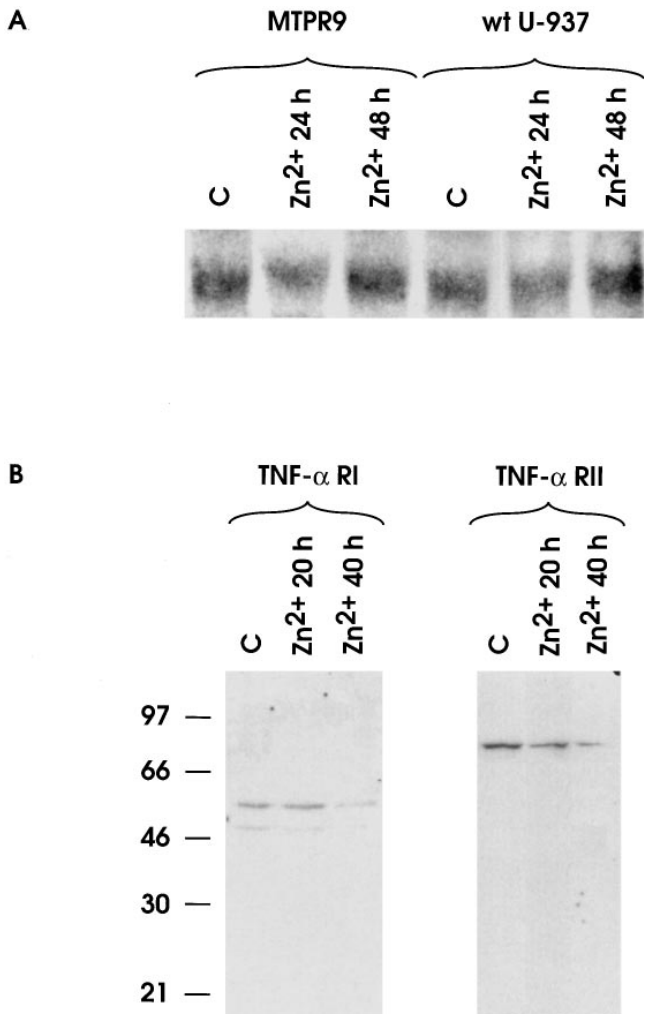

Figure 5. Northern blot analysis of TNF-αRI mRNA (*A*) and Western blot analysis of TNF-αRI and TNF-αRII (*B*) in wt U937 and MTPR9 cells grown in the absence or in the presence of Zn^{2+}.

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

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

Fresh APL blasts are resistant to TNF-α–mediated apoptosis and do not bind TNF-α. Five cases of newly diagnosed PML/RARα$^+$ 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-αRs (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-αR (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-αR and sensitivity of APL cell line NB4 to TNF-α–mediated cytotoxicity depends on the synthesis of PML/RARα 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/RARα 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/RARα 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-αRs (TNF-αRI and TNF-αRII) is TNF-α–sensitive (27, 28). Here we investigated the effect of PML/RARα 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 Zn^{2+} these clones are only scarcely sensitive to TNF-α–triggered cytotoxicity. This phenomenon is specifically related to the induction of PML/RARα protein expression, in that: (*a*) the clones grown in the absence of Zn^{2+} displayed a sensitivity to TNF-α cytotoxicity comparable to that of wt U937 cells; (*b*) wt cells grown in absence or presence of Zn^{2+} showed comparable levels of sensitivity to TNF-α–triggered cytotoxicity.

A specific role for PML/RARα 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/RARα.

More importantly, fresh APL blasts exhibit a virtually absent TNF-αR 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/RARα$^+$ U937 cells induced by Zn^{2+}. 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-αR 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-

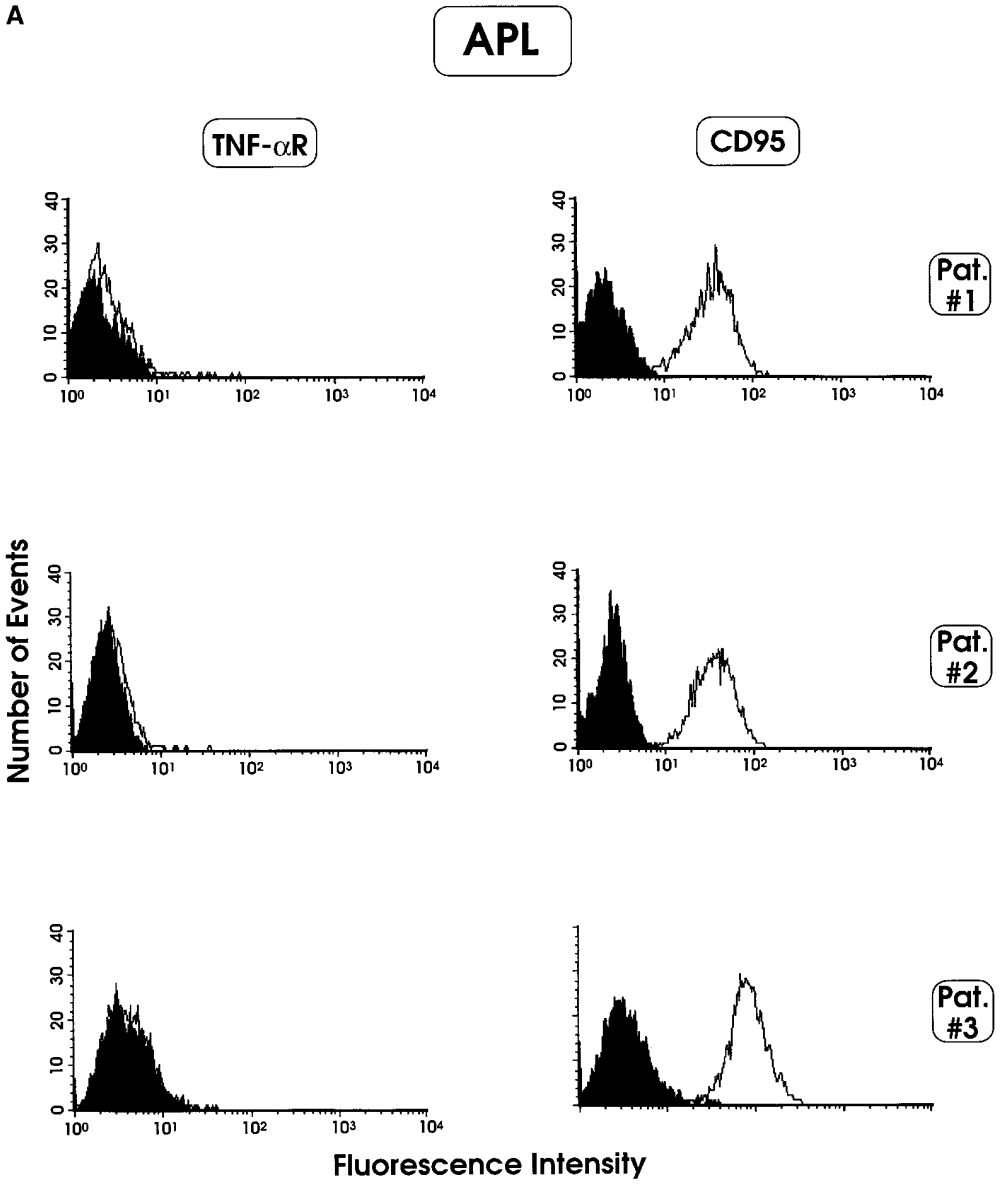

Figure 6. (*A*) Expression of TNF-αR and CD95 in leukemic blasts derived from three representative APL patients. TNF-αR expression was measured by analysis of TNF-α biotinylated binding. (*B*) Expression of TNF-αR and CD95 in leukemic blasts derived from three representative non-APL AML patients (*M1*, *M2*, and *M4* FAB types). (*C*) Expression of TNF-αR 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/RARα on TNF-αR 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-αR 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-αRI may explain the reduced sensitivity to TNF-α–mediated cytotoxicity, in that U937 cells are particularly sensitive to apoptotic mechanisms triggered through TNF-αRI (28, 32, 33 and the present study). Our results, as well as previous studies (28, 32), further indicate that TNF-αRII cooperates with TNF-αRI in inducing TNF-α–mediated cytotoxicity. It may be hence assumed that the reduced expression of TNF-αRII induced by PML/RARα also contributes to the decreased sensitivity to the cytotoxic action of TNF-α. Altogether, we suggest that the reduced cytotoxic 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-αR induced by PML/RARα expression is seemingly mediated through posttranscriptional mechanisms: indeed, PML/RARα$^+$ cells show an unmodified TNF-αRI and TNF-αRII 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-αRs 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.,

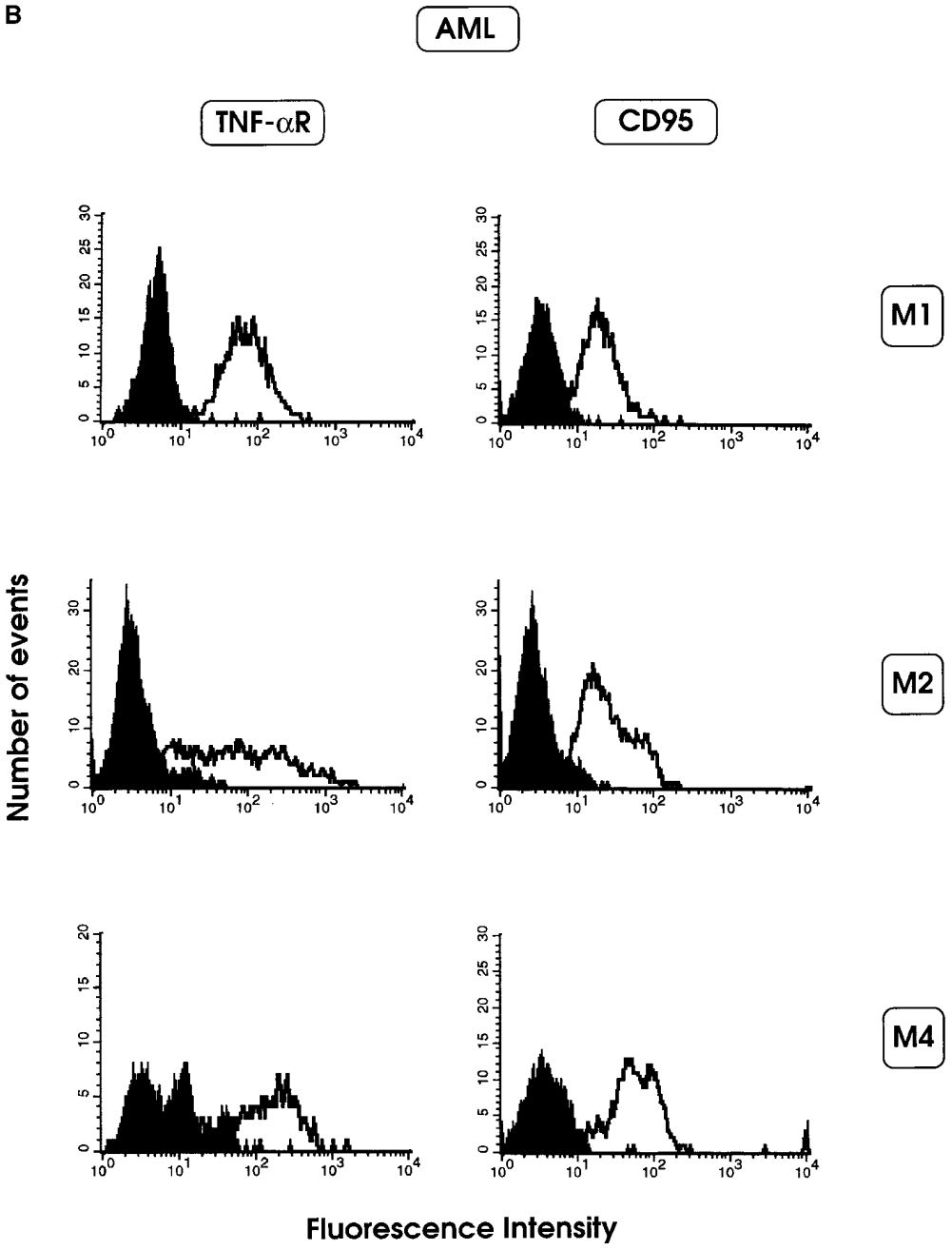

Figure 6 (Continued)

the fusion PML/RARα protein in APL, may specifically modulate the number of TNF-αRs.

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 physiologic regulator of the early hematopoietic cell compartment. Finally, TNF-α is produced by both myelodysplastic (57) and AML cells (58), and inhibits the clonogenetic 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-

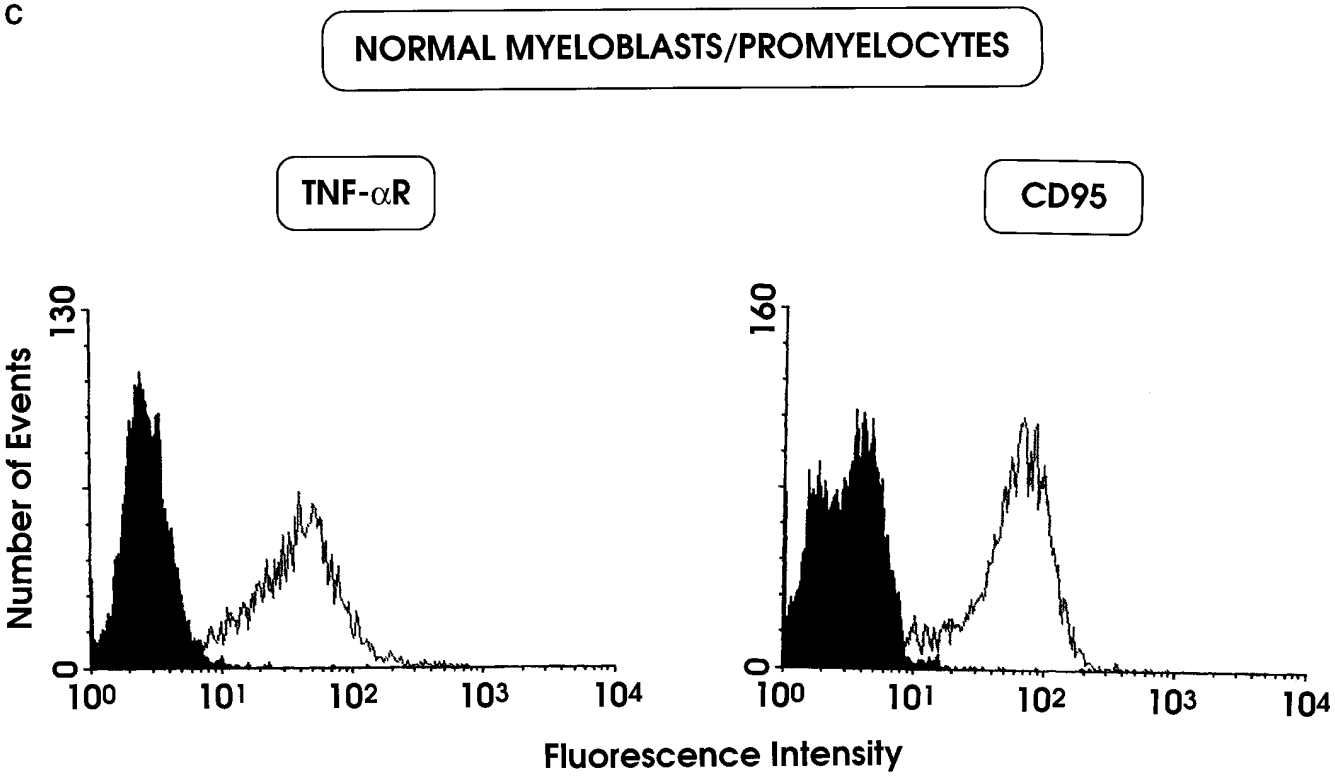

Figure 6 (Continued)

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

References

1. Grignani, F., M. Fagioli, M. Alcalay, L. Longo, P.P. Pandolfi, E. Donti, A. Biondi, F. Lo Coco, F. Grignani, and P.G. Pelicci. 1994. Acute promyelocytic leukemia: from genetics to treatment. *Blood.* 83:10–25.

2. Warrell, R.P., H. De Thé, Z.Y. Wang, and L. Degos. 1993. Acute promyelocytic leukemia. *N. Engl. J. Med.* 329:177–189.

3. Raza, A., Y. Maheshwari, and H.D. Preisler. 1987. Differences in cell cycle characteristics among patients with acute nonlymphocytic leukemia. *Blood.* 69:1647–1653.

4. Raza, A., N. Yousuf, A. Abbas, A. Umerani, A. Mehdi, S.A. Bokhari, Y. Sheikh, K. Qadir, J. Freeman, M. Masterson, et al. 1992. High expression of transforming growth factor-beta long cell cycle times and a unique clustering of S-phase cells in patients with acute promyelocytic leukemia. *Blood.* 79:1037–1048.

5. De Thé, H., C. Chomienne, M. Lanotte, L. Degos, and A. Dejean. 1990. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. *Nature.* 347:558–561.

6. Borrow, J., A.D. Goddard, D. Sheer, and E. Solomon. 1990. Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. *Science.* 249:1577–1580.

7. Longo, L., P.P. Pandolfi, A. Biondi, A. Rambaldi, A. Mencarelli, F. Lo Coco, D. Diverio, L. Pegoraro, G. Avanzi, A. Tabilio, et al. 1990. Rearrangements and aberrant expression of the retinoic acid receptor alpha gene in acute promyelocytic leukemias. *J. Exp. Med.* 172:1571–1575.

8. Alcalay, M., D. Zangrilli, P.P. Pandolfi, L. Longo, A. Mencarelli, A. Giacomucci, M. Rocchi, A. Biondi, A. Rambaldi, F. Lo Coco, et al. 1991. Translocation breakpoint of acute promyelocytic leukemia lies within the retinoic acid receptor alpha locus. *Proc. Natl. Acad. Sci. USA.* 88:1977–1981.

9. Giguere, V., E.S. Ong, P. Segui, and R.M. Evans. 1987. Identification of a receptor for the morphogene retinoic acid. *Nature.* 330:624–629.

10. Petkovich, M., N.J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature.* 330:444–450.

11. Goddard, A.D., J. Borrow, P.S. Freemont, and E. Solomon. 1991. Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. *Science.* 254:1371–1374.

12. Dyck, J.A., G.G. Maul, W.H. Miller, J.D. Chen, A. Kakizuka, and R.M. Evans. 1994. A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell.* 76:333–343.

13. Weis, K., S. Rambaud, C. Lavau, J. Jansen, T. Carvalho, M. Carmo-Fonseca, A. Lamond, and A. Dejean. 1994. Retinoic acid regulates aberrant nuclear localization of PML-RARα in acute promyelocytic leukemia cells. *Cell.* 76:345–356.

14. Pandolfi, P.P., F. Grignani, M. Alcalay, A. Mencarelli, A. Biondi, F. LoCoco, F. Grignani, and P.G. Pelicci. 1991. Structure and origin of the acute promyelocytic leukemia myl/RARα cDNA and characterization of its retinoid-binding and transactivation properties. *Oncogene.* 6:1285–1292.

15. Grignani, F., P.F. Ferrucci, U. Testa, G. Talamo, M. Fagioli, M. Alcalay, A. Mencarelli, F. Grignani, C. Peschle, I. Nicoletti, and P.G. Pelicci. 1993. The acute promyelocytic leukemia-specific PML/RARα fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell.* 74:423–431.

16. Grignani, F., U. Testa, M. Fagioli, T. Barberi, R. Masciulli, G. Mariani, C. Peschle, and P.G. Pelicci. 1995. Promyelocytic leukemia-specific PML-retinoic acid alpha receptor fusion protein interferes with erythroid differentiation of human erythroleukemia K562 cells. *Cancer Res.* 55:440–443.

17. Testa, U., F. Grignani, T. Barberi, M. Fagioli, R. Masciulli, P.F. Ferrucci, D. Seripa, A. Camagna, M. Alcalay, P.G. Pelicci, and C. Peschle. 1994. PML/RARα$^+$ U937 mutant and NB4 cell lines: retinoic acid restores the monocytic differentiation response to vitamin D3. *Cancer Res.* 54:4508–4515.

18. Grignani, F., U. Testa, D. Rogaia, P.F. Ferrucci, P. Samoggia, A. Pinto, D. Aldinucci, V. Gelmetti, M. Fagioli, M. Alcalay, et al. 1996. Effects on differentiation by the promyelocytic leukemia PML/RARα protein depend on the fusion of the PML protein-dimerization and RARα DNA binding domains. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:4949–4958.

19. Grisolano, J.L., R.L. Wesselschmidt, P.G. Pelicci, and T.J. Ley. 1997. Altered myeloid development and acute leukemia in transgenic mice expressing PML-RARα under control of cathepsin G regulatory sequences. *Blood.* 89:376–387.

20. Altabef, M., M. Garcia, C. Lavau, S.C. Bae, A. Dejean, and J. Samarut. 1996. A retrovirus carrying the promyelocyte-retinoic acid receptor PML-RAR alpha fusion gene transforms haematopoietic progenitors in vitro and induces acute leukaemias. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2707–2716.

21. Dubois, C., M.H. Schlageter, A. De Gentile, N. Balitrand, M.E. Toubert, I. Krawice, P. Fenaux, S. Castaigne, Y. Najean, L. Degos, and C. Chomienne. 1994. Modulation of IL-8, IL-1β and G-CSF secretion by all-trans retinoic acid in acute promyelocytic leukemia. *Leukemia.* 8:1750–1757.

22. Dubois, C., M.H. Schlageter, A. De Gentile, F. Guidez, N. Balitrand,

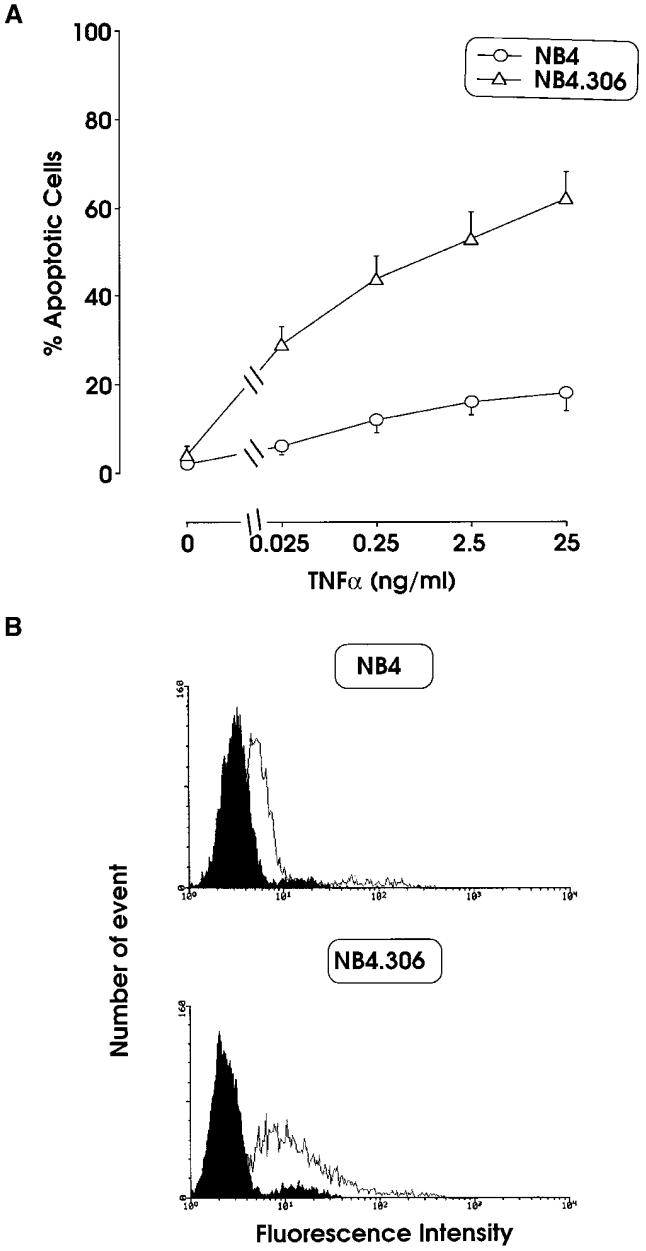

Figure 7. TNF-α–binding capacity and sensitivity of wt NB4 and NB4.306 cells to TNF-α–mediated cell apoptosis. (*A*) Cells were incubated with biotinylated TNF-α and then stained with FITC-Avidin. (*B*) NB4 and NB4.306 cells were grown for 24 h in absence or presence of increasing TNF-α concentrations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

M.E. Toubert, I. Krawice, P. Fenaux, S. Castaigne, Y. Najean, L. Degos, and C. Chomienne. 1994. Hematopoietic growth factor expression and ATRA sensitivity in acute promyelocytic blast cells. *Blood.* 83:3264–3270.

23. Fiers, W. 1991. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. *FEBS Lett.* 285:199–212.

24. Ruggiero, V., K. Latham, and C. Baglioni. 1987. Cytostatic and cytotoxic activity of tumor necrosis factor on human cancer cells. *J. Immunol.* 138: 2711–2717.

25. Hernandez-Caselles, T., and O. Stutman. 1993. Immune functions of tumor necrosis factor. I. Tumor necrosis factor induces apoptosis of mouse thymocytes and can also stimulate or inhibit IL-6-induced proliferation depending on the concentration of mitogenic costimulation. *J. Immunol.* 151:3999–4012.

26. Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell.* 76:959–962.

27. Thoma, B., M. Grell, K. Pfizenmaier, and P. Scheurich. 1990. Identification of a 60-kD tumor necrosis factor (TNF) receptor as the major signal transducing component in TNF responses. *J. Exp. Med.* 172:1019–1023.

28. Shalaby, M.R., A. Sundan, H. Loetscher, M. Brockhaus, W. Lesslauer, and T. Espevik. 1990. Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. *J. Exp. Med.* 172:1517–1520.

29. Grell, M., G. Zimmermann, D. Hulser, K. Pfizenmaier, and P. Scheurich. 1994. TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. *J. Immunol.* 153:1963–1972.

30. Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich. 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell.* 83:793–802.

31. Barbara, J.A., W.B. Smith, J.R. Gamble, X. Van Ostade, P. Vandenabeele, J. Tavernier, W. Fiers, M.A. Vadas, and A.F. Lopez. 1994. Dissociation of TNF-α cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-α mutants. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:843–850.

32. Greenblatt, M.S., and L. Elias. 1992. The type B receptor for tumor necrosis factor-α mediates DNA fragmentation in HL-60 and U937 cells and differentiation in HL-60 cells. *Blood.* 80:1339–1346.

33. Chambaut-Guérin, A.M., M. Guerrier, and P. Thomopoulos. 1993. Role of the 75 KD- and 55 KD-receptors in tumour necrosis factor mediated cytotoxicity and its regulation by dexamethasone and by 1,25-dihydroxyvitamin D3 in U937 cells. *Eur. Cytokine Net.* 4:285–292.

34. Lanotte, M., V. Martin-Thouvenin, S. Najman, P. Balerini, F. Valensi, and R. Berger. 1991. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood.* 77:1080–1086.

35. Dermime, S., F. Grignani, M. Clerici, C. Nervi, G. Sozzi, G.P. Talamo, E. Marchesi, F. Formelli, G. Parmiani, P.G. Pelicci, and C. Gambacorti-Passerini. 1993. Occurrence of resistance to retinoic acid in the acute promyelocytic leukemia cell line NB4 is associated with altered expression of the PML/RAR alpha protein. *Blood.* 82:1573–1577.

36. Gabbianelli, M., M. Sargiacomo, E. Pelosi, U. Testa, G. Isacchi, and C. Peschle. 1990. "Pure" human hematopoietic progenitors: permissive action of basic fibroblast growth factor. *Science.* 249:1561–1564.

37. Labbaye, C., M. Valtieri, T. Barberi, E. Meccia, B. Masella, E. Pelosi, G. Condorelli, U. Testa, and C. Peschle. 1995. Differential expression and functional role of GATA-2, NF-E2 and GATA-1 in normal adult hematopoiesis. *J. Clin. Invest.* 95:2346–2358.

38. Gorczyca, W., J. Gong, and Z. Darzynkiewicz. 1993. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 53:1945–1951.

39. Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Meth.* 139:271–279.

40. Grell, M., P. Scheurich, A. Meager, and K. Pfizenmaier. 1993. TR60 and TR80 tumor necrosis factor (TNF)-receptors can independently mediate cytolysis. *Lymphokine Cytokine Res.* 12:143–148.

41. Cramer, R., M.R. Soranzo, and P. Patriarca. 1981. Evidence that eosinophils catalyze the bromide-dependent decarboxylation of amino acids. *Blood.* 58:1112–1118.

42. Butera, S.T., B.D. Roberts, K. Leung, G.J. Nabel, and T.M. Folks. 1993. Tumor necrosis factor receptor expression and signal transduction in HIV-1-infected cells. *AIDS.* 7:911–918.

43. Chirwing, T.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of a biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5302.

44. Rogaia, D., F. Grignani, F. Grignani, I. Nicoletti, and P.G. Pelicci. 1995. The acute promyelocytic leukemia-specific PML/RARα fusion protein reduces the frequency of commitment to apoptosis upon growth factor deprivation of GM-CSF-dependent myeloid cells. *Leukemia.* 9:1467–1472.

45. Fenaux, P., and C. Chomienne. 1996. Biology and treatment of acute promyelocytic leukemia. *Curr. Opin. Oncology.* 8:3–12.

46. Chomienne, C., P. Fenaux, and L. Degos. 1996. Retinoid differentiation therapy in promyelocytic leukemia. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 10: 1025–1030.

47. Martin, S.J., J.G. Bradley, and T.G. Cotter. 1990. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin. Exp. Immunol.* 79:448–453.

48. Tosi, P., G. Visani, D. Gibellini, G. Zauli, E. Ottaviani, A. Cenacchi, B. Gamberi, S. Manfroi, M. Marchisio, and S. Tura. 1994. All-trans retinoic acid and induction of apoptosis in acute promyelocytic leukemia cells. *Leuk. Lymphoma.* 14:503–507.

49. Hestdal, K., P. Aukrust, F. Müller, E. Lien, V. Bjerkeli, T. Espevik, and S.S. Froland. 1997. Dysregulation of membrane-bound tumor necrosis factor-α and tumor necrosis factor receptors on mononuclear cells in human immunodeficiency virus type 1 infection: low percentage of p75-tumor necrosis factor receptor positive cells in patients with advanced disease and high viral load.

Blood. 90:2670–2679.

50. Aggarwal, B.B., T.E. Eessalu, and P.E. Hass. 1985. Characterization of receptors for human tumor necrosis factor and their regulation by gamma-interferon. *Nature.* 318:665–667.

51. Holtmann, H., and D. Wallach. 1987. Down regulation of the receptors for tumor necrosis factor by interleukin 1 and 4 beta-phorbol-12-myristate-13-acetate. *J. Immunol.* 139:1161–1167.

52. Higuchi, M., and B.B. Aggarwal. 1993. Okadaic acid induces down-modulation and shedding of tumor necrosis factor receptors. Comparison with another tumor promoter, phorbol ester. *J. Biol. Chem.* 268:5624–5631.

53. Dubravec, D.B., D.R. Spriggs, J.A. Mannick, and M.L. Rodrick. 1990. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. *Proc. Natl. Acad. Sci. USA.* 87:6758–6761.

54. Caux, C., C. Favre, S. Saeland, V. Duvert, I. Durand, P. Mannoni, and J. Bancherau. 1991. Potentiation of early hematopoiesis by tumor necrosis factor-alpha is followed by inhibition of granulopoietic differentiation and proliferation. *Blood.* 78:635–644.

55. Murray, J., J.A. Barbara, S.A. Dunkley, A.F. Lopez, X.V. Ostada, A.M. Condliffe, I. Dransfield, C. Haslett, and E.R. Chilvers. 1997. Regulation of neutrophil apoptosis by tumor necrosis factor-α: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood.* 90:2772–2783.

56. Zhang, Y., A. Harada, H. Bluethmann, J.B. Wang, S. Nakao, N. Mukaida, and K. Matsushima. 1995. Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNFα in vitro. *Blood.* 86:2930–2937.

57. Shetty, V., S. Mundle, S. Alvi, M. Showel, L. Broady-Robinson, S. Dar, R. Borok, J. Showel, S. Gregory, S. Rifkin, et al. 1996. Measurement of apoptosis, proliferation and three cytokines in 46 patients with myelodysplastic syndromes. *Leuk. Res.* 20:891–900.

58. Kobari, L., D. Weil, F.M. Lemoine, C. Dubois, D. Thiam, C. Baillou, M. Guigon, N.C. Gorin, and A. Najman. 1990. Secretion of tumor necrosis factor-alpha by fresh human acute nonlymphoblastic leukemic cells: role in the disappearance of normal CFU-GM progenitors. *Exp. Hematol.* 18:1187–1192.

59. Munker, R., and P. Koeffler. 1987. In vitro action of tumor necrosis factor on myeloid leukemia cells. *Blood.* 69:1102–1108.

60. Inaba, T., T. Inukai, T. Yoshihara, H. Seyschab, R.A. Ashmun, C.E. Canman, S.J. Laken, M.B. Kastan, and A.T. Look. 1996. Reversal of apoptosis by the leukaemia-associated E2A-HLF chimaeric transcription factor. *Nature.* 382:541–544.