# TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL

Roberta Riccioni
Luca Pasquini
Gualtiero Mariani
Ernestina Saulle
Annalisa Rossini
Daniela Diverio
Elvira Pelosi
Antonella Vitale
Anna Chierichini
Michele Cedrone
Robin Foà
Francesco Lo Coco
Cesare Peschle
Ugo Testa

Background and Objectives. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is regarded as a potential anticancer agent. However, many cancer cells are resistant to apoptosis induction by TRAIL. The present study was designed to evaluate the sensitivity to TRAIL-induced apoptosis in acute myeloblastic leukemias (AML).

Design and Methods. TRAIL/TRAIL receptor (TRAIL-R) expression and sensitivity to TRAIL-mediated apoptosis were explored in 79 AML patients, including 17 patients with acute promyelocytic leukemia (APL).

Results. In non-APL AML we observed frequent expression of TRAIL decoy receptors (TRAIL-R3 and TRAIL-R4), while TRAIL-R1 and TRAIL-R2 expression was restricted to AML exhibiting monocytic features. Total leukemic blasts, as well as AML colony-forming units (AML-CFU), were invariably resistant to TRAIL-mediated apoptosis. APL express membrane-bound TRAIL on their surface and exhibit a pattern of TRAIL-R expression similar to that observed in the other types of AML. Before, during and after retinoic acid treatment APL cells are TRAIL-resistant. The induction of granulocytic maturation of APL cells by retinoic acid was associated with a marked decline of TRAIL expression.

Interpretation and Conclusions. The analysis of experimental APL models (i.e., U937 cells engineered to express PML/RAR-α and NB4 cells) provided evidence that PML/RAR-α expression was associated with downmodulation of TRAIL-R1 and with resistance to TRAIL-mediated apoptosis. We suggest that AML blasts, including APL blasts, are resistant to TRAIL-mediated apoptosis, a phenomenon seemingly related to the expression of TRAIL decoy receptors on these cells. Finally, APL blasts express membrane-bound TRAIL that could confer an immunologic privilege to these cells.

Key words: apoptosis, leukemia, TRAIL, TRAIL receptors, acute promyelocytic leukemia.

Haematologica 2005; 90:612-624



*From the *Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy; °Hematology, Department of Cellular Biotechnologies and Hematology, University "La Sapienza", Rome, Italy; §Department of Biopathology, Università Tor Vergata, Rome, Italy; #Hematology Section, Ospedale San Giovanni, Romme, Italy.*

*Correspondence:*
*Ugo Testa, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Roma. E-mail: u.testa@iss.it*

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), otherwise known as Apo2Ligand (Apo2L) is a member of the tumor necrosis factor family of ligands capable of initiating apoptosis through triggering of their death receptors.[1,2] TRAIL induces apoptosis in a variety of tumor cells by interacting with its cell surface receptors TRAIL-R1 and TRAIL-R2, also known as DR4 and DR5, respectively. Both TRAIL-R1 and TRAIL-R2 contain a region known as the *death domain* in their cytoplasmic tail; this domain is responsible for the transduction of the death signal. Binding of TRAIL to TRAIL-R1 and TRAIL-R2 induces the recruitment of a cytoplasmic adaptor molecule FADD to the death domains of the two receptors, inducing the activation of first caspase-8 and then effector caspases-3, -6 and –7 and finally apoptosis.[3] Besides binding to TRAIL-R1 and TRAIL-R2, TRAIL also binds to two other membrane receptors, TRAIL-R3 and TRAIL-R4, which lack a functional death domain and therefore act as decoy receptors (in fact, TRAIL-R3 and TRAIL-R4 are also known as DcR1 and DcR2, respectively).[3] These decoy receptors cannot transmit the apoptotic signal, because they lack a death domain, and inhibit TRAIL-induced apoptosis by competing with TRAIL-R1 and TRAIL-R2 for TRAIL binding. All the normal tissues that express TRAIL decoy receptors and real TRAIL death receptors at the same time have been found to be resistant to TRAIL-induced apoptosis.[4] The physiological role of TRAIL is not fully understood. However, the ligand's ability to trigger apoptosis in a variety of transformed cancer cells suggests that it may be a physiological modulator of tumor cell apoptosis.[5] In line with this hypothesis, studies with TRAIL

gene knockout mice confirm that TRAIL has a role in anti-tumor immune surveillance, specifically in host defenses against tumor initiation and metastasis.[5]

The anti-tumor activity of TRAIL has been explored in detail during the last few years. It has been shown that TRAIL exerts a cytotoxic activity against the majority of tumor cell lines, while it spares normal tissues.[5,6] TRAIL is constitutively expressed in many human tissues. TRAIL-R1 and TRAIL-R2 are also expressed in many normal tissues, suggesting the existence of physiological mechanisms that protect normal cells from TRAIL-induced apoptosis.[5,6] Importantly, TRAIL-induced apoptosis is independent of p53 status, a property that makes it potentially effective against tumors that are resistant to chemotherapy.[5,6]

The anti-tumor activity of TRAIL has been investigated in hematologic malignancies, including multiple myeloma cells and Philadelphia chromosome-positive leukemia in which it was shown that TRAIL is able to induce apoptosis.[7-9] Only a few studies have explored the sensitivity of acute myeloid leukemia (AML) cells to TRAIL. These studies, carried out on a limited number of cases, have shown a very low sensitivity of AML blasts to the apoptotic effects of TRAIL.[10] In contrast, some continuous cell lines derived from AML were observed to have a pronounced sensitivity to the TRAIL-mediated apoptotic effects.[11] Furthermore, in some of these cell lines, TRAIL was shown to act as an inducer of monocytic differentiation.[12] Studies on acute promyelocytic leukemia (APL) cells have documented that APL blasts are sensitive to TRAIL-mediated apoptosis and that retinoic acid induces TRAIL expression and thereafter killing of the leukemic cells.[13]

In the present study we explored the expression of TRAIL and TRAIL-R on 79 samples of primary AML, including 15 cases of APL.

## Design and Methods

### Cells

The wild type (wt) U937 myeloid cell line was maintained in RPMI 1640 medium and 10% fetal calf serum (FCS) (both from GIBCO, Grand Island, NY, USA). The detailed cloning methodology to obtain the PML/RARα$^+$ U937 (clone MTPR9) cells has been reported previously.[13] In order to induce PML/RAR-α expression, MTPR9 cells were incubated in the presence of 100 μM $Zn_2SO_4$. A clear induction of PML/RARα protein synthesis was observed 16-20h after $Zn^{2+}$ addition.[14] The wt PML/RARα$^+$ NB4 promyelocytic cell line[15] was grown in RPMI 1640 medium containing 10% FCS. In some experiments, either NB4 cells or fresh M3 blasts were grown in the presence of 1 μM all trans retinoic acid (ATRA, Sigma, St Louis, USA). Fresh leukemic blasts from 72 consecutive AML patients diagnosed and managed at the Hematology Center of "La Sapienza" University of Rome, were obtained after informed consent. The cells were isolated from bone marrow by Ficoll Hypaque density centrifugation. Of the 72 patients, 8 were diagnosed as having FAB M3, PML/RAR-α$^+$ acute promyelocytic leukemias. Nine additional APL cases were studied only at the RNA level. For the analysis of the sensitivity to TRAIL-mediated apoptosis, leukemic blasts were grown in Iscove's modified minimal essential medium (IMDM, Gibco, NY, USA) containing 10% FCS at a cell density of $5\times10^5$ cells/mL.

Peripheral blood hematopoietic progenitor cells (HPC) were obtained from three normal adult donors (three young males aged 25, 31 and 42 years) after informed consent. The HPC, purified as reported,[16] were grown in liquid suspension cultures under conditions allowing selective growth of either granulopoietic-neutrophilic cells (i.e., in the presence of 1 U/mL IL-3, 0.1 ng/mL GM-CSF, and 500 U/mL G-CSF) or monocytic precursors (i.e., in the presence of 10 ng/mL M-CSF, 100 ng/mL flt3 ligand), as previously reported in detail.[17,18]

### Treatment of cells with chemotherapeutic agents

In some experiments, MTPR9 cells induced or not to express PML/RAR-α were incubated for 48h *in vitro* in the absence (control) or in the presence of one of the following drugs: cytosine arabinoside (25 μM); hydroxyurea (100 μM); etoposide (5 μM) and the percentage of apoptotic cells was evaluated by the annexin-V binding assay. All drugs were purchased from Sigma Co, USA.

### Analysis of cell growth, cell cycle distribution, and apoptosis

Cell growth was determined by counting the number of viable cells after the trypan blue exclusion test.

Apoptosis was determined by two different methods: (i) evaluation of hypodiploid cells by flow cytometric analysis of PI-stained nuclei;[19] (ii) annexin V binding assay using a kit from R&D Systems (Minneapolis, MN, USA).

To trigger apoptotic damage, cells were incubated for 24-96 hours in the presence of recombinant human TRAIL, using concentrations ranging from 10 to 300 ng/mL (R&D Systems).

### AML colony assay

The AML-CFU assay was carried out according to a procedure previously described.[20] Briefly, AML mononuclear blood cells were plated in α-methylcellulose in IMDM medium containing 3U/mL recombinant human (rh) erythropoietin, 20 ng/mL rhIL-3, 20 ng/mL rhG-CSF, 20 ng/mL rhGM-CSF, 50 ng/mL Kit Ligand and 50 ng/mL flt3 ligand. After 14 days of incubation at

37°C in a 5% $CO_2$ humidified incubator, AML blast clusters (10 to 20 cells) or colonies (>50 cells) were counted and the numbers were pooled to obtain the CFU number.

### TRAIL-R characterization

In order to analyze the expression of TRAIL-R the cells were incubated with 5 μg/mL of phycoerythrin-labeled anti TRAIL-R1, -R2, -R3 or –R4 monoclonal antibodies (mAb) (all obtained from R & D Systems), washed with cold PBS, and then analyzed for fluorescence emission by a flow cytometer (FACSCAN, Becton Dickinson, San José, CA, USA).

Membrane-bound TRAIL was detected using a phycoerythrin-labeled anti-TRAIL mAb (Becton-Dickinson/Pharmingen, San José, CA, USA).

### Caspase activity

Caspase-3 and caspase-8 activities were determined by a colorimetric assay. The principle of the assay is based on the incubation of the cell lysate with a caspase-specific peptide (specific for either caspase-8 or caspase-3) that is conjugated to the color reporter molecule p-nitroaniline (pNA) (R&D Systems). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantified spectrophotometrically at a wavelength of 405 nm.

### RNA extraction and polymerase chain reaction (PCR) analysis

Total RNA was extracted by the guanidium isothiocyanate-cesium chloride method in the presence of 12 μg of *E. coli* rRNA as the carrier. Total RNA was reverse transcribed ( Moloney murine leukemia virus RT, BRL) with oligo (dT) as primer.

The amplification procedure consisted of denaturation at 95°C for 30 s, annealing for 30 s at 54°C for β2-microglobulin and extension at 72°C for 45 s; denaturation at 94°C for 30 s, annealing for 45 s at 58°C for TRAIL R1, R2, R4, and extension at 72°C for 1 min; denaturation at 94°C for 30 s, annealing for 45 s at 55°C for TRAIL R3 and TRAIL ligand, and extension at 72°C for 60 s. The sequences used for the primers and probes are shown in Table 1. PCR was performed in a total volume of 50 μL; 10 μL of each sample were separated in a 2% agarose gel and transferred to a nylon filter. Filters were hybridized with a probe end-labeled with ($^{32}$P) ATP and polynucleotide kinase.

**Table 1.** Oligomers used for RT-PCR studies.

| Gene | Type of sequence | Sequence |
|---|---|---|
| $\beta_2$-m | sense | 5'-AACCACGTGACTTTGTCACAGC-3' |
| | antisense | 5'- CTGCTCATACATCAAACATG-3' |
| | probe | from 311bp to 360 bp |
| TRAIL-R1 | sense | 5'- CTGAGCAACGCAGACTCGCTGTCC-3' |
| | antisense | 5'-TCCAAGGACACGGCAGAGCCTGTGCCAT-3' |
| | probe | 5'-TGAGGCAGCTGGACCTCACGA-3' |
| TRAIL-R2 | sense | 5'-GCCTCATGGACAATGAGATAAAGGTGGCT-3' |
| | antisense | 5'-CAAACCAAATCTCAAAGTACGCACAAACGG-3' |
| | probe | 5'-TCCAGTCAGTAGGAAAGTGCCAC-3' |
| TRAIL-R3 | sense | 5'-CACCAACGCTTCCAACAATG-3' |
| | antisense | 5'-GAGAGGTAATGAGAAGAGGC-3' |
| | probe | 5'-CACCAGCCCAGGGACTCCTGC-3' |
| TRAIL-R4 | sense | 5'-GGTCAAGGTCAGTAATTGTAC-3' |
| | antisense | 5'-CCACCAGTTGGTCCTGAATTG-3' |
| | probe | 5'-GTAACAGATACTTGCAGCCCAC-3' |
| TRAIL | sense | 5'-GGAACCCAAGGTGGGTAGAT-3' |
| | antisense | 5'-TCTCACCACACTGCAACCTC-3' |
| | probe | 5'-GTCCCAGCTACTTGAGAGGCTG-3' |

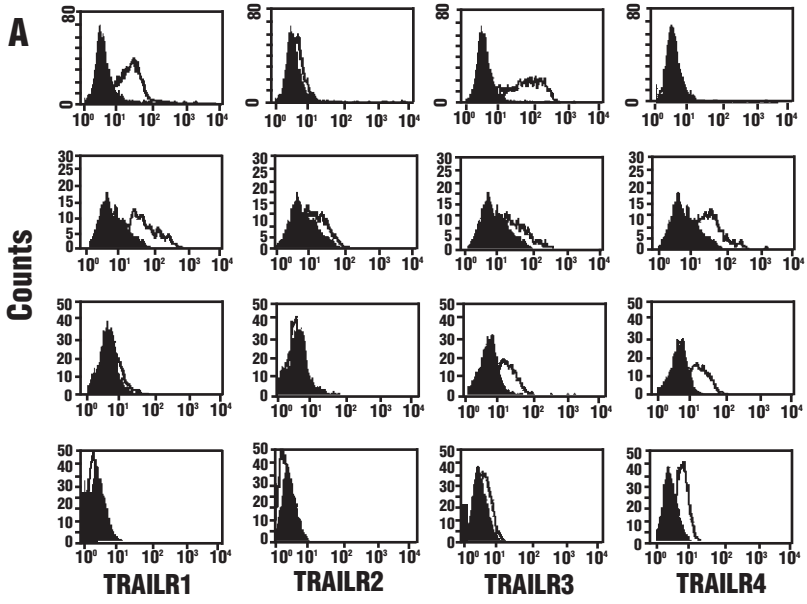
## Results

### Expression of TRAIL receptors on AML cells

The expression of TRAIL-R on freshly isolated AML cells was studied by flow cytometry using specific monoclonal antibodies directly conjugated with phycoerythrin (Table 2). Control experiments carried out on peripheral blood monocytes showed that these antibodies exhibited the expected pattern of reactivity: in fact, in line with a previous report[21] these cells were positive for TRAIL-R2 and TRAIL-R3 expression, while TRAIL-R1 and TRAIL-R4 were expressed at lower levels (*data not shown*). Similarly, normal peripheral blood neutrophils clearly expressed TRAIL-R3 and TRAIL-R4, but had virtually undetectable levels of TRAIL-R1 and TRAIL-R2 (*data not shown*). TRAIL-R3 and TRAIL-R4 were expressed in the majority of cases of non-APL AML, while the expression of TRAIL-R1 was observed in about 50% of cases; finally, TRAIL-R2 expression was observed only in a minority (about 10%) of the 64 cases analyzed (Table 2 and Figure 1A). APL expressed TRAIL-R3 and TRAIL-R4 in the majority of cases, while TRAIL-R1 and TRAIL-R2 were usually undetectable (in only 1/8 cases was TRAIL-R1 detectable at low levels) (Table 2 and Figure 1A). Membrane-bound TRAIL was observed in the majority of APL cases (7 out of 8) (Table 3). Interestingly, membrane-bound Fas Ligand (FasL) was clearly detected on cells from all the APL patients (Table 3). In parallel, we evaluated TRAIL and TRAIL-R expression at mRNA level by RT-PCR. This analysis showed that: (i) in non-APL AML (the analysis was carried out in 22 cases) TRAIL, TRAIL-R3 and TRAIL-R4 were frequently expressed, while the expression of TRAIL-R1 and TRAIL-R2 was confined to a minority of cases (Figurre 1B); (ii) in M3 AML (the analysis was carried out in 14 cases), TRAIL mRNA was observed in the large majority of cases, TRAIL-R3

**Table 2.** Biological characteristics and TRAIL receptor expression in 64 non-APL AML patients.

| *Patient* | *Age* | *FAB* | *WBC (×10⁹/L)* | *% Blasts* | *TRAIL-R1* | *TRAIL-R2* | *TRAIL-R3* | *TRAIL-R4* | *CD14 (%)* | *CD11b (%)* |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 58 | M1 | 96.8 | 99 | – | – | – | + | 0 | 7 |
| 2 | 41 | M2 | 129.5 | 80 | – | – | + | – | 2 | 2 |
| 3 | 18 | M6 | 1.5 | 41 | – | – | + | + | 0 | 8 |
| 4 | 27 | M1 | 35 | 90 | – | – | – | – | 2 | 32 |
| 5 | 51 | M4 | 26.1 | 50 | – | – | ++ | – | N.D. | 30 |
| 6 | 55 | M4 | 108 | 80 | ++ | – | + | + | 32 | 33 |
| 7 | 50 | M1 | 119.9 | 90 | – | – | – | – | 1 | 96 |
| 8 | 39 | M2 | 20 | 70 | – | – | – | ++ | 0 | 14 |
| 9 | 48 | M5a | 43 | 49 | ++ | – | + | – | 42 | 45 |
| 10 | 45 | M2 | 16 | 88 | + | – | – | – | 8 | 6 |
| 11 | 37 | M4 | 67.8 | 43 | ++ | – | +++ | – | 72 | 85 |
| 12 | 36 | M2 | 15 | 78 | – | – | + | + | 2 | 11 |
| 13 | 38 | M4 | 77 | 34 | – | – | – | – | 28 | 52 |
| 14 | 21 | M4 | 54.1 | 85 | ++ | – | + | ++ | 68 | 67 |
| 15 | 33 | M4 | 43.4 | 68 | + | – | + | + | 32 | 35 |
| 16 | 53 | M2 | 26.4 | 51 | – | – | + | + | 5 | 23 |
| 17 | 46 | M5a | 97.3 | 95 | ++ | – | + | ++ | 23 | 71 |
| 18 | 35 | M1 | 28 | 95 | – | – | – | – | 2 | 16 |
| 19 | 33 | M4 | 69 | 90 | ++ | – | + | + | 50 | 44 |
| 20 | 43 | M5b | 64 | 87 | ++ | – | + | ++ | 48 | 72 |
| 21 | 44 | M1 | 21.7 | 80 | – | – | – | – | 0 | 0 |
| 22 | 53 | M5b | 14.4 | 40 | ++ | – | ++ | ++ | 56 | 93 |
| 23 | 33 | M4 | 25 | 80 | – | – | + | + | 2 | 9 |
| 24 | 43 | M4 | 30 | 88 | – | – | + | + | 3 | 23 |
| 25 | 44 | M4 | 44.8 | 90 | ++ | + | ++ | +++ | 27 | 43 |
| 26 | 53 | M2 | 21 | 90 | – | – | + | – | 1 | 25 |
| 27 | 31 | M1 | 135 | 90 | + | – | + | + | 12 | 31 |
| 28 | 48 | M5a | 63.6 | 95 | + | – | + | + | 5 | 13 |
| 29 | 29 | M4Eo | 188 | 90 | – | + | – | + | 7 | 12 |
| 30 | 33 | M4 | 225 | 95 | – | – | – | – | 1 | 24 |
| 31 | 53 | M0 | 90 | 87 | – | – | – | – | 1 | 7 |
| 32 | 25 | M1 | 165 | 90 | – | – | – | – | 3 | 6 |
| 33 | 38 | M4 | 204 | 99 | ++ | – | ++ | ++ | 41 | 74 |
| 34 | 59 | M4 | 56 | 60 | + | – | + | + | 36 | 26 |
| 35 | 58 | M0 | 8.8 | 93 | – | – | – | – | 1 | 4 |
| 36 | 21 | M5b | 148 | 95 | ++ | – | ++ | ++ | 78 | 94 |
| 37 | 25 | M2 | 4.4 | 50 | – | – | – | – | 0 | 2 |
| 38 | 20 | M1 | 358 | 99 | – | – | – | + | 2 | 54 |
| 39 | 30 | M1 | 1.3 | 98 | – | – | – | + | 0 | 9 |
| 40 | 33 | M2 | 65.5 | 95 | – | – | – | – | 3 | 14 |
| 41 | 31 | M5b | 49.3 | 90 | ++ | – | ++ | ++ | 75 | 81 |
| 42 | 24 | M4 | 31 | 68 | + | – | ++ | – | 20 | 15 |
| 43 | 59 | M5b | 56.6 | 72 | – | – | – | – | 3 | 66 |
| 44 | 45 | M5b | 178 | 93 | ++ | + | + | +++ | 91 | 90 |
| 45 | 47 | M1 | 38.0 | 99 | – | – | – | - | 0 | 2 |
| 46 | 51 | M5b | 215 | 95 | ++ | + | ++ | ++ | 61 | 68 |
| 47 | 26 | M2 | 332 | 95 | + | ++ | + | ++ | 24 | 31 |
| 48 | 51 | M5b | 215 | 95 | + | – | + | ++ | 42 | 58 |
| 49 | 35 | M0 | 200 | 95 | – | + | - | + | 3 | 16 |
| 50 | 58 | M4 | 232 | 98 | ++ | + | ++ | +++ | 57 | 79 |
| 51 | 28 | M1 | 59 | 90 | + | – | – | – | 7 | 28 |
| 52 | 39 | M4 | 84.8 | 84 | – | ++ | – | + | 58 | 80 |
| 53 | 54 | M2 | 2.3 | 88 | – | – | + | + | 0 | 3 |
| 54 | 33 | M4Eo | 39 | 80 | ++ | – | – | + | 23 | 27 |
| 55 | 28 | M2 | 56 | 71 | + | – | – | – | 6 | 20 |
| 56 | 33 | M1 | 7.1 | 84 | – | – | + | + | 5 | 8 |
| 57 | 50 | M0 | 49.7 | 86 | + | – | + | + | 2 | 14 |
| 58 | 61 | M2 | 14.2 | 59 | ++ | – | ++ | ++ | 13 | 29 |
| 59 | 36 | M1 | 330 | 98 | + | – | + | + | 3 | 4 |
| 60 | 52 | M5b | 20.9 | 82 | + | – | + | + | 21 | 26 |
| 61 | 31 | M2 | 183 | 91 | – | – | – | – | 1 | 12 |
| 62 | 48 | M4 | 7 | 85 | + | – | + | + | 5 | 7 |
| 63 | 55 | M4 | 21 | 88 | + | – | ++ | + | 18 | 54 |
| 64 | 48 | M5a | 175 | 92 | ++ | – | + | ++ | 58 | 94 |



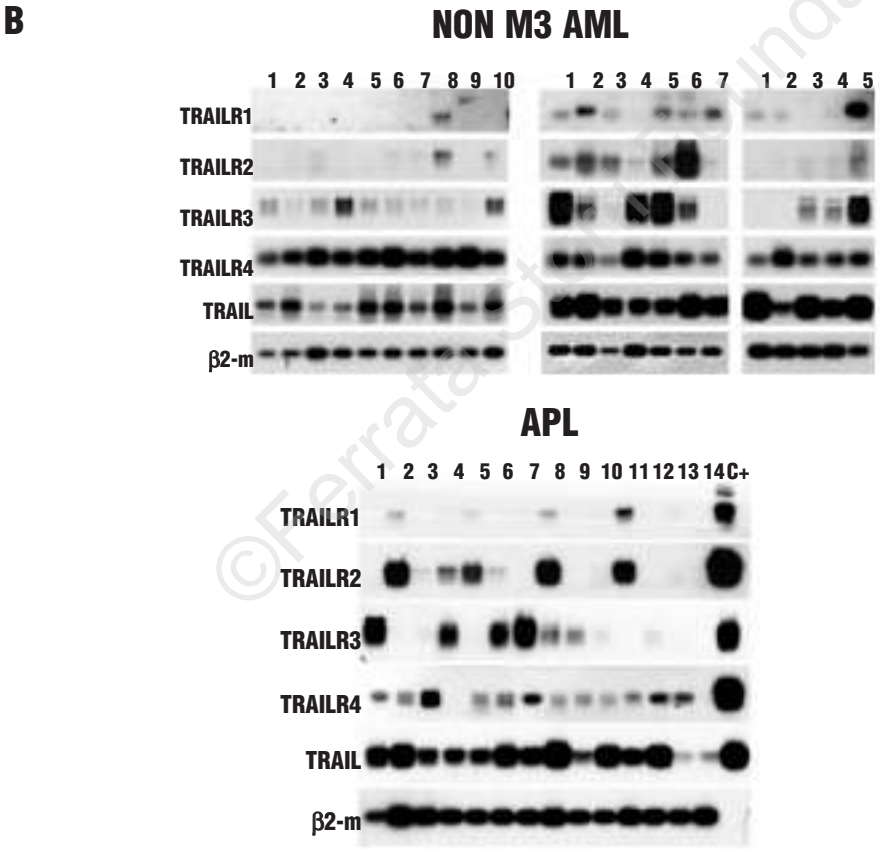




**Figure 1. A.** Flow cytometry analysis of TRAIL-R expression in fresh AML blasts isolated from 4 different patients. The first two rows from the top to the bottom correspond to 2 AML with monocytic features; the third row corresponds to an AML patient not exhibiting monocytic markers; finally, the bottom row corresponds to an M3 AML. **B.** 1B. RT-PCR analysis of TRAIL and TRAIL-R mRNA levels in 22 non-M3 AMLs (top) and 14 APLs (bottom). Samples were normalized according to the β2-microglobulin levels.



and TRAIL-R4 were frequently expressed, while TRAIL-R2 and particularly TRAIL-R1 were expressed in only a few cases (Figure 1B). Interestingly, the expression of TRAIL-R1 was limited to AML characterized by monocytic features, as supported by the presence of an elevated percentage of cells expressing the CD14 and Cd11b antigens. In our study, 29 out of 33 cases of AML expressing TRAIL-R1 displayed monocytic features; on the other hand, none of the remaining 32 TRAIL-R1$^-$ cases of AML displayed monocytic markers. In line with this observation, the majority of the TRAIL-R1$^+$ cases of AML were of the M4/M5 FAB

Table 3. Biological characteristics and TRAIL receptor expression in APL patients.

| Patient | FAB | WBC | % Blasts | TRAIL-R1 | TRAIL-R2 | TRAIL-R3 | TRAIL-R4 | TRAIL | CD34 (%) | c-kit (%) | CD33 (%) | CD11b (%) | CD14 (%) | FasL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M3 | 1.3 | 80 | – | – | – | – | + | 26 | 94 | 98 | 3 | 0 | N.D. |
| 2 | M3 | 13 | 84 | + | – | + | ++ | + | 0.5 | 72 | 99 | 79 | 21 | + |
| 3 | M3 | 5.2 | 85 | – | – | + | ++ | – | 1.5 | 41 | 100 | 26 | 1 | + |
| 4 | M3 | 3.9 | 91 | – | – | + | – | ++ | 4 | 71 | 96 | 12 | 9 | ++ |
| 5 | M3 | 95 | 95 | – | – | – | – | + | 17 | 80 | 99 | 42 | 0 | + |
| 6 | M3 | 8 | 88 | – | + | ++ | – | + | 6 | 75 | 99 | 22 | 5 | ++ |
| 7 | M3 | 2.2 | 92 | – | – | ++ | + | + | 78 | 89 | 98 | 29 | 2 | + |
| 8 | M3 | 2.5 | 92 | + | – | ++ | ++ | ++ | 49 | 83 | 100 | 80 | 6 | ++ |

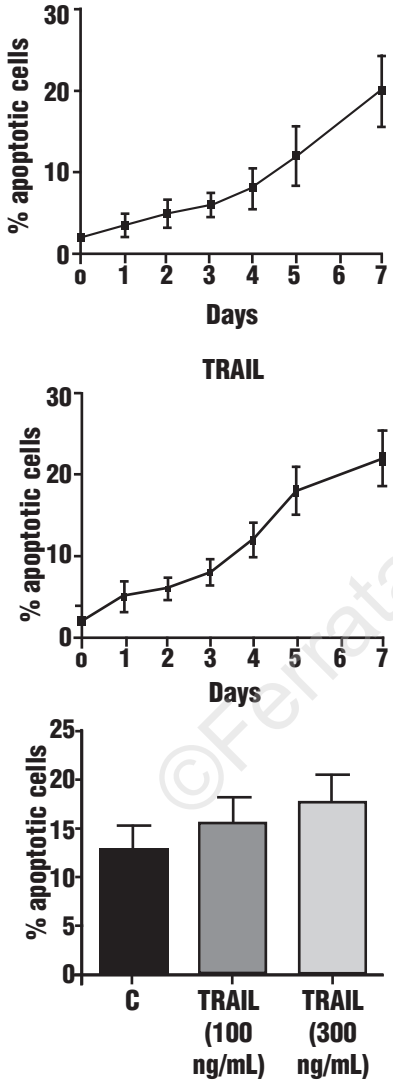



Figure 2. Sensitivity of AML blasts to TRAIL-mediated apoptosis. AML blasts derived from 8 different patients were incubated *in vitro* for different time periods in the absence or in the presence of 100 ng/mL TRAIL and the proportion of apoptotic cells was evaluated using the annexin V binding assay. The values represent mean values ± standard errors. The difference between the percentage of apoptotic cells observed for samples treated or not with TRAIL was not statistically significant.

types (Table 2). Monocytic TRAIL-R1$^+$ AML expressed also at least one of the two decoy receptors, TRAIL-R3 and/or TRAIL-R4. In some of these AML the effect of 100 ng/mL TRAIL was investigated by the annexin V binding assay of apoptosis. All the AML samples analyzed displayed a very low sensitivity to TRAIL-mediated apoptotis (Figure 2). In contrast, treatment of U937 cells with 100 ng/mL TRAIL induced a high level of apoptosis (*see below*), confirming the ability of the TRAIL preparation to induce cell killing in sensitive leukemic cells. In 6 additional AML patients we also evaluated the apoptotic effect of a high TRAIL dose (300 ng/mL). Even at this concentration TRAIL was unable to induce a significant rate of apoptosis of leukemic blasts (Figure 2).

In addition to the above studies carried out on total leukemic cells grown in liquid suspension we also investigated whether TRAIL could affect the growth of leukemic progenitors, i.e. of the leukemic cells that form colonies *in vitro* when grown in semisolid medium (AML-CFU). These studies, carried out in 6 cases of non-APL AML and in 1 case of APL showed that TRAIL at 100 or 300 ng/mL failed to inhibit AML-CFU significantly (Table 4).

Since it was previously reported that TRAIL may act as an inducer of differentiation on some leukemic cell lines,[10] we explored whether it could display a similar effect also on fresh AML blasts. However, the analysis of cell morphology as well as of membrane immunophenotype of fresh leukemic blasts grown for 4-7days in the presence of 50-100-300 ng/mL TRAIL failed to demonstrate any significant effect on cell maturation along the granulocytic or monocytic lineages (*data not shown*).

### TRAIL-R expression and TRAIL sensitivity during normal granulocytic and monocytic differentiation

Taking advantage of the development of unilineage cell culture methods allowing the selective and progressive *in vitro* differentiation of hematopoietic progenitor cells along the different cell lineages,[16-18] we explored TRAIL-R expression during normal granulocytic and

**Table 4.** Effect of TRAIL on AML-CFU growth.

| Patient | C | AML-CFU/$10^5$ cells TRAIL (100 ng/mL) | TRAIL (200 ng/mL) |
|---|---|---|---|
| 1 | 592 | 561 | 542 |
| 2 | 648 | 556 | 505 |
| 3 | 62 | 74 | 60 |
| 4 | 10 | 11 | 8 |
| 5 | 22 | 20 | 17 |
| 6 | 18 | 15 | 20 |
| 7 | 45 | 40 | 48 |

*AML-CFU, leukemic clusters (10 to 20 cells) plus colonies (> 20 cells) scored after 14 days in methylcellulose per $10^4$ cells plated. Patient #1 corresponds to patient #56 of Table 2, patients #2 to 5 to the patients 57 to 61 of Table 2, respectively, patient #6 to patient #6 of Table 3, and patient #7 to patient #64 of Table 2.*

**Table 5.** Effect of PML/RAR-α induction on the sensitivity of MTPR9 cells to chemotherapeutic agents.

| Treatment | C | TRAIL | HU | Etoposide | ARAC |
|---|---|---|---|---|---|
| C | 6 | 80 | 25 | 15 | 72 |
| $Zn^{2+}$ | 8 | 12 | 39 | 14 | 61 |

*MTPR9 cells were grown for 48 hours either in the absence ( C ) or in the presence of TRAIL (100 ng/mL) or hydroxyurea (HU,100 μM) or etoposide (5 μM ) or cytosine arabinoside (ARAC, 25 μM ) and then the percentage of apoptotic cells was dertmined by the annexin-V binding assay. Mean values observed in three separate experiments.*

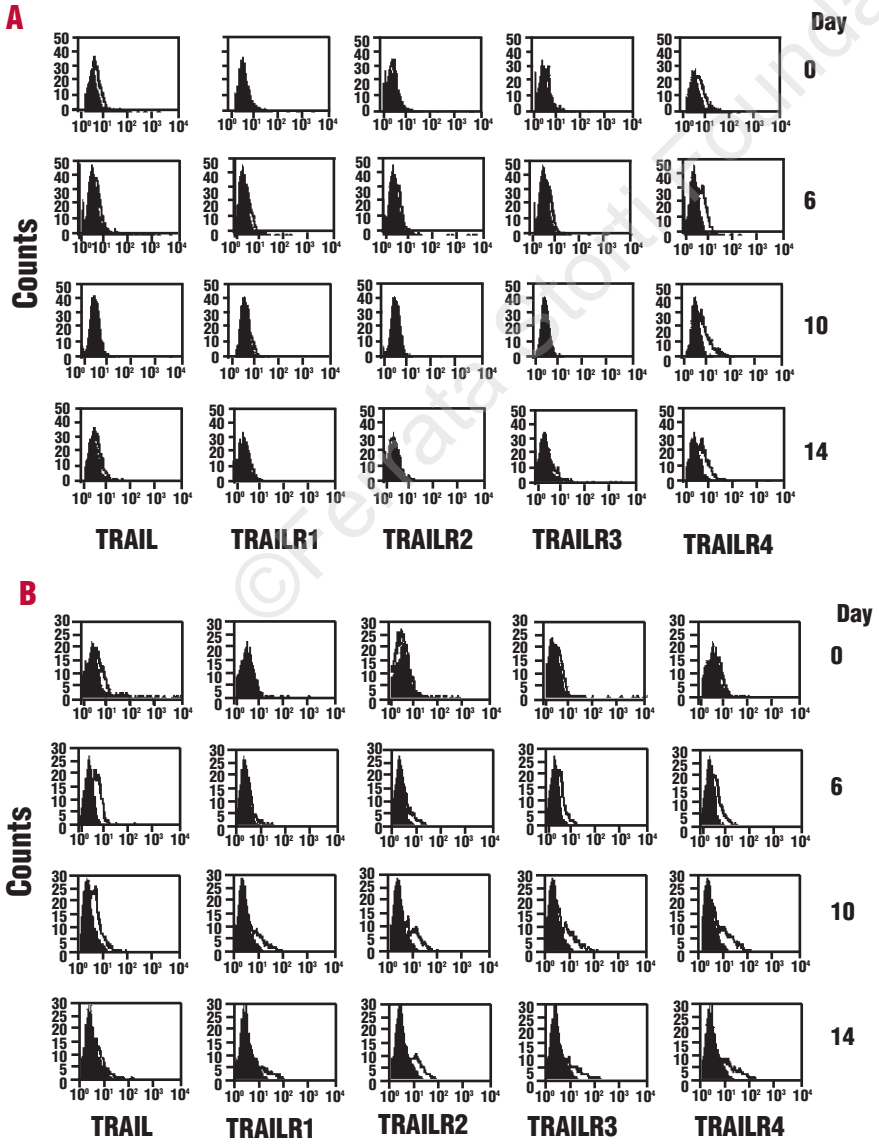



**Figure 3.** Flow cytometry analysis of TRAIL and TRAIL-R expression in purified hematopoietic progenitors induced to selective unilineage differentiation along the granulocytic (G, A) and the monocytic (Mo, B) lineages.

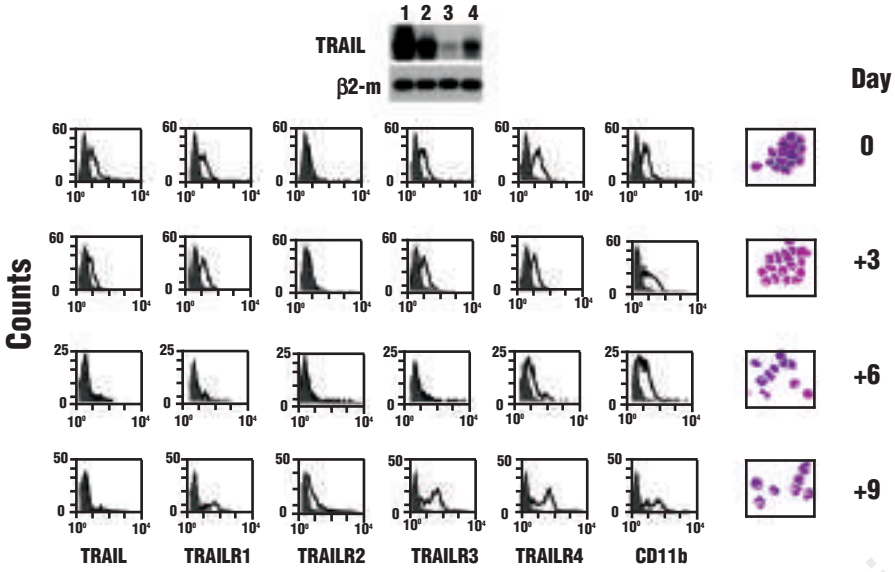



**Figure 4.** Flow cytometry analysis of TRAIL and TRAIL-R expression of PB cells derived from the blood of an APL patient undergoing treatment with ATRA+ idarubicin (from the top to the bottom: before therapy, day +3, day +6 and day +9). The morphology of PB cells at these days of treatment is shown in the right-hand panels.

monocytic differentiation. All four TRAIL-R were undetectable on quiescent CD34+ HPC and remained at very low levels during the first stages of differentiation of these cells along the granulocytic and monocytic pathways (Figure 3). During the entire granulocytic differentiation pathway, TRAIL-R1, TRAIL-R2 and TRAIL-R3 remained undetectable, while the expression of TRAIL-R4 was observed in immature as well as mature granulocytic elements (Figure 3A). Starting from day 7 onwards all four TRAIL-R were expressed in cells differentiating along the monocytic pathway (Figure 3B). Membrane TRAIL was detectable on monocytic, but not on granulocytic precursors (Figure 3A and Figure 3B).

### *TRAIL-R expression and TRAIL sensitivity in APL*

Following the observation that AML blasts are resistant to TRAIL-mediated apoptosis we explored the possible mechanisms involved in this phenomenon. For such analysis we selected the model of APL, characterized by the t(15;17) translocation and by the formation of the PML/RAR-α fusion protein. As reported above, APL blasts frequently express TRAIL-R3 and TRAIL-R4, but usually failed to express the functional cytotoxic TRAIL-R1 and TRAIL-R2. This pattern of TRAIL-R expression was not significantly modified by retinoic acid (Figure 4 and *data not shown*). Furthermore, APL cells usually expressed significant levels of membrane-bound TRAIL that were downmodulated after retinoic acid treatment (Figure 4 and *data not shown*). Figure 4 ports the analysis of TRAIL and TRAIL-R expression, at different days of treatment, in an APL patient during induction therapy with ATRA and idarubicin. It was clearly evident in this patient that the induction of granulocytic maturation of APL blasts was associated with a marked decrease of TRAIL expression and with a moderate increase of all four TRAIL-R concomitantly with the induction of terminal maturation (Figure 4). A similar observation was made in three additional APL patients (patients #6, 7 and 8 of Table 3) (*data not shown*).

In parallel, we evaluated the sensitivity of APL cells to TRAIL-mediated apoptosis. Basically, we observed that 100-300 ng/mL TRAIL elicited only very weak apoptotic activity on APL blasts. ATRA treatment elicited a small increase in the proportion of apoptotic cells, which was not further potentiated by the addition of TRAIL (*data not shown*).

We then explored a possible role of the fusion PML/RAR-α protein in this pattern of TRAIL/TRAIL-R expression. To this end we first carried out a set of experiments in U937 cells transduced with the PML/RAR-α gene under the control of the metallothionein gene promoter.[12] Using this cellular system we showed that the induction of the synthesis of the PML/RAR-α protein in these cells was associated with: (a) downmodulation of TRAIL-R1, while the expression of TRAIL-R3 and TRAIL-R4 remained unmodified (Figure 5A); TRAIL-R2 expression always remained at very low levels; (b) unmodified expression of membrane-bound TRAIL, which always remained expressed at very low levels (*data not shown*); (c) a marked decrease of the sensitivity to the apoptotic effects elicited by TRAIL (Figure 5B). It is important to note that the effect of PML/RAR-α on TRAIL-R1 seems to be specific in that the expression of Fas, as well as the sensitivity of the cells to Fas ligand, were not modified by the fusion protein (Figure 5A and *data not shown*). Furthermore, the effect of $Zn^{2+}$ used to stimulate PML/RAR-α expression seemed to be specifically related to the induction of the fusion protein, since the addi-

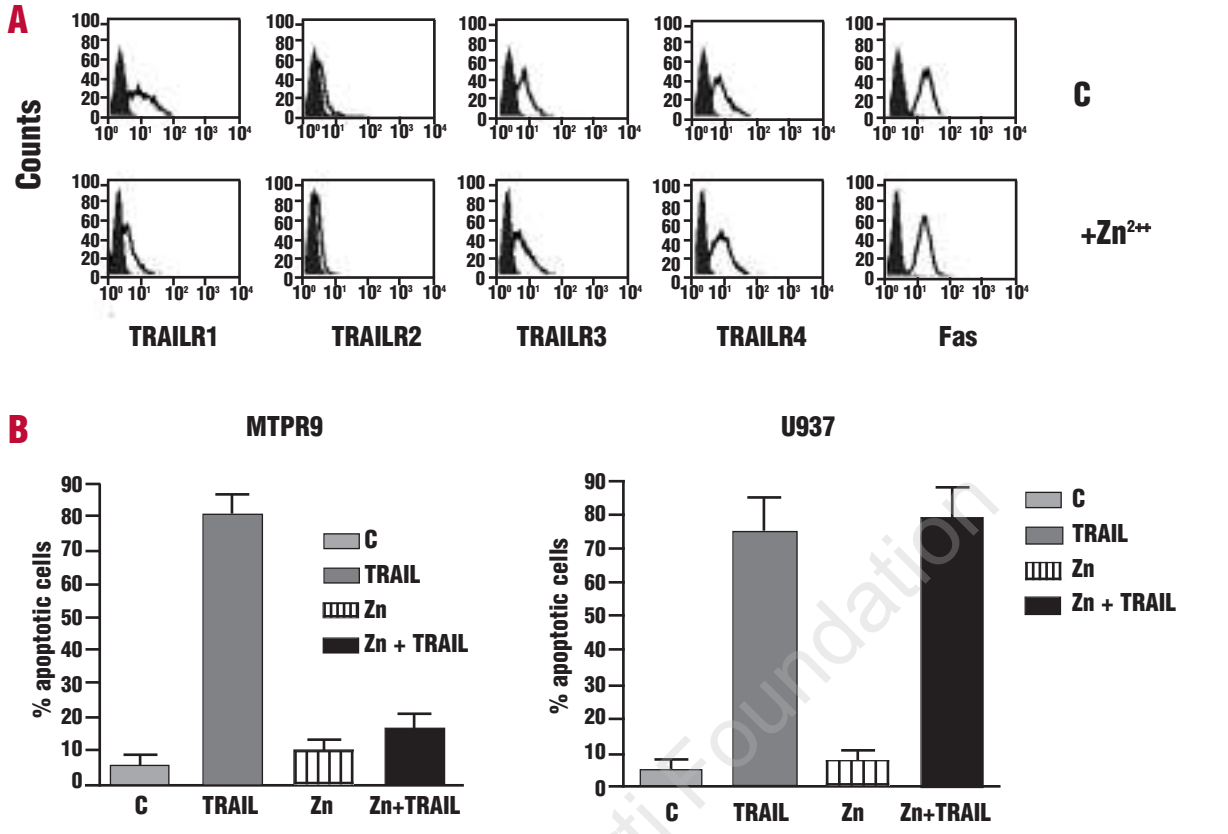



Figure 5. A. Flow cytometry analysis of TRAIL-R and Fas expression in MTPR9 cells grown for 48 hrs in the absence (upper row) or in the presence (lower row) of 100 μM ZnSO4. This pattern is representative of 4 separate experiments. B. Sensitivity of wt U937 and MTPR9 cells to TRAIL-mediated apoptosis. U937 and MTPR9 cells were grown for 24h either in the absence or in the presence of 100 μM $ZnSO_4$ and then analyzed for their sensitivity to TRAIL-mediated apoptosis. The proportion of apoptotic cells was determined by the annexin V binding assay (mean value±SD observed in 3 separate experiments).



tion of this metal to wtU937 cells did not modify their sensitivity to TRAIL-mediated apoptosis (Figure 5B). Finally, we noted that MTPR9 cells treated with $Zn^{2+}$ were sensitive, like the control untreated cells, to apoptsis induced by chemiotherapeutic agents such as doxorubicin, hydroxyurea or etoposide, thus suggesting that the anti-apoptotic effect of PML/RAR-α is specific to the TRAIL pathway (*data not shown*).

The protective effect of PML/RAR-α on TRAIL-mediated apoptosis seems to be related to the downmodulation of TRAIL-R1 on U937 cells. We explored the activation of caspase-8, an upstream acting caspase, following the addition of TRAIL to MTPR9 cells grown for 24 hrs either in the absence or in the presence of $Zn^{2+}$. At early time points after TRAIL addition, caspase-8 was clearly activated in control MTPR9 cells, but not in MTPR9 cells in which PML/RARα expression had been activated by the addition of $Zn^{2+}$ (Figure 6). Similarly, caspase-3 was activated by TRAIL in MTPR9 cells grown without $Zn^{2+}$, but not in cells grown in the presence of this metal (Figure 6).

In a second set of experiments, we explored the sensitivity of NB4 cells, a leukemic cell line derived from an APL patient, to TRAIL before and after ATRA treatment. NB4 cells before and at different times after ATRA treatment (from day 1 to day 4) were resistant to TRAIL-mediated apoptosis when we used this cytokine at 100 ng/mL (Figure 7). Using higher TRAIL concentrations (i.e., 300 ng/mL) a slight increase in the rate of apoptosis was observed compared to in the respective controls (Figure 7). This resistance was observed in two different experimental conditions involving the addition of TRAIL either in simultaneous combination with ATRA or given subsequently after four days of treatment with ATRA. In parallel, the study of TRAIL-R provided evidence that both in control and ATRA-treated NB4 cells TRAIL-R1 expression was virtually undetectable, TRAIL-R2 was expressed at low levels, and TRAIL-R3 and TRAIL-R4 were expressed at significant levels (Figure 8). This pattern of TRAIL-R expression is similar to that observed in normal neutrophils and is consistent with the lack of sensitivity of NB4 cells to TRAIL-mediated cytotoxicity. In a last set of experiments we evaluated TRAIL/TRAIL-R

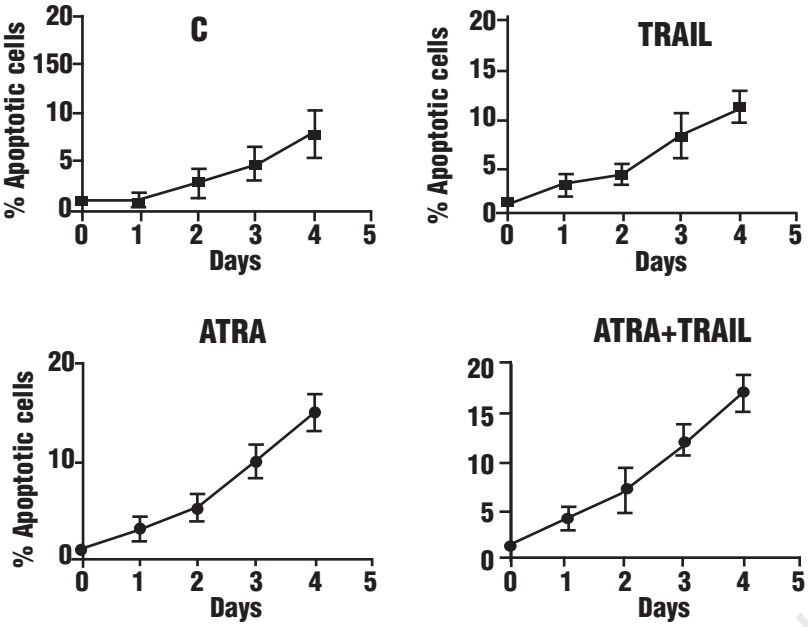



Figure 7. Sensitivity of NB4 cells to TRAIL-mediated cytotoxicity. NB4 cells were grown in four different cell culture conditions: without any addition (control); ATRA; TRAIL (100 or 300 ng/mL); ATRA+TRAIL. At each day of culture, one cellular aliquot was removed and the proportion of apoptotic cells was determined using the annexin V binding assay.

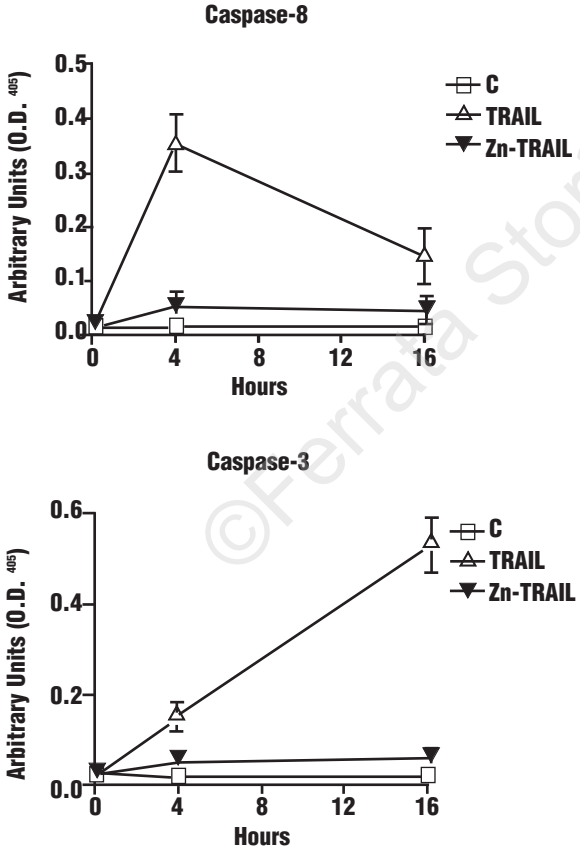



Figure 6. Kinetics of caspase-8 and caspase-3 activation following addition of TRAIL to MTPR9 cells. MTPR9 cells were grown in the absence C) or in the presence of $ZnSO_4$ (Zn) for 48 h. After this time the cells were incubated in the presence of 100 ng/mL TRAIL and at different time points (0, 4 and 16 h) cell aliquots were removed, lysed and analyzed for caspase-8 and caspase-3 activity, as reported in the *Materials and Methods*.

expression in the non-APL myeloid cell line HL60. Interestingly, HL60 cells distinctly express all four TRAIL-R, as well as membrane TRAIL (Figure 9). Following monocytic induction by 1α25OH-VitD3, TRAIL and TRAIL-R expression remained virtually unmodified (Figure 9). After granulocytic induction by ATRA there was a clear decline of TRAIL and TRAIL-R2 expression, while the other TRAIL-R remained virtually unmodified, being similar to that in controls (Figure 9). Interestingly, the addition of exogenous TRAIL (100 ng/mL) induced the selective downmodulation of TRAIL-R2 (Figure 9). In parallel we evaluated the sensitivity of HL60 cells to TRAIL-mediated apoptosis. Uninduced HL60 cells were moderately sensitive to the apoptotic effects induced by TRAIL (at day 1 of incubation 27±3% of HL60 cells incubated with TRAIL were apoptotic compared to 2±0.5% of apoptotic cells in control cultures). HL60 cells induced to monocytic differentiation displayed a slightly decreased sensitivity to TRAIL-mediated apoptosis (i.e., 17±3.8% cells were apoptotic). Finally, HL60 cells induced to granulocytic differentiation by 4 days of treatment with ATRA displayed a significant proportion of apoptotic cells (17±3.8), slightly increased by TRAIL addition (27±4.9).

## Discussion

The results of this study indicate that malignant cells isolated from the large majority of AML patients are resistant to apoptosis induction by TRAIL. This phenomenon was accompanied by and possibly related to

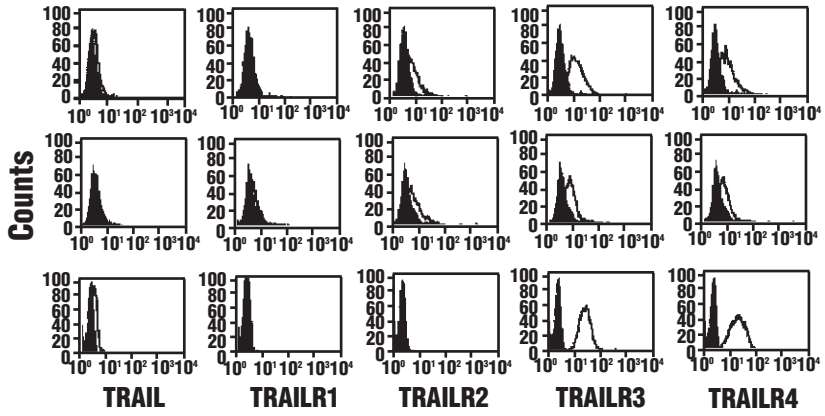



Figure 8. Flow cytometric analysis of TRAIL and TRAIL-R expression in NB4 cells grown in standard conditions (upper row) or in the presence of ATRA (1 μM) for four days (middle row). For comparison TRAIL and TRAIL-R expression on normal neutrophils is shown in the bottom row.

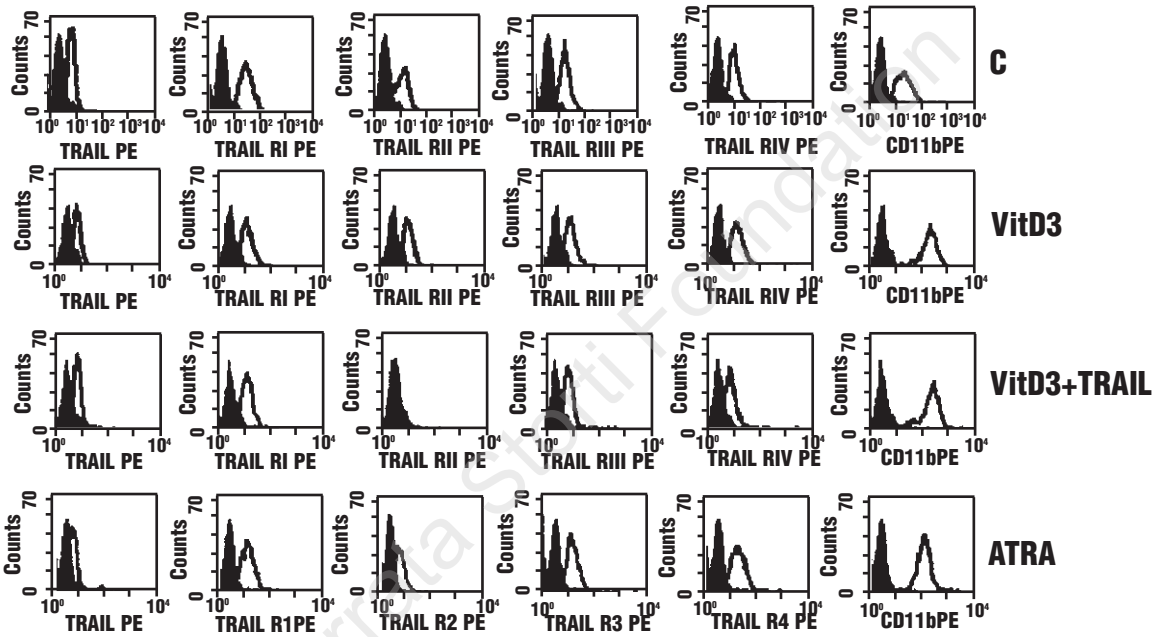



Figure 9. Flow cytometric analysis of TRAIL, TRAIL-R and CD11b expression in HL60 cells grown grown under standard conditions (C) or in the presence of either 1·25OH-VitD3 (VitD3) or 1·25OHVitD3+TRAIL (VitD3+TRAIL) or ATRA (ATRA).



the frequent expression of TRAIL decoy receptors (TRAIL-R3 and TRAIL-R4). Studies in APL provided evidence that the PML/RAR-α fusion protein downmodulates TRAIL-R1 expression. Based on these findings we suggest that AML cells are resistant to TRAIL due to the expression of TRAIL decoy receptors and to the downmodulation of TRAIL-R1.

The expression of TRAIL decoy receptors is involved in the mechanisms of resistance of several tumor cell types to TRAIL, including osteosarcoma,[23] breast cancer,[24] myeloma[25] and prostate cancer.[26] We suggest that AML could represent an additional neoplastic condition in which TRAIL resistance is mediated by the expression of TRAIL decoy receptors. It is also of interest that TRAIL-R1 was expressed only in AML exhibiting monocytic markers. This finding is related to the expression of TRAIL-R1 in normal monocytes.[21] However, in leukemic monocytic cells, unlike in their normal counterpart, TRAIL-R3 and TRAIL-R4 expression predominates over TRAIL-R1 expression. The predominant expression of the decoy receptors TRAIL-R3 and TRAIL-R4 is a feature observed also in normal cells of the granulocytic lineage[27] (*and results of the present study*), as well as in HL60 cells induced to granulocytic maturation.[28] In these latter cells the resistance to TRAIL-mediated apoptosis was related to the high expression of TRAIL decoy receptors[28] and to the downmodulation of TRAIL-R2 (*present study*). Previous studies have already reported the resistance of AML blasts to TRAIL-mediated apoptosis, but have failed to

provide any explanation for this phenomenon.[10,22] In these studies TRAIL-R expression was explored only at the mRNA level and no relationship was observed between the pattern of TRAIL-R expression and TRAIL sensitivity.[22] In the present study we explored TRAIL-R expression at the protein level by flow cytometry and found that the expression of decoy receptors is very frequent in AML cells and may explain the resistance of these cells to TRAIL-mediated apoptosis. This interpretation is directly supported by the observation that TRAIL failed to induce caspase-8, an upstream acting caspase, in AML blasts. However, we cannot exclude that other mechanisms could contribute to the resistance of leukemic cells to TRAIL (i.e., high expression of the apoptosis inhibitors cFLIP and/or XIAP).[4] Resistance to TRAIL-mediated cytotoxicity has also been described in other leukemic subsets. B-cell chronic lymphocytic leukemia (B-CLL) cells are resistant to TRAIL, due in part to a low expression of TRAIL-R1 and TRAIL-R2 and also to a high expression of the caspase-8 inhibitor c-FLIP;[29] chemotherapeutic agents active on B-CLL, such as chlorambucil and fludarabine, induce a marked upmodulation of TRAIL-R1 and TRAIL-R2, and through this mechanism they induce apoptosis of B-CLL cells.[30] As to chronic myeloid leukemia, the BCR/ABL fusion protein was shown to inhibit TRAIL transcription thereby promoting leukemia cell survival.[31] In addition to chemotherapeutic agents, retinoids in breast cancer cells[32] and histone deacetylase inhibitors in AML[33] are also able to induce TRAIL expression. Finally, adult T-cell leukemias[34] and lymphomas[35] are resistant to TRAIL-mediated apoptosis. Interestingly, these cells, like APL cells, constitutively express TRAIL on their surface.[34,35]

A previous study provided evidence that APL blasts are sensitive to TRAIL-mediated apoptosis and that TRAIL-induced expression most likely caused blast apoptosis.[13] We investigate this topic in detail both in fresh APL blasts and in experimental models (i.e., PML/RAR-α expressing cell lines). Studies in U937 cells engineered to express PML/RAR-α provided clear evidence that induction of the expression of the fusion protein was associated with TRAIL-R1 downmodulation and induction of resistance to TRAIL-mediated apoptosis. Studies in the promyelocytic NB4 cell line showed resistance to TRAIL-mediated apoptosis in both untreated and ATRA-induced cells, a phenomenon seemingly related to the scarce expression of TRAIL-R1 and TRAIL-R2 in these cells, associated with the expression of both TRAIL decoy receptors.

On the other hand, studies on fresh APL blasts showed that these cells usually fail to express TRAIL-R1 and TRAIL-R2, while they express both TRAIL decoy receptors and are resistant to TRAIL-mediated apoptosis before, during and after ATRA treatment. Finally, APL blasts express membrane-bound TRAIL, which is markedly downmodulated after ATRA induction.

The cause of discrepancy between our observations and those previously reported by Altucci *et al*. are unclear to us.[13] In this context, it is important to note that Altucci *et al*. reported TRAIL-mediated apoptosis only at very high doses of TRAIL (i.e., 200-500 ng/mL) and that they evaluated TRAIL and TRAIL-R expression in APL cells only at the mRNA level. However, even using 300 ng/mL TRAIL we failed to induce apoptosis in APL blasts.

The expression of TRAIL by APL blasts deserves some comments. First, unlike other reports,[13,36] our findings do not support the hypothesis that TRAIL is related to the ATRA-induced apoptosis of APL cells. In fact, we constantly observed that ATRA markedly downmodulates the expression of membrane-bound TRAIL on APL cells. Similarly, ATRA downmodulates TRAIL expression in HL60 cells. We propose that TRAIL could confer a sort of immunologic privilege to APL cells, as reported for other tumor cells expressing membrane-bound TRAIL.[37,38] This hypothesis is further supported by the observation that APL cells constantly express FasL on their membrane. However, the induction of TRAIL and TRAIL-R1 and –R2 expression by chemotherapy and other anticancer agents, concomitantly with a decreased expression of anti-apoptotic proteins such as c-FLIP and XIAP, may render leukemic cells sensitive to TRAIL-mediated cytotoxicity. We previously reported that the PML/RAR-α fusion protein was capable of downmodulating TNF-RI expression and through this mechanism of inducing resistance of APL blasts to TNF-α-mediated apoptosis.[39] On the other hand, other studies have shown that PML/RAR-α protects hematopoietic cells from apoptosis after growth factor deprivation.[14,40] All these studies suggest that the PML/RARα fusion protein induces a resistance to apoptosis elicited by the death receptors TNF-R and TRAIL-R and by growth factor deprivation: these mechanisms contribute to confering leukemic blasts a growth advantage over the normal hematopoietic counterpart.[41]

*All authors (RR, LP. GM, ES, AR, EP, AV, AC, MC, RF, FLC, CP and UT) gave substantial contributions to the conception and design of the study, analysis and interpretation of data, drafting and revising the article, and gave the final approval of the present version of the manuscript. Particularly, RR, LP, GM, ES, AR, DD, EP and UT performed most of the laboratory analyses. UT, RR, CP, FLC and RF supervised the study. DD, MC and AV performed the analysis of all the clinical and biological patients data. The authors declare that they have no potential conflict of interest.*

*Manuscript received October 27, 2004. Accepted March 24, 2005.*

## References


1. Gaur U, Aggarwal BB. Regulation of proliferation, survival amd apoptosis by members of the TNF superfamily. Biochem Pharmacol 2003;66:1403-8.
2. Wang S, El-Deiry WS. TRAIL and apoptosis by TNF-family death receptors. Oncogene 2003;22:8628-33.
3. Askenazi A, Dixit V. Death receptors: signaling and modulation. Science 1999; 281:1305-8.
4. Zhang L, Faug B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Therapy 2005, in press.
5. Kelley SK, Ashkenazi A. Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol 2004;4:333-9.
6. Younes A, Kadin ME. Emerging applications of the tumor necrosis factor family of ligands and receptors in cancer therapy. J Clin Invest 2003;21:3526-34.
7. Mitsiades CS, Treon SP, Mitsiades N, Shima Y, Richardson P, Schlossman R, et al. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. Blood 2001;98:795-804.
8. Chen Q, Gong B, Mahmoud-Ahmed S, Zhou A, Hsi ED, Hussein M, et al. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. Blood 2001; 98:2183-92.
9. Uno K, Inukai T, Kayagaki N, Goi K, Sato H, Nemoto A, et al. TNF-related apoptosis-inducing ligand (TRAIL) frequently induces apoptosis-inducing ligand (TRAIL) frequently induces apoptosis in Philadelphia chromosome positive leukemia cells. Blood 2003;101:3658-67.
10. Jones DT, Ganeshaguru K, Mitchell WA, Foroni L, Baker RT, Prentice HG, et al. Cytotoxic drugs enhance the ex vivo sensitivity of malignant cells from a subset of acute myeloid leukemia patients to apoptosis induction by tumour necrosis factor receptor-related apoptosis-inducing ligand. Br J Haematol 2003;121:713-20.
11. Wen J, Ramadevi N, Nguyen D, Perkins C, Worthington E, Bhalia K. Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of acute human leukemia cells. Blood 2000;96:3900-6.
12. Secchiero P, Gonelli A, Mirandola P, Melloni E, Zamai L, Celeghini C, et al. Tumor necrosis factor-related apoptosis-inducing ligand induces monocytic maturation of leukemic and normal myeloid precursors through a caspase-dependent pathway. Blood 2002;100: 2421-9.
13. Altucci L, Rossin A, Raffelsberger W, Retmair A, Chomienne C, Gronmeyer H. Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. Nature Medicine 2001;7:680-6.
14. Grignani F, Ferrucci PF, Testa U, Talamo M, Fagioli M, Alcalay M, et al. The acute promyelocytic leukemia-specific PML/RARα fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 1993;74: 423-31.
15. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 1991;77:1080-6.
16. Gabbianelli M, Sargiacomo M, Pelosi E, Testa U, Isacchi G, Peschle C. "Pure" human hematopoietic progenitors: permissive action of basic fibroblast growth factor. Science 1990;249:1561-4.
17. Labbaye C, Valtieri M, Barberi T, Meccia E, Masella B, Pelosi E, et al. Differential expression and functional role of GATA-2, NF-E2 and GATA-1 in normal adult hemopoiesis. J Clin Invest 1995;95:2346-58.
18. Gabbianelli M, Pelosi E, Montesoro E, Valtieri M, Luchetti L, Samoggia P, et al. Multi-level effects of flt3 ligand on human hematopoiesis: expansion of putative stem cells and proliferation of granulomonocytic progenitors/monocytic precursors. Blood 1995;86:1661-70.
19. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 1991;139:271-9.
20. Guar Y, Gerhard B, Hogge DE. Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML). Blood 2003; 101:3142-9.
21. Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR and Fanger NA. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J Exp Med 1999;189:1343-53.
22. Wechter C, Kroppmann D, Cai Z, Ruppert V, Schedereit C, Dorkem C, et al. In vitro susceptibility to TRAIL-induced apoptosis of acute leukemia cells in the context of TRAIL receptors gene express high and constitutive NF-κ B activity. Leukemia 2001;15:921-8.
23. Bouralexis S, Findlay DM, Atkins GJ, Labrinidis A, Hay S, Evdokiou A. Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. Br J Cancer 2003;89:206-14.
24. De Almodovar CR, Ruiz-Ruiz C, Rodriguez A, Ortiz-Ferron G, Reo JM, Lopez-Rivas A. Tumor necrosis factor-related apoptsis-inducing ligand (TRAIL) decoy receptor TRAIL-R3 is up-regulated by p53 in breast tumor cells through a mechanism involving an intron-p53-binding site. J Biol Chem 2004;279:4093-101.
25. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res 2003; 63:912-6.
26. Holen I, Croucher PI, Mamdy FC, Eaton CL. Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells. Cancer Res 2002;62:1619-23.
27. Renshaw SA, Parmar JS, Singleton V, Rowe SJ, Dockrell DH, Dower SK, et al. Acceleration of human neutrophil apoptosis by TRAIL. J Immunol 2003; 170:1027-33.
28. Shiki K, Yoshikawa H, Kinoshita H, Takeda M, Ueano A, Nakajima Y, et al. Potential mechanisms of resistance to TRAIL/ApoL-induced apoptosis in human promyelocytic leukemia HL-60 cells during granulocytic differentiation. Cell Death Diff 2000; 7:936-46.
29. MacFarlane M, Harper N, Snowden RT, Dyer MJS, Barnett GA, Pringle JH, et al. Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. Oncogene 2002;21:6809-18.
30. Johnston JB, Kabore AF, Strutinsky J, Hu X, Paul JT, Kropp DM, et al. Role of the TRAIL/APO2-L death receptors in chlorambucil- and fludarabine-induced apoptosis in chronic lymphocytic leukemia. Oncogene 2003; 22:8356-69.
31. Ghaffari S, Jagani Z, Kitidis C, Lodish HF, Khosravi-Far R. Cytokines and BCR-ABL mediate suppression of TRAIL-induced apoptosis through inhibition of forkhead FOXO3a transcription factor. Proc Natl Acad Sci USA 2003;100:6523-8.
32. Clarke N, Jemenez-lora AM, Voltz E, Gronmeyer H. Tumor suppressor IRF-1 mediates retinoid and interferon anticancer signaling to death ligand TRAIL. EMBO J 2004;23:3051-60.
33. Insinga A, Monestirali S, Ronzoni S, Gelmetti V, Marchesi F, Altucci L, et al. Inhibitors of histone deacetylases induce apoptosis selectively in leukemic cells through activation of the death receptor pathway. Nat Med 2004; 11:71-6.
34. Matsuda T, Almasan A, Tomita M, Uchihara J, Masuda M, Ohshiro K, et al. Resistance to Apo2 ligand (Apo2L)/ tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis and constitutive expression of Apo2L/TRAIL in human T-cell leukemia virus type 1-infected T-cell lines. J Virol 2005;79:1367-8.
35. Hasegawa H, Yamada Y, Harasawa H, Tsuji T, Murata K, Sugahara K, et al. Sensitivity of adult T-cell leukemia lymphoma cells to tumor necrosis factor-related apoptosis-inducing ligand. Br J Haematol 2004;128:253-5.
36. Jiménez-Lara AM, Clarke N, Altucci L, Gronemeyer H. Retinoic-acid-induced apoptosis in leukemia cells. Trend Mol Med 2004;10:508-15.
37. Giovarelli M, Musiani G, Garotta R, Ebner R, Di Carlo E, Kim Y, et al. A "stealth effect": adenocarcinoma cells engineered to express TRAIL elude tumor-specific and allogeneic T cell reactions. J Immunol 1999;163:4886-92.
38. Martinez-Lorenzo MJ, Anel A, Alava MA, Pineiro A, Naval J, Lasierra P, et al. The human melanoma cell line MelJuSo secretes bioactive FasL and APO2L/TRAIL on the surface of microvesicles. Possible contribution to tumor counterattack. Exp Cell Res 2004; 295: 315-29.
39. Grignani F, Valtieri M, Gabbianelli M, Gelmetti V, Botta R, Luchetti L, et al. PML/RARα fusion protein expression in normal human hematopoietic progenitors dictates myeloid commitment and the promyelocytic phenotype. Blood 2000;96:1531-7.
40. Testa U, Grignani F, Samoggia P, Zanetti C, Riccioni R, Lo Coco F, et al. The PML/RARα fusion protein inhibits tumor necrosis factor-α-induced apoptosis in U937 cells and acute promyelocytic leukemia blasts. J Clin Invest 1998; 101:2278-89.
41. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukemia. Nature Rev Cancer 2002; 2: 502-13.