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Regulation of the resistance to TRAIL-induced apoptosis in human primary T lymphocytes: Role of NF-κB inhibition

Jorge Carlos Morales, María José Ruiz-Magaña, Carmen Ruiz-Ruiz *

Departamento de Bioquímica y Biología Molecular 3 e Inmunología, Facultad de Medicina, Universidad de Granada, Avda. de Madrid 11, 18012 Granada, Spain

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Abstract

Several combined strategies have been recently proposed to overcome the resistance to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) showed by some tumor cells, thus improving the use of this death ligand in antitumor therapy. However, the molecular mechanisms of the tumor selective activity of TRAIL are not completely understood and hence the effects of the combined therapy on normal cells are unknown. Here, we have studied the resistance of primary T lymphocytes to TRAIL-mediated apoptosis. No significant differences were found in the expression of proteins involved in TRAIL-mediated apoptosis between resting and activated T cells. The low expression of death receptors TRAIL-R1/-R2 as well as the high levels of the antiapoptotic proteins TRAIL-R4 and cellular Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) may explain the lack of caspase-8 activation observed upon TRAIL treatment in both cell types. We have also analyzed the effect of different sensitizing agents such as genotoxic drugs, phosphatidylinositol-3 kinase (PI3K) inhibitors, proteasome inhibitors, microtubule depolymerizing agents, histone deacetilase inhibitors (HDACi), and NF-κB inhibitors. Although some of them induced T cell death, only NF-κB inhibitors sensitized activated T cells to TRAIL-induced apoptosis, maybe through the regulation of the antiapoptotic proteins TRAIL-R4, c-FLIP$_S$ and members of the inhibitors of apoptosis proteins (IAP) family. These results question the safety of the combined treatments with TRAIL and NF-κB inhibitors against tumors.

Keywords: Apoptosis; TRAIL; T lymphocytes; Antitumor therapy

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL/APO-2L) and CD95 ligand (CD95L or FasL/APO-1L) are type II transmembrane proteins that belong to the tumour necrosis factor (TNF) superfamily. They are potent inducers of apoptosis upon binding to their respective death domain-containing receptors, also known as death receptors (Suda et al., 1993; Wiley et al., 1995). Cytoplasmic death domains serve to recruit intracellular adapter molecules such as Fas-associated death domain protein (FADD) that in turn engage procaspase-8, thereby forming the death-inducing signalling complex (DISC). Caspase-8 is activated in the DISC allowing the initiation of a cascade of events that leads to apoptotic cell death (Chinnaiyan et al., 1995; Sprick et al., 2000). Such direct connection to the cell's death machinery suggests a therapeutic potential for CD95L and TRAIL in cancer treatment. Concerning CD95L, although it can efficiently induce apoptosis in a variety of tumour cells, its administration may not be a useful strategy as it is associated with severe liver toxicity (Ogasawara et al., 1993). In contrast, TRAIL has no systemic toxicity in preclinical studies with mice and non-human primates when administered at doses that inhibit the growth of breast and colon cancer xenografts (Walczak et al., 1999).

TRAIL can bind to four specific type I membrane receptors. TRAIL-R1/DR4 and TRAIL-R2/DR5 are death receptors, as they contain the intracellular death domain essential for transmitting the apoptotic signals (MacFarlane et al., 1997; Pan et al., 1997b; Walczak et al., 1997). TRAIL-R3/DcR1 and TRAIL-R4/DcR2 are known as antiapoptotic or decoy receptors because they do not contain an intact death domain. They act as non-functional binding partners for TRAIL, thereby attenuating its apoptotic activity (Degli-Esposti et al., 1997; MacFarlane et al., 1997; Pan et al., 1997a). TRAIL receptors appear to be ubiquitously expressed with transcripts detected in most human

* Corresponding author. Tel.: +34 958 24 35 22; fax: +34 958 24 90 15.
E-mail address: mcarmenr@ugr.es (C. Ruiz-Ruiz).

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tissues as well as in tumor cell lines of different lineages. Moreover, unlike CD95L, TRAIL is expressed constitutively and it is widely distributed in normal organs and tissues (Wiley et al., 1995), which is in relation with its lack of toxicity to normal cells. Even though a recombinant form of human TRAIL has been shown to cause apoptosis in human hepatocytes (Jo et al., 2000), more recent data have demonstrated that different recombinant versions of TRAIL vary considerably in toxicity towards normal human cells, but all of them maintain their antitumor properties (Lawrence et al., 2001).

To date, the mechanisms of TRAIL resistance in normal cells and some tumor cells are not completely understood. As regulation of TRAIL-mediated apoptosis is exerted at several stages along the signalling pathway, resistance can occur by different means. Initially, the degree of TRAIL sensitivity or resistance was proposed to be dependent on the levels of expression, localization and function of death and decoy receptors (Sheridan et al., 1997; Zhang et al., 2000). However, a correlation between cell survival and the expression of decoy or death receptors has not been conclusively demonstrated. On the other hand, several intracellular molecules can block the apoptotic effect of TRAIL. The cellular Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) modulates the apoptotic pathway right at the beginning because it competes with caspase-8 for binding to FADD (MacFarlane et al., 2002; Siegmund et al., 2002). Bcl-2 and Bcl-X_L impede the activation of the mitochondrial pathway thereby blocking the release of cytochrome *c* from mitochondria to cytosol (Ruiz de Almodovar et al., 2001). The inhibitors of apoptotic proteins XIAP, c-IAP1 and c-IAP2 act by inhibiting active caspases (Cummins et al., 2004; Li et al., 2004). The antiapoptotic signals induced by NF-κB (Ravi et al., 2001), protein kinase B (PKB)/Akt (Whang et al., 2004), protein kinase C (PKC) or mitogen-activated protein kinase (MAPK) (Ortiz-Ferron et al., 2006; Sarker et al., 2001) have also been involved in the resistance to TRAIL-induced apoptosis of different types of cells.

Despite the controversial results about the physiological role of TRAIL in vivo, several studies point to a role for TRAIL in the suppression of autoimmune diseases and the immune surveillance of developing and metastatic tumors (Cretney et al., 2006). In this respect, it has been reported that TRAIL is implicated in the antitumor cytotoxicity of dendritic cells, monocytes, NK cells and even B cells (Fanger et al., 1999; Griffith et al., 1999; Kemp et al., 2004; Takeda et al., 2001). Moreover, TRAIL is induced on the surface of human T lymphocytes upon activation through the T cell antigen receptor (TCR) or treatment with type I interferons, mediating an important part of the cytotoxic activity of T cells (Dorothee et al., 2002; Kayagaki et al., 1999). The resistance of immune cells to TRAIL-induced apoptosis is essential for them to act as mediators of antitumoral response. In this work, we have investigated the mechanisms of TRAIL resistance in resting and activated primary human T lymphocytes. We have analyzed the expression of several proteins involved in the TRAIL signalling pathway, such as TRAIL receptors and components of the DISC, in both cell types. Furthermore, we have studied their susceptibility to TRAIL upon combination with agents that regulate TRAIL sensitivity in tumor cells. Our results show that NF-κB inhibitors are able to sensitize primary activated T lymphocytes to TRAIL-mediated apoptosis and regulate the expression of several antiapoptotic proteins in these cells.

2. Materials and methods

2.1. Reagents and antibodies

Human recombinant TRAIL was prepared as described previously (MacFarlane et al., 1997). Phytohemagglutinin, doxorubicin, LY294002, nocodazole, valproic acid, BAY 11-7085, sulfasalazine and mouse anti-α-tubulin mAb were from Sigma–Aldrich (St. Louis, MO). Velcade (bortezomib) was from Janssen-Cilag SA (Madrid, Spain). Z-VAD-FMK was provided by Bachem (Bubendorf, Switzerland). Parthenolide, CAPE, anti-cFLIP monoclonal antibody NF6 and mouse anti-human TRAIL receptor antibodies for flow cytometry studies were purchased from Alexis Biochemicals (San Diego, CA). Mouse anti-human CD28 and polyclonal anti-human NF-κB p50 antibodies were from eBioscience (San Diego, CA). Anti-human caspase-8 monoclonal antibody was purchased from Cell Diagnostica (Münster, Germany). Mouse anti-FADD was obtained from Transduction Laboratories (Lexington, KY). Monoclonal antibodies against poly(ADP-ribose) polymerase (PARP), XIAP and c-IAP2 were obtained from BD Biosciences (San Jose, CA). Mouse anti-p65 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal antibody anti-Bcl-2 was from Dako (Glostrup, Denmark).

2.2. Cells and cell culture

Blood samples were obtained from healthy donors by informed consent and collected into citrate tubes. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll–Histopaque density gradient centrifugation (Sigma–Aldrich) and adherent monocytes were depleted by culture on plastic dishes for 1 h at 37 °C. Peripheral blood T lymphocytes were then isolated by negative selection with an indirect magnetic labelling system consisting of a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123, and CD235a (Human Pan T Cells Isolation Kit II, Miltenyi Biotec, GmbH). Purity of T cells was >95% $CD3^+$ as determined by flow cytometry. Purified resting T lymphocytes were resuspended in RPMI 1640 medium (BioWhittaker Inc.) containing 10% fetal bovine serum, 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated as indicated at 37 °C in a humidified 5% CO_2, 95% air incubator. For activation, resting T cells were cultured at 2×10^6 cells/ml with 5 μg/ml PHA and 1 μg/ml anti-CD28 for 20 h. After washing, cells were incubated in complete medium supplemented with 25 U/ml IL-2 for an additional 5 days.

The human cell lines Jurkat, CEM and SKBR3 were all maintained in culture in RPMI 1640 medium with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin at 37 °C in a humidified 5% CO_2, 95% air incubator.

2.3. Determination of apoptotic cells

Hypodiploid apoptotic cells were detected by flow cytometry according to published procedures (Gong et al., 1994). Briefly, cells were washed with phosphate-buffered saline (PBS), fixed in cold 70% ethanol, and then stained with propidium iodide while treating with RNase. Quantitative analysis of sub-G_1 cells was carried out in a FACScan cytometer using the Cell Quest software (BD Biosciences).

2.4. Cytofluorometric analysis of TRAIL receptors

To detect TRAIL receptors at the cell surface, control or treated cells were incubated with primary antibodies (5 μg/ml) at 4 °C for 30 min. After washing with PBS to remove unbound primary antibody, cells were incubated with goat anti-mouse fluorescein isothiocyanate-conjugated antibody (Caltag Laboratories) for 30 min at 4 °C. Cells were then washed again, resuspended in PBS and analyzed in a FACScan flow cytometer.

2.5. Immunoblot detection of proteins

For detection of cytosolic proteins, cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–Cl, 1% NP-40) for 30 min. Proteins of cytosolic supernatants were resolved on 10% SDS-PAGE gels and detected as reported previously (Ruiz-Ruiz and Lopez-Rivas, 1999).

For nuclear proteins extractions, cells were lysed with 300 μl of hypotonic buffer (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Na_2MoO_4, and protease inhibitors) containing 0.6% of Nonidet P-40. Nuclei were then centrifuged and incubated in 50 μl of high salt-containing buffer (20 mM HEPES, pH 7.6, 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 mM Na_2MoO_4, and protease inhibitors) for 30 min on a rocking platform at 4 °C. Nuclei were centrifuged at 13,000 × *g* for 10 min, to get the supernatants containing the nuclear extracts. Proteins were then separated through 10% SDS-PAGE gel and detected as described (Ruiz-Ruiz and Lopez-Rivas, 1999).

2.6. Reverse transcription (RT)-PCR

Total RNA was extracted from T lymphocytes with Trizol Reagent (Invitrogen) as recommended by the supplier. cDNAs were synthesized from 3 μg of total RNA by using M-MLV reverse transcriptase (Invitrogen) and oligo(dT) primer in a total volume of 20 μl. Reverse transcription was performed at 37° for 50 min followed by 15 min at 70° for inactivation. PCR reactions were performed with 0, 1–2 μl cDNA (depending on the gene) in a 50-μl volume and using the following primer pairs (fragments size indicated in brackets): TRAIL-R1 (506 bp), forward 5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′ and reverse 5′-TCCAAGGACACGGCAGAGCCTGTGCCAT-3′; TRAIL-R2 (502 bp), forward 5′-GCCTCATGGACAATGAGATAAAGGTGGCT-3′ and reverse 5′-CCAAATCTCAAAGTACGCACAAACGG-3′; TRAIL-R3 (612 bp), forward 5′-GAAGAATTTGGTGCCAATGCCACTG-3′ and reverse 5′-CTCTTGGACTTGGCTGGGAGATGTG-3′; TRAIL-R4 (453 bp), forward 5′-CTTTTCCGGCGGCGTTCATGTCCTTC-3′ and reverse 5′-GTTTCTTCCAGGCTGCTTCCCTTTGTAG-3′; c-$FLIP_L$ (227 bp), forward 5′-AATTCAAGGCTCAGAAGCGA-3′ and reverse 5′-GGCAGAAACTCTGCTGTTCC-3′; c-$FLIP_S$ (100 bp), forward 5′-AATGTTCTCCAAGCAGCAATCC-3′ and reverse 5′-CCAAGAATTTTCAGATCAGGACAAT-3′; Bcl-2 (367 bp), forward 5′-AGATGTCCAGCCAGCTGCACCTGAC-3′ and reverse 5′-AGATAGGCACCAGGGTGAGCAAGCT-3′; Bcl-x (257 bp), forward 5′-CATGGCAGCAGTAAAGCAAGC-3′ and reverse 5′-CTGCGATCCGACTCACCAATAC-3′; Mcl-1 (211 bp), forward 5′-CTTAGTTGATATTTTGGGCTTGGG-3′ and reverse 5′-AGAAGTCAAAAAGTAGTCACTGGG-3′; XIAP (858 bp), forward 5′-GGCCATCTGAGACACATGCAG-3′ and reverse 5′-GCATTCACTAGATCTGCAACC-3′; c-IAP1 (171 bp), forward 5′-CCAGTTCTTTTCTACACTATAAT-3′ and reverse 5′-CTTAATCTGTTTATTTACAAGGG-3′; c-IAP2 (150 bp), forward 5′-CAACATGGAGATTCGAAATCC-3′ and reverse 5′-CACATCACTCTTCTGTGAAGGG-3′; β-actin (661 bp), forward 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and reverse 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′. PCR cycle conditions were as follows: 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C for TRAIL-R1, TRAIL-R2 and TRAIL-R3; 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C for TRAIL-R4, Bcl-2 and β-actin; 40 seg at 95 °C, 40 seg at 60 °C, and 40 seg at 72 °C for c-$FLIP_S$ and c-$FLIP_L$; 1 min at 95 °C, 1 min at 57 °C, and 1 min at 72 °C for Bcl-x, Mcl-1, XIAP, c-IAP1 and c-IAP2. The number of cycles varied between 30 and 35, depending on the gene analyzed. The products were resolved on a 1% agarose gel and visualized with ethidium bromide.

3. Results

3.1. Resistance of resting and activated primary human T lymphocytes to TRAIL-mediated apoptosis

The resistance of different peripheral blood cell subpopulations to TRAIL-mediated cytotoxicity has recently been reported (Hasegawa et al., 2004; Mirandola et al., 2004). To further characterize the effect of TRAIL on primary human T lymphocytes we first analyzed the induction of apoptosis on resting and activated T cells from 10 different healthy donors after treatment for 24 h with 250 ng/ml recombinant TRAIL. As shown in Fig. 1A, all of the resting and activated primary T cell samples were resistant to TRAIL-induced apoptosis as assessed by sub-G1 DNA content. Moreover, we did not observe any effect on primary T cell viability after incubation either for 2 days or with higher doses of TRAIL (data not shown). In this set of experiments, we used TRAIL-sensitive Jurkat cells as a positive control (Fig. 1). We also examined the activation of apical caspase-8 as it is the first biochemical event that occurs upon ligation of TRAIL receptors at the cell surface. To this end, we analyzed the processing of the procaspase into the 43–41 kDa intermediate proteolytic fragments corresponding to the cleavage of

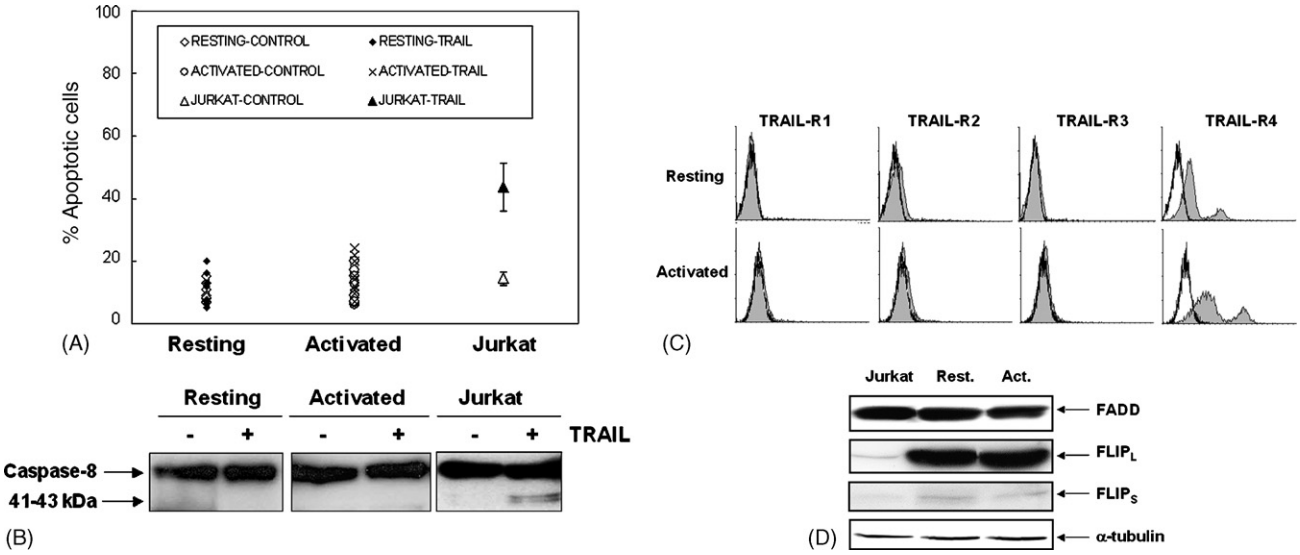

Fig. 1. Primary T cells are resistant to TRAIL-induced apoptosis. (A) Resting and activated T cells from 10 different healthy donors were treated for 24 h with 250 ng/ml recombinant TRAIL. Apoptosis was assessed by analysis of the percentage of cells with sub-G_1 content. (B) Resting and activated T lymphocytes were incubated for 15 h with 250 ng/ml TRAIL and caspase-8 activation was determined by Western blot as described in Section 2. As a positive control, apoptosis (A) and caspase-8 activation (B) were determined in Jurkat T cells after treatment with 100 ng/ml TRAIL. (C) Cell surface TRAIL receptors expression was analyzed by flow cytometry in resting and activated T lymphocytes (shaded peaks). Solid lines represent background fluorescence with secondary antibody alone. (D) Expression of FADD and c-FLIP was determined by Western blot in Jurkat, resting and activated T cells. α-Tubulin was used as a control of loaded protein. In B–D, results shown are representative of at least three different donors.

procaspase-8a and -8b. Data presented in Fig. 1B shows that TRAIL was not able to induce activation of caspase-8 in either resting or activated primary T lymphocytes, which indicates an apical block of the TRAIL signalling pathway in both types of cells.

These results led us to examine the expression of proteins known to participate in the formation of the DISC and thus in the activation of caspase-8. Firstly, we analyzed the surface expression of TRAIL receptors in resting and activated T lymphocytes. In agreement with previous data (Hasegawa et al., 2004), we observed that the expression of death receptors TRAIL-R1 and -R2, as well as decoy receptor TRAIL-R3, was barely detectable in all analyzed resting T cells, with very weak differences among donors. In contrast, when we examined the expression of TRAIL-R4, two subsets of cells with different fluorescence intensities were detected (Fig. 1C). The highly positive TRAIL-R4 peak seems to correspond to the $CD8^+$ T cell subpopulation (Hasegawa et al., 2004; Mirandola et al., 2004) while the less fluorescent peak, which varies from very low to moderate intensity among different donors, corresponds to $CD4^+$ T cells. T cell activation did not change significantly the expression of TRAIL receptors except for TRAIL-R4 (Fig. 1C). For this receptor, we found a slight enhancement in the fluorescence intensity of the two subpopulations, as well as an increase in the percentage of TRAIL-R4 highly positive cells, which is likely due to the observed increment of the $CD8^+/CD4^+$ ratio within the population of activated T cells compared to the resting one (data not shown). Regarding the expression of intracellular proteins involved in the activation of caspase-8, we observed that the levels of FADD in resting and activated T cells were similar to that found in the TRAIL-sensitive Jurkat cell line (Fig. 1D). However, it is also known that human T lymphocytes express the inhibitory protein c-FLIP (Bosque et al., 2005; Schmitz et al., 2003). When we compared the expression of this protein between Jurkat and primary T cells we detected a much higher level of the long isoform, c-$FLIP_L$, in both resting and activated T lymphocytes than in Jurkat cells. The expression of the c-$FLIP_S$ isoform was low in primary T cells but barely perceptible in Jurkat cells (Fig. 1D). Taken together, these results suggest that several factors may be responsible for the resistance of resting and activated T lymphocytes to TRAIL-induced apoptosis.

3.2. *Effect of different modulators of TRAIL sensitivity on the resistance of human primary T lymphocytes*

Deregulated expression of TRAIL receptors and intracellular antiapoptotic proteins seem to cause resistance to TRAIL-induced apoptosis in several types of tumor cells, which reduces the effectiveness of TRAIL in cancer therapy. However, most of these TRAIL-resistant cancer cells can be sensitized by several therapeutic approaches such as conventional chemotherapeutic drugs, histone deacetylase inhibitors (HDACi), inhibitors of survival pathways or proteasome inhibitors (Inoue et al., 2004; Kandasamy and Srivastava, 2002; Sayers and Murphy, 2006; Shankar and Srivastava, 2004; Singh et al., 2003). We analyzed the effect of some agents belonging to these functional groups on the resistance of primary T lymphocytes to TRAIL-mediated apoptosis. As shown in Fig. 2, we specifically tested the genotoxic drug doxorubicin, the PI3K inhibitor LY294002, the HDACi valproic acid (VPA), the microtubule depolymerizing agent nocodazole and the proteasome inhibitor Velcade (bortezomib). Working concentrations of these agents were chosen according to previous reports and on the basis of experiments with the T-lymphoblastic leukemic CEM cell line and the breast

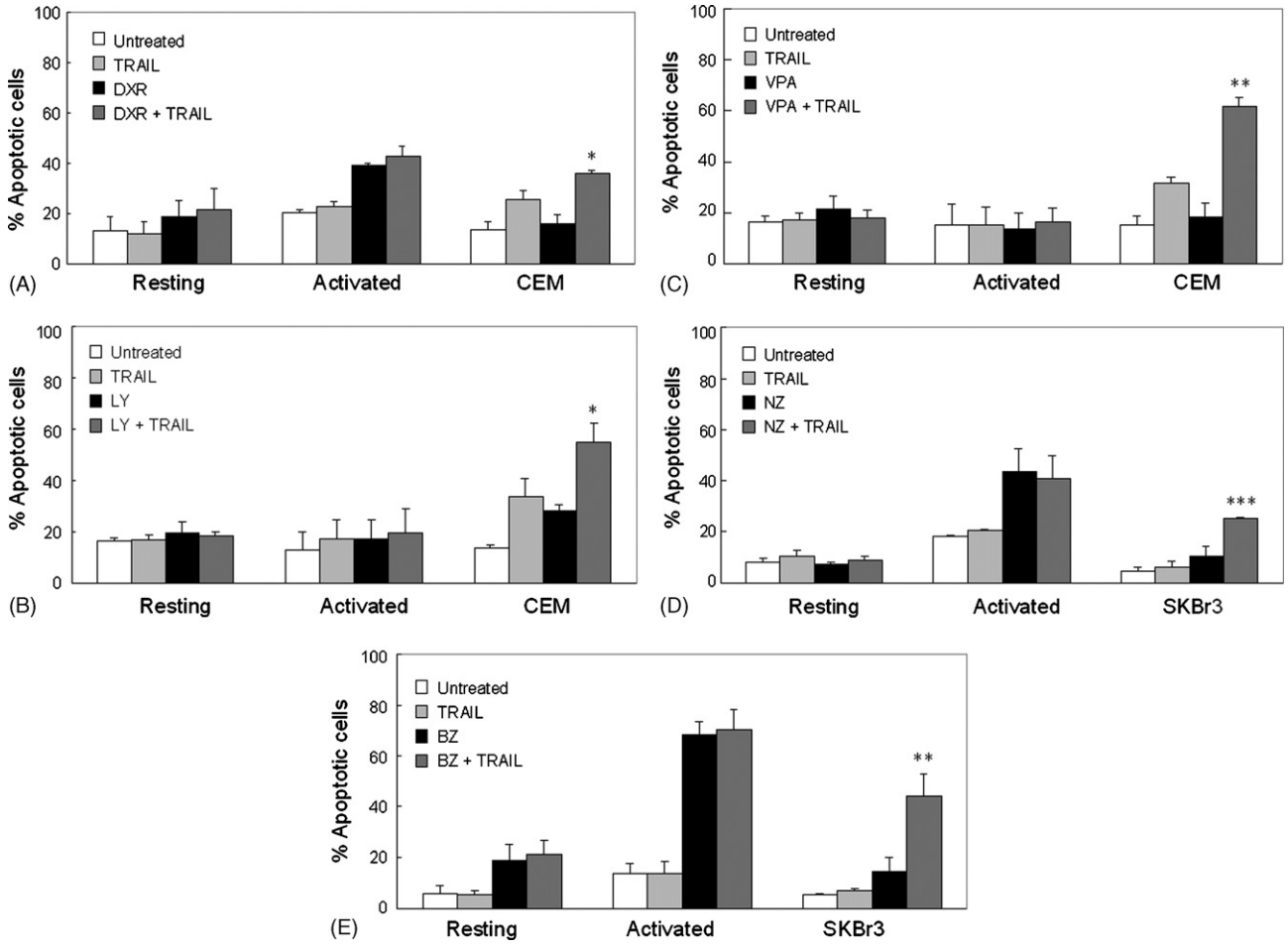

Fig. 2. Primary T lymphocytes remain resistant to TRAIL upon treatment with different sensitizing agents. Resting and activated T lymphocytes were preincubated with (A) 100 ng/ml doxorubicin (DXR), (B) 10 μM LY 294002 or (E) 50 nM bortezomib (BZ) for 1 h, or with (C) 1mM valproic acid (VPA) or (D) 400 ng/ml nocodazole (NZ) for 7 h. After preincubation, cells were treated with or without 250 ng/ml recombinant TRAIL for 24 h. CEM (A–C) and SKBr3 (D and E) cells were preincubated in the same conditions before treatment with 50 and 250 ng/ml recombinant TRAIL, respectively, for 20 h. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Error bars show S.D. from three independent experiments. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ by unpaired Student's *t*-test (two-tailed).

cancer SKBr3 cell line, which show a moderate sensitivity and a high resistance to TRAIL, respectively. Pretreatment with either doxorubicin, LY294002 or VPA increased the susceptibility of CEM cells to TRAIL while bortezomib and nocodazole clearly sensitized TRAIL-resistant SKBr3 cells. However, we did not find TRAIL sensitization in either resting or activated T cells in response to pretreatment with any of the indicated agents. Interestingly, doxorubicin, nocodazole and bortezomib showed a remarkable toxicity against activated T cells, whereas only bortezomib was slightly toxic to resting ones (Fig. 2A, D and E).

3.3. NF-κB inhibitors sensitize human activated T lymphocytes to TRAIL-induced apoptosis

In addition to the above agents, we also analyzed the effect of the NF-κB inhibitor BAY 11-7085. Data presented in Fig. 3A indicates that the response of resting T lymphocytes to this inhibitor varies among different donors. BAY 11-7085 alone was toxic to varying degrees to 9/10 samples. Surprisingly, while it caused a significant sensitization to TRAIL-induced apoptosis in 4/10 samples, in five different ones the percentage of apoptotic cells decreased following treatment with both BAY 11-7085 and TRAIL compared to cells treated with the NF-κB inhibitor alone. In the case of activated T lymphocytes, a variable toxicity was also found in response to BAY 11-7085 alone (Fig. 3B). However, we observed that preincubation with the NF-κB inhibitor induced a different but substantial sensitization to TRAIL-mediated apoptosis in all donors analyzed, except for one in which the inhibitor alone showed an extreme toxicity.

To further establish that the death process promoted by the NF-κB inhibitor was apoptosis, resting and activated T lymphocytes from donors previously proved to be sensitized to TRAIL (Fig. 3A and B, respectively) were preincubated with the general caspase inhibitor Z-VAD-FMK before treatment with BAY 11-7085 and TRAIL. Representative samples in Fig. 3C and D show that not only TRAIL-mediated cell death but also the cytotoxic effect of BAY 11-7085 were almost completely inhibited by Z-VAD. It is worth mentioning that Z-VAD was

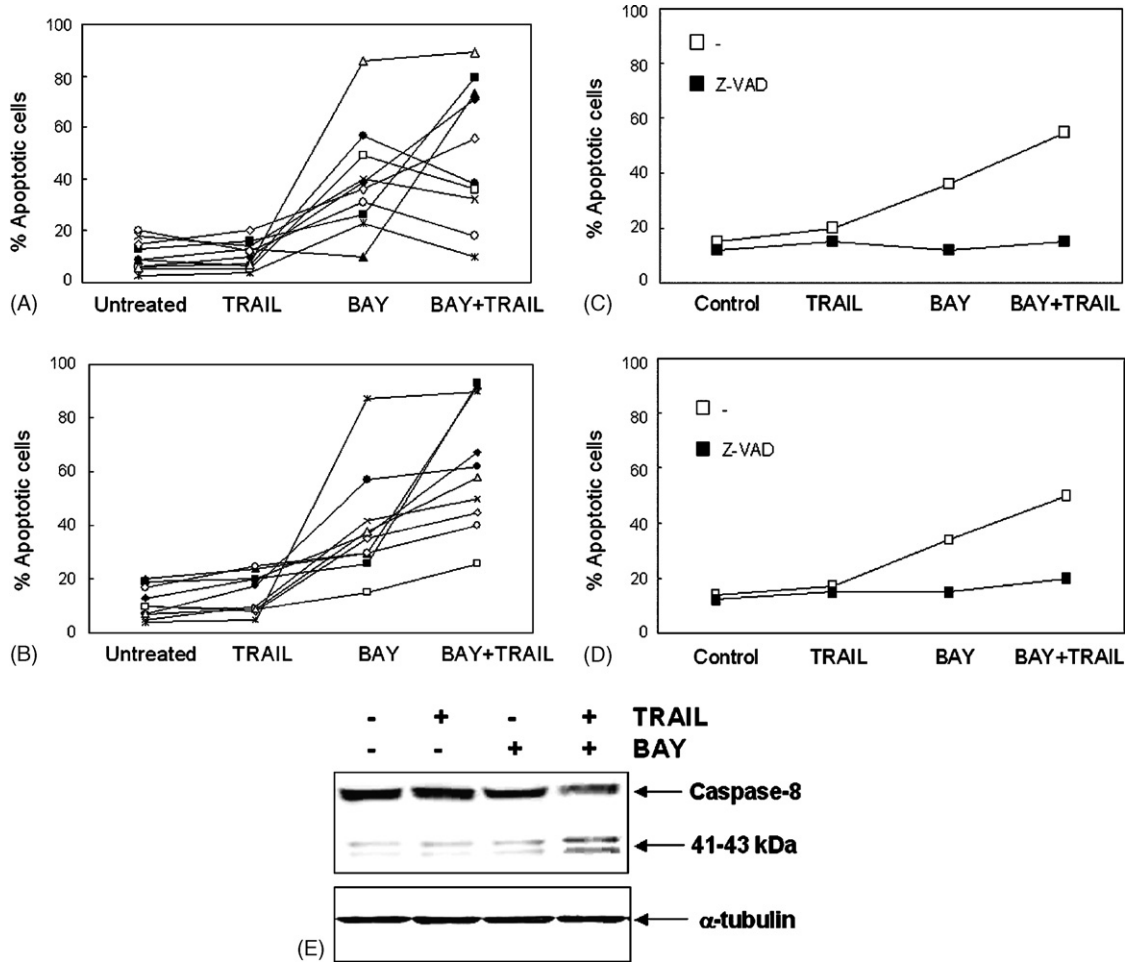

Fig. 3. BAY 11-7085 sensitizes activated T lymphocytes to TRAIL-induced apoptosis. Resting (A and C) and activated (B and D) T lymphocytes from 10 different healthy donors (A and B) and one representative donor (C and D) were preincubated for 1 h with 2.5 μM BAY 11-7085 and then treated with or without 250 ng/ml TRAIL for 24 h. In C and D, preincubation was carried out in the presence or in the absence of 50 μM Z-VAD. Apoptosis was determined by flow cytometry. Results in C and D are representative of three different experiments. (E) Activation of caspase-8 was determined by Western blot in activated T lymphocytes incubated with or without 250 ng/ml TRAIL for 20 h after pretreatment for 1 h with 2.5 μM BAY 11-7085. α-Tubulin was used as a control of loaded protein. Data shown are representative of three different experiments.

not able to inhibit cell death in the samples in which BAY 11-7085 alone was extremely toxic (data not shown). This suggests that BAY 11-7085 may activate both caspase-dependent and caspase-independent cell death signalling pathways in T lymphocytes. On the other hand, we observed the processing of apical procaspase-8 in response to TRAIL in activated T lymphocytes preincubated with BAY 11-7085 (Fig. 3E), which confirm the induction of the TRAIL signalling pathway in T cells sensitized by treatment with the NF-κB inhibitor.

To determine the specific involvement of NF-κB in the mechanism of BAY 11-7085-induced sensitization, we examined the effects of different NF-κB inhibitors on the resistance of activated T cells to TRAIL-mediated cell death. Sulfasalazine, parthenolide and caffeic acid phenetyl ester (CAPE) have all been reported to be specific NF-κB inhibitors (Bork et al., 1997; Natarajan et al., 1996; Wahl et al., 1998). We observed that activated T lymphocytes were significantly sensitized to TRAIL-induced apoptosis upon pretreatment with sulfasalazine. However, parthenolide and CAPE showed a very week and no effect, respectively, on the resistance to TRAIL (Fig. 4A). We next confirmed that all these compounds effectively inhibited NF-κB at the concentrations used in this study. To this end, we analyzed the presence of the p65 (RelA) and the p50 (NF-κB1) subunits of NF-κB in nuclear extracts of activated T lymphocytes following treatment with the NF-κB inhibitors. Western blot analysis showed that BAY 11-7085, sulfasalazine and parthenolide significantly reduced the levels of p65 and p50 in the nucleus of activated T cells, while CAPE only had a slight effect on the nuclear translocation of the NF-κB subunits (Fig. 4B), which may explain the lack of sensitization to TRAIL-induced apoptosis showed by this agent.

3.4. BAY 11-7085 regulates the expression of TRAIL receptors and antiapoptotic genes in activated T lymphocytes

The transcription factor NF-κB has been reported to regulate the expression of some TRAIL receptors as well as that of

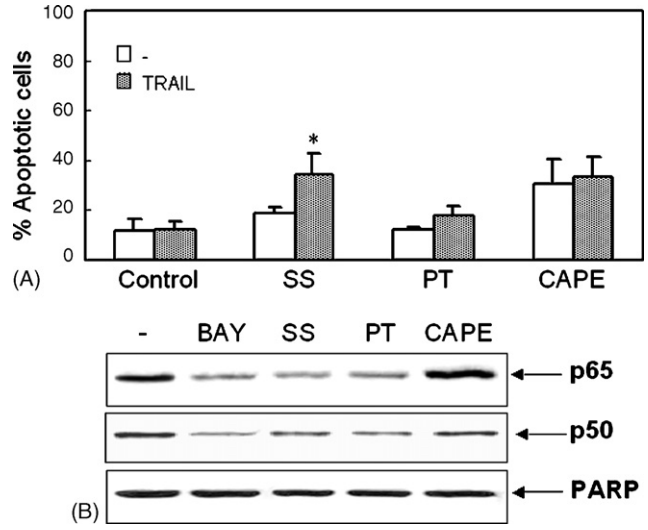

Fig. 4. Effect of different NF-κB inhibitors on the resistance of activated T cells to TRAIL. (A) Activated T cells were preincubated for 1 h with 1.5 mM sulfasalazine (SS), 2.5 μM parthenolide (PT) or 50 μg/ml CAPE, before treatment with 250 ng/ml TRAIL for 24 h. Sub-G1 apoptotic cells were analyzed by flow cytometry. Error bars show S.D. from three independent experiments. $^{*}p < 0.05$ by unpaired Student's *t*-test (two-tailed). (B) Expression of the p50 and p65 subunits of NF-κB was determined by Western blot in nuclear extracts of activated T cells after treatment for 4 h with the indicated inhibitors. PARP was used as control of loaded protein. Results shown are representative of three independent experiments.

several antiapoptotic proteins involved in TRAIL signalling apoptotic pathway (Micheau et al., 2001; Ravi et al., 2001; Stehlik et al., 1998; Wang et al., 1998). In order to understand the mechanism for NF-κB inhibition-induced sensitization of human primary T lymphocytes to TRAIL-mediated apoptosis, we first analyzed the expression of TRAIL-R1, -R2 and -R4 on the surface of activated T lymphocytes upon treatment with BAY 11-7085. As shown in Fig. 5A, we did not observe any change in the expression of death receptors, although the surface level of the antiapoptotic receptor TRAIL-R4 was reduced in response to the NF-κB inhibitor in all samples analyzed. Moreover, when we determined the expression of TRAIL receptors at the mRNA level we observed a clear time-dependent down-regulation of all of them after treatment with BAY 11-7085 (Fig. 5B). TRAIL-R3 expression was undetectable at the mRNA level (data not shown).

We then examined the effects of BAY 11-7085 on the expression of different antiapoptotic genes, namely c-FLIP, Bcl-2, Bcl-x, Mcl-1, XIAP, c-IAP1 and c-IAP2, in activated T lymphocytes. We found no marked changes in the mRNA levels of c-FLIP$_L$, Bcl-x and Mcl-1 for up to 9 h following treatment with BAY 11-7085 (Fig. 5C). In contrast, a significant and time-dependent decrease in the mRNA expression of c-FLIP$_S$, Bcl-2, XIAP, c-IAP1 and c-IAP2 was observed in T cells exposed to the NF-κB inhibitor (Fig. 5C). We further analyzed whether the decrease of these antiapoptotic genes was paralleled by changes in the levels of the corresponding proteins. Results in Fig. 5D indicate that all the proteins analyzed, with the exception of Bcl-2, were down-regulated after 24 h of treatment with BAY 11-7085. The absence of down-regulation of Bcl-2 at this time may be due to the long half-life of the protein (Reed, 1996). Altogether, regulation of these antiapoptotic proteins and changes in the expression of TRAIL-R4, may account for the sensitization of T lymphocytes to TRAIL-mediated apoptosis observed in response to NF-κB inhibition.

4. Discussion

The significance of TRAIL as an anti-cancer therapeutic agent is supported by its known involvement in immune surveillance against tumors. Surface expression of TRAIL is upregulated in virtually all cell types of the immune system in response to activation signals, mediating to a great degree the cytotoxic activity of these cells (Fanger et al., 1999; Griffith et al., 1999; Kayagaki et al., 1999; Kemp et al., 2004, 2005; Takeda et al., 2001). The lack of toxicity to most normal cells is an important feature for TRAIL to act as an anti-tumor agent although the molecular mechanisms of TRAIL resistance in normal cells are not completely understood. Here, we have analyzed the response of resting and activated T lymphocytes to TRAIL. We have shown for the first time that the TRAIL signalling pathway is inhibited at the most apical level in both, resting and activated T cells, as no caspase-8 activation was observed in response to TRAIL. The analysis of the expression of the most important proteins involved in the formation of the DISC indicate that: (1) long-term T cell activation (day 6) does not modulate the expression levels of FADD, caspase-8, c-FLIP and TRAIL receptors, with the exception of TRAIL-R4; (2) when comparison with the TRAIL sensitive Jurkat cells, the expression of the antiapoptotic protein c-FLIP is much higher in T lymphocytes than in this cell line; (3) the levels of expression of TRAIL-R1, -R2 and -R3 receptors on the surface of T cells are barely detectable while the expression of TRAIL-R4 shows two peaks with low and high fluorescence intensity, respectively. From these data we can infer that several factors may be responsible for the lack of caspase-8 activation upon treatment with TRAIL and hence for the resistance of resting and activated T lymphocytes to TRAIL-induced apoptosis. It has been recently described that c-FLIP is involved the resistance of NK cells to TRAIL-induced apoptosis (Mirandola et al., 2004). Our results about the expression of c-FLIP, in agreement with previous reports (Bosque et al., 2005; Mirandola et al., 2004; Schmitz et al., 2003), suggest that this inhibitory protein could also play a role in the resistance of T lymphocytes.

Regarding the expression of TRAIL receptors, there are some discrepancies between authors. Previous studies have reported that resting T cells do not show significant expression of TRAIL receptors, with the exception of TRAIL-R4 on the CD8$^+$ subpopulation (Hasegawa et al., 2004; Mirandola et al., 2004). However, we have observed that the intensity of the TRAIL-R4 less fluorescence peak, which seems to correspond to the CD4$^+$ cells, may vary among donors. On the other hand, those reports show that activation of CD8$^+$ T cells up-regulate the expression of either TRAIL-R1 and -R2 (Hasegawa et al., 2004) or TRAIL-R2, -R3 and -R4 (Mirandola et al., 2004) while no changes are detected in the expression of TRAIL receptors upon activation of CD4$^+$ T cells (Hasegawa et al., 2004). In contrast to Miran-

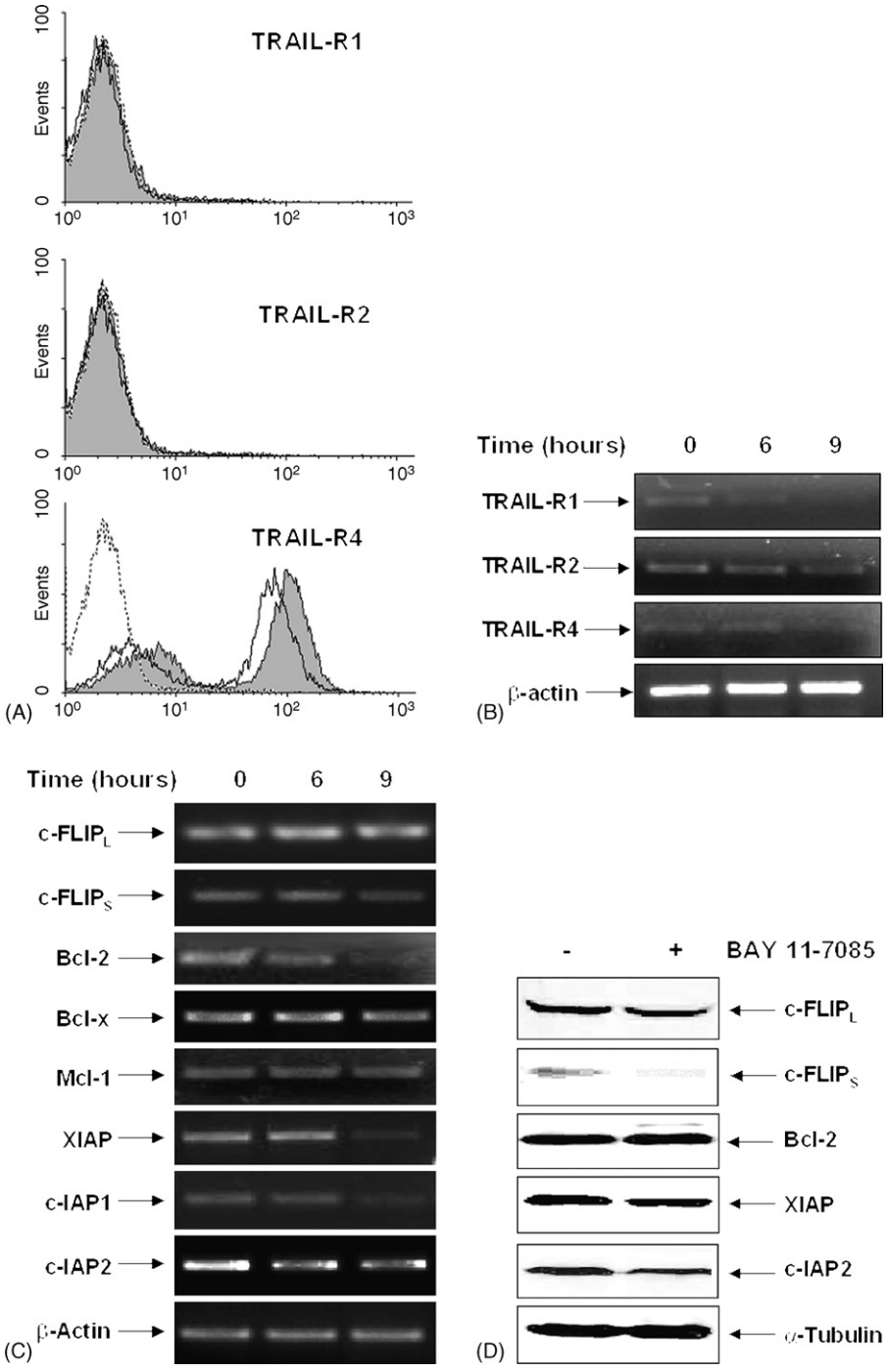

Fig. 5. Expression of TRAIL receptors and antiapoptotic genes in activated T lymphocytes in response to BAY 11-7085. (A) Activated T cells were incubated with (unshaded peaks) or without (shaded peaks) 2.5 μM BAY for 15 h and cell surface TRAIL-R1, -R2 and -R4 receptors expression was assessed by flow cytometry. Dashed lines show background fluorescence with secondary antibody. mRNA levels of TRAIL-R1, -R2, -R4 (B), c-FLIP$_L$, c-FLIP$_S$, Bcl-2, Bcl-x, Mcl-1, XIAP, c-IAP1 and c-IAP2 (C) were determined in activated T cells after treatment with 2.5 μM BAY for 6 and 9 h by RT-PCR as described under Section 2. β-Actin was used as control of RNA input. (D) Expression of c-FLIP$_L$, c-FLIP$_S$, Bcl-2, XIAP and c-IAP2 was analyzed by Western blot in activated T lymphocytes treated with or without 2.5 μM BAY for 24 h. α-Tubulin was used as control of loaded protein. Data shown are representative of at least three independent experiments with different donors.

dola et al., we have worked with the whole population of CD3$^+$ T lymphocytes as CD4$^+$ and CD8$^+$ T cells subpopulations coexist and may influence each other in physiological conditions. Moreover, not only CD8$^+$ cytotoxic T cells but also CD4$^+$ T cells seem to be involved in antitumor immune response via TRAIL (Dorothee et al., 2002; Kayagaki et al., 1999). Contradictory data concerning the expression of TRAIL receptors in activated T lymphocytes may also be due to differences in the way of T cell activation. Independently of the reported profile of TRAIL receptors expression, it is important to emphasize that all studies agree in the resistance of activated T lymphocytes to TRAIL-induced apoptosis.

NF-κB inhibition has been recently proposed as a mechanism for sensitization of some tumor resistant cells to TRAIL-induced apoptosis since this transcription factor is constitutively active in certain tumors modulating the expression of several antiapoptotic proteins (Huerta-Yepez et al., 2004; Karacay et al., 2004; Kasuga et al., 2004). Our findings reveal that several inhibitors of NF-κB are also able to sensitize activated T lymphocytes to TRAIL-mediated apoptosis. Specifically, we have observed a clear sensitization to TRAIL with the NF-κB inhibitors BAY 11-7085 and sulfasalazine while the effect of parthenolide is too low to be significant. As all these agents were able to inhibit the translocation of p65 and p50 subunits to the nucleus, we speculate that the weak sensitization observed with parthenolide may be due to the additional activities that have been reported for this compound, such as JNK activation and STAT3 inhibition (Nakshatri et al., 2004; Sobota et al., 2000), which can interfere in some way with the signals derived from NF-κB inhibition. Interestingly, BAY 11-7085 reduces the mRNA expression of TRAIL-R1 and TRAIL-R2 receptors in activated T lymphocytes, although we could not estimate this decrease at the level of surface protein expression. Despite that the levels of death receptors are too low to be detected by FACS analysis, we have clearly observed activation of the initiator caspase-8 in response to TRAIL upon pretreatment of T lymphocytes with BAY 11-7085. Thus, the above data suggest that TRAIL-R1, TRAIL-R2 or both receptors must be present on the surface of activated T lymphocytes and are able to transmit the apoptotic signals. In addition, we have demonstrated for the first time that NF-κB inhibition not only down-regulates the expression of TRAIL-R4 at the mRNA level but also modestly reduces TRAIL-R4 expression at the cell surface. TRAIL-R4 has been recently proposed to be a regulatory rather than a decoy receptor as it associates with TRAIL-R2 forming a ligand-independent, death-inhibitory complex (Clancy et al., 2005). Moreover, authors demonstrate that this complex regulates the sensitivity of $CD8^+$ T cells to TRAIL-induced apoptosis. Therefore, down-regulation of TRAIL-R4 by NF-κB inhibition may be involved in the sensitization of activated T lymphocytes to TRAIL. We have further observed that BAY 11-7085 down-regulates the expression levels of some antiapoptotic proteins known to be transcriptionally modulated by NF-κB, such as c-$FLIP_S$, XIAP and c-IAP2. Although the contribution of each factor in mediating TRAIL resistance of activated T lymphocytes is unknown, the overall results provide a mechanism for the sensitization of activated T lymphocytes to TRAIL-mediated apoptosis by NF-κB inhibition. Regarding Bcl-2, even though there is a clear decrease of mRNA levels, our findings suggest that this antiapoptotic factor must not be involved in the effect of the NF-κB inhibitor as the protein levels do not change at the time we observe sensitization of T lymphocytes.

Our data indicate that the response of resting T lymphocytes to TRAIL-induced apoptosis upon NF-κB inhibition vary among different donors. While NF-κB is actively involved in T cell proliferation and activation, only low levels of constitutive NF-κB binding activity are usually found in resting T cells. Moreover, homodimeric complexes of the NF-κB p50 subunit, which generally function as transcriptional repressors, have been reported to bind to the DNA in the nuclei of resting T cells, in sharp contrast with the transcriptionally active p65/p50 heterodimers found in activated T lymphocytes (Algarte et al., 1995; Kang et al., 1992). These special features of NF-κB activity in resting T lymphocytes, together with individual singularities, may influence the effect of BAY 11-7085. Furthermore, TRAIL has been reported to activate NF-κB through TRAIL-R1 and TRAIL-R2 in many cell types (Franco et al., 2001; Hao et al., 2003; Schneider et al., 1997). Our striking findings about the blockade of cell death observed in some resting T cells upon treatment with both, BAY 11-7085 and TRAIL, might be due to the activation of NF-κB by TRAIL, which could counteract the cytotoxic effect of the NF-κB inhibitor. It would be interesting to determine whether NF-κB activation is the predominant effect of TRAIL receptors engagement in resting T lymphocytes. On the other hand, although we have detected similar levels of pro- and anti-apoptotic genes in resting and activated T lymphocytes (Fig. 1 and data not shown) it is known that only the last ones are able to undergo activation-induced cell death (AICD) (Wesselborg et al., 1993) indicating that different mechanisms of apoptosis resistance work in both types of cells, which may also influence the sensitization to TRAIL.

Although proteasome inhibitors block NF-κB activation, we have not observed any effect of either bortezomib or MG132 on the resistance of T lymphocytes to TRAIL-mediated apoptosis (Fig. 2E and data not shown). This is not surprising as the biological effects of proteasome inhibitors are multiple and the final result of all of them may be not suitable for the sensitization of T cells to TRAIL. In spite of that, our results indicate that bortezomib is highly toxic especially for activated T lymphocytes. Similarly, other modulators of TRAIL sensitivity in tumor cells, such as doxorubicin and nocodazole, show a moderate toxicity against activated T cells. In contrast, and in agreement with previous reports, our findings suggest that HDAC inhibitors may be a valuable therapeutic strategy to sensitize tumor cells to TRAIL-induced apoptosis as they seem to be non-toxic and they do not regulate TRAIL resistance in primary T cells (Inoue et al., 2004). PI3K/Akt inhibitors may also be useful in the treatment of tumors with high level of activity of this survival pathway that seems to block the apoptotic signals derived from TRAIL receptors engagement (Nam et al., 2003; Nesterov et al., 2001).

NF-κB activation is essential to numerous physiological processes and plays a crucial role in the immune response, e.g. in lymphocyte proliferation and cytokine production, which suggests that general NF-κB inhibitors will probably produce a myriad of side-effects. In the present study, we have demonstrated that NF-κB inhibitors not only sensitize primary activated T lymphocytes to TRAIL-induced apoptosis but also show a variable toxicity to resting and activated T cells. It is worth mentioning a recent report describing that the mechanism of TRAIL resistance in normal human urothelial cells is mainly dependent on the NF-κB pathway (Steele et al., 2006). Together with these data, our results suggest a likely role for NF-κB in the resistance of some normal cells types to TRAIL-induced apoptosis and challenge the use of NF-κB inhibitors as a useful approach in antitumor therapy.

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References

Algarte, M., Lecine, P., Costello, R., Plet, A., Olive, D., Imbert, J., 1995. In vivo regulation of interleukin-2 receptor alpha gene transcription by the coordinated binding of constitutive and inducible factors in human primary T cells. EMBO J. 14, 5060–5072.

Bork, P.M., Schmitz, M.L., Kuhnt, M., Escher, C., Heinrich, M., 1997. Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF-kappaB. FEBS Lett. 402, 85–90.

Bosque, A., Pardo, J., Martinez-Lorenzo, M.J., Iturralde, M., Marzo, I., Pineiro, A., Alava, M.A., Naval, J., Anel, A., 2005. Down-regulation of normal human T cell blast activation: roles of APO-2L/TRAIL, FasL, and c- FLIP, Bim, or Bcl-x isoform expression. J. Leukoc. Biol. 77, 568–578.

Clancy, L., Mruk, K., Archer, K., Woelfel, M., Mongkolsapaya, J., Screaton, G., Lenardo, M.J., Chan, F.K., 2005. Preligand assembly domain-mediated ligand-independent association between TRAIL receptor 4 (TR4) and TR2 regulates TRAIL-induced apoptosis. Proc. Natl. Acad. Sci. U.S.A. 102, 18099–18104.

Cretney, E., Shanker, A., Yagita, H., Smyth, M.J., Sayers, T.J., 2006. TNF-related apoptosis-inducing ligand as a therapeutic agent in autoimmunity and cancer. Immunol. Cell Biol. 84, 87–98.

Cummins, J.M., Kohli, M., Rago, C., Kinzler, K.W., Vogelstein, B., Bunz, F., 2004. X-linked inhibitor of apoptosis protein (XIAP) is a non-redundant modulator of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Res. 64, 3006–3008.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., Dixit, V.M., 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81, 505–512.

Degli-Esposti, M.A., Dougall, W.C., Smolak, P.J., Waugh, J.Y., Smith, C.A., Goodwin, R.G., 1997. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 7, 813–820.

Dorothee, G., Vergnon, I., Menez, J., Echchakir, H., Grunenwald, D., Kubin, M., Chouaib, S., Mami-Chouaib, F., 2002. Tumor-infiltrating $CD4^+$ T lymphocytes express APO2 ligand (APO-2L)/TRAIL upon specific stimulation with autologous lung carcinoma cells: role of IFN-alpha on APO-2L/TRAIL expression and -mediated cytotoxicity. J. Immunol. 169, 809–817.

Fanger, N.A., Maliszewski, C.R., Schooley, K., Griffith, T.S., 1999. Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J. Exp. Med. 190, 1155–1164.

Franco, A.V., Zhang, X.D., Van Berkel, E., Sanders, J.E., Zhang, X.Y., Thomas, W.D., Nguyen, T., Hersey, P., 2001. The role of NF-kappaB in TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of melanoma cells. J. Immunol. 166, 5337–5345.

Gong, J.P., Traganos, F., Darzynkiewicz, Z., 1994. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal. Biochem. 218, 314–319.

Griffith, T.S., Wiley, S.R., Kubin, M.Z., Sedger, L.M., Maliszewski, C.R., Fanger, N.A., 1999. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J. Exp. Med. 189, 1343–1354.

Hao, X.S., Hao, J.H., Liu, F.T., Newland, A.C., Jia, L., 2003. Potential mechanisms of leukemia cell resistance to TRAIL-induced apoptosis. Apoptosis 8, 601–607.

Hasegawa, H., Yamada, Y., Harasawa, H., Tsuji, T., Murata, K., Sugahara, K., Tsuruda, K., Masuda, M., Takasu, N., Kamihira, S., 2004. Restricted expression of tumor necrosis factor-related apoptosis-inducing ligand receptor 4 in human peripheral blood lymphocytes. Cell. Immunol. 231, 1–7.

Huerta-Yepez, S., Vega, M., Jazirehi, A., Garban, H., Hongo, F., Cheng, G., Bonavida, B., 2004. Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-kappaB and inhibition of Bcl-xl expression. Oncogene 23, 4993–5003.

Inoue, S., MacFarlane, M., Harper, N., Wheat, L.M., Dyer, M.J., Cohen, G.M., 2004. Histone deacetylase inhibitors potentiate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in lymphoid malignancies. Cell Death Differ. 11 (Suppl. 2), S193–S206.

Jo, M., Kim, T.H., Seol, D.W., Esplen, J.E., Dorko, K., Billiar, T.R., Strom, S.C., 2000. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat. Med. 6, 564–567.

Kandasamy, K., Srivastava, R.K., 2002. Role of the phosphatidylinositol 3′-kinase/PTEN/Akt kinase pathway in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in non-small cell lung cancer cells. Cancer Res. 62, 4929–4937.

Kang, S.M., Tran, A.C., Grilli, M., Lenardo, M.J., 1992. NF-kappa B subunit regulation in non-transformed $CD4^+$ T lymphocytes. Science 256, 1452–1456.

Karacay, B., Sanlioglu, S., Griffith, T.S., Sandler, A., Bonthius, D.J., 2004. Inhibition of the NF-kappaB pathway enhances TRAIL-mediated apoptosis in neuroblastoma cells. Cancer Gene Ther. 11, 681–690.

Kasuga, C., Ebata, T., Kayagaki, N., Yagita, H., Hishii, M., Aral, H., Sato, K., Okumura, K., 2004. Sensitization of human glioblastomas to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by NF-kappaB inhibitors. Cancer Sci. 95, 840–844.

Kayagaki, N., Yamaguchi, N., Nakayama, M., Eto, H., Okumura, K., Yagita, H., 1999. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J. Exp. Med. 189, 1451–1460.

Kemp, T.J., Ludwig, A.T., Earel, J.K., Moore, J.M., VanOosten, R.L., Moses, B., Leidal, K., Nauseef, W.M., Griffith, T.S., 2005. Neutrophil stimulation with *Mycobacterium bovis* bacillus calmette-guerin (BCG) results in the release of functional soluble TRAIL/APO-2L. Blood 106, 3474–3482.

Kemp, T.J., Moore, J.M., Griffith, T.S., 2004. Human B cells express functional TRAIL/Apo-2 ligand after CpG-containing oligodeoxynucleotide stimulation. J. Immunol. 173, 892–899.

Lawrence, D., Shahrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Totpal, K., DeForge, L., Schow, P., Hooley, J., Sherwood, S., Pai, R., Leung, S., Khan, L., Gliniak, B., Bussiere, J., Smith, C.A., Strom, S.S., Kelley, S., Fox, J.A., Thomas, D., Ashkenazi, A., 2001. Differential hepatocyte toxicity of recombinant APO-2L/TRAIL versions. Nat. Med. 7, 383–385.

Li, L., Thomas, R.M., Suzuki, H., De Brabander, J.K., Wang, X., Harran, P.G., 2004. A small molecule Smac mimic potentiates TRAIL-and TNFalpha-mediated cell death. Science 305, 1471–1474.

MacFarlane, M., Ahmad, M., Srinivasula, S.M., Fernandes-Alnemri, T., Cohen, G.M., Alnemri, E.S., 1997. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J. Biol. Chem. 272, 25417–25420.

MacFarlane, M., Harper, N., Snowden, R.T., Dyer, M.J., Barnett, G.A., Pringle, J.H., Cohen, G.M., 2002. Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia. Oncogene 21, 6809–6818.

Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., Tschopp, J., 2001. NF-kappaB signals induce the expression of c-FLIP. Mol. Cell. Biol. 21, 5299–5305.

Mirandola, P., Ponti, C., Gobbi, G., Sponzilli, I., Vaccarezza, M., Cocco, L., Zauli, G., Secchiero, P., Manzoli, F.A., Vitale, M., 2004. Activated human NK and $CD8^+$ T cells express both TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors but are resistant to TRAIL-mediated cytotoxicity. Blood 104, 2418–2424.

Nakshatri, H., Rice, S.E., Bhat-Nakshatri, P., 2004. Antitumor agent parthenolide reverses resistance of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand through sustained activation of c-Jun N-terminal kinase. Oncogene 23, 7330–7344.

Nam, S.Y., Jung, G.A., Hur, G.C., Chung, H.Y., Kim, W.H., Seol, D.W., Lee, B.L., 2003. Upregulation of FLIP(S) by Akt, a possible inhibition mecha-

nism of TRAIL-induced apoptosis in human gastric cancers. Cancer Sci. 94, 1066–1073.

Natarajan, K., Singh, S., Burke Jr., T.R., Grunberger, D., Aggarwal, B.B., 1996. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappaB. Proc. Natl. Acad. Sci. U.S.A. 93, 9090–9095.

Nesterov, A., Lu, X., Johnson, M., Miller, G.J., Ivashchenko, Y., Kraft, A.S., 2001. Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. J. Biol. Chem. 276, 10767–10774.

Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., Nagata, S., 1993. Lethal effect of the anti-Fas antibody in mice. Nature 364, 806–809.

Ortiz-Ferron, G., Tait, S.W., Robledo, G., de Vries, E., Borst, J., Lopez-Rivas, A., 2006. The mitogen-activated protein kinase pathway can inhibit TRAIL-induced apoptosis by prohibiting association of truncated Bid with mitochondria. Cell Death Differ. 13, 1857–1865.

Pan, G., Ni, J., Wei, Y.F., Yu, G., Gentz, R., Dixit, V.M., 1997a. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 277, 815–818.

Pan, G., O'Rourke, K., Chinnaiyan, A.M., Gentz, R., Ebner, R., Ni, J., Dixit, V.M., 1997b. The receptor for the cytotoxic ligand TRAIL. Science 276, 111–113.

Ravi, R., Bedi, G.C., Engstrom, L.W., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E.J., Bedi, A., 2001. Regulation of death receptor expression and TRAIL/APO-2L-induced apoptosis by NF-kappaB. Nat. Cell Biol. 3, 409–416.

Reed, J.C., 1996. A day in the life of the Bcl-2 protein: does the turnover rate of Bcl-2 serve as a biological clock for cellular lifespan regulation? Leuk. Res. 20, 109–111.

Ruiz-Ruiz, M.C., Lopez-Rivas, A., 1999. p53-Mediated up-regulation of CD95 is not involved in genotoxic drug-induced apoptosis of human breast tumor cells. Cell Death Differ. 6, 271–280.

Ruiz de Almodovar, C., Ruiz-Ruiz, C., Munoz-Pinedo, C., Robledo, G., Lopez-Rivas, A., 2001. The differential sensitivity of Bc1-2-overexpressing human breast tumor cells to TRAIL or doxorubicin-induced apoptosis is dependent on Bc1-2 protein levels. Oncogene 20, 7128–7133.

Sarker, M., Ruiz-Ruiz, C., Lopez-Rivas, A., 2001. Activation of protein kinase C inhibits TRAIL-induced caspases activation, mitochondrial events and apoptosis in a human leukemic T cell line. Cell Death Differ. 8, 172–181.

Sayers, T.J., Murphy, W.J., 2006. Combining proteasome inhibition with TNF-related apoptosis-inducing ligand (APO-2L/TRAIL) for cancer therapy. Cancer Immunol. Immunother. 55, 76–84.

Schmitz, I., Krueger, A., Baumann, S., Schulze-Bergkamen, H., Krammer, P.H., Kirchhoff, S., 2003. An IL-2-dependent switch between CD95 signaling pathways sensitizes primary human T cells toward CD95-mediated activation-induced cell death. J. Immunol. 171, 2930–2936.

Schneider, P., Thome, M., Burns, K., Bodmer, J.L., Hofmann, K., Kataoka, T., Holler, N., Tschopp, J., 1997. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity 7, 831–836.

Shankar, S., Srivastava, R.K., 2004. Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: mechanisms and clinical implications. Drug Resist. Update 7, 139–156.

Sheridan, J.P., Marsters, S.A., Pitti, R.M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C.L., Baker, K., Wood, W.I., Goddard, A.D., Godowski, P., Ashkenazi, A., 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277, 818–821.

Siegmund, D., Hadwiger, P., Pfizenmaier, K., Vornlocher, H.P., Wajant, H., 2002. Selective inhibition of FLICE-like inhibitory protein expression with small interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis. Mol. Med. 8, 725–732.

Singh, T.R., Shankar, S., Chen, X., Asim, M., Srivastava, R.K., 2003. Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma in vivo. Cancer Res. 63, 5390–5400.

Sobota, R., Szwed, M., Kasza, A., Bugno, M., Kordula, T., 2000. Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. Biochem. Biophys. Res. Commun. 267, 329–333.

Sprick, M.R., Weigand, M.A., Rieser, E., Rauch, C.T., Juo, P., Blenis, J., Krammer, P.H., Walczak, H., 2000. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity 12, 599–609.

Steele, L.P., Georgopoulos, N.T., Southgate, J., Selby, P.J., Trejdosiewicz, L.K., 2006. Differential susceptibility to TRAIL of normal versus malignant human urothelial cells. Cell Death Differ. 13, 1564–1576.

Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J.A., Binder, B.R., Lipp, J., 1998. Nuclear factor (NF)-kappa B-regulated X-chromosome-linked IAP gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J. Exp. Med. 188, 211–216.

Suda, T., Takahashi, T., Golstein, P., Nagata, S., 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75, 1169–1178.

Takeda, K., Hayakawa, Y., Smyth, M.J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H., Okumura, K., 2001. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat. Med. 7, 94–100.

Wahl, C., Liptay, S., Adler, G., Schmid, R.M., 1998. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J. Clin. Invest. 101, 1163–1174.

Walczak, H., Degli-Esposti, M.A., Johnson, R.S., Smolak, P.J., Waugh, J.Y., Boiani, N., Timour, M.S., Gerhart, M.J., Schooley, K.A., Smith, C.A., Goodwin, R.G., Rauch, C.T., 1997. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. 16, 5386–5397.

Walczak, H., Miller, R.E., Ariail, K., Gliniak, B., Griffith, T.S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R.G., Rauch, C.T., Schuh, J.C., Lynch, D.H., 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat. Med. 5, 157–163.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., Baldwin Jr., A.S., 1998. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281, 1680–1683.

Wesselborg, S., Janssen, O., Kabelitz, D., 1993. Induction of activation-driven death (apoptosis) in activated but not resting peripheral blood T cells. J. Immunol. 150, 4338–4345.

Whang, Y.E., Yuan, X.J., Liu, Y., Majumder, S., Lewis, T.D., 2004. Regulation of sensitivity to TRAIL by the PTEN tumor suppressor. Vitam. Horm. 67, 409–426.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., Sutherland, G.R., Smith, T.D., Rauch, C., Smith, C.A., Goodwin, R.G., 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3, 673–682.

Zhang, X.D., Franco, A.V., Nguyen, T., Gray, C.P., Hersey, P., 2000. Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. J. Immunol. 164, 3961–3970.