

Apo2L/TRAIL is an indirect mediator of apoptosis induced by interferon- α in human myeloma cells

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Abstract Interferon- α (IFN- α) is currently used for the therapy of multiple myeloma (MM) though it is only effective in some patients. IFN- α induces apoptosis in some MM cell lines and it has been proposed to occur through an autocrine loop involving Apo2L/TRAIL. We have analysed the sensitivity to IFN- α and Apo2L/TRAIL of five MM cell lines and found no correlation between the apoptosis inducing ability of both cytokines. IFN- α -induced apoptosis in MM cells was not prevented by a caspase-8 selective inhibitor (Z-IETD-fmk) or blocking Apo2L/TRAIL. However, human monocytes treated with IFN- α release bioactive Apo2L/TRAIL to culture media which was cytotoxic for MM cells resistant to IFN- α . We propose that Apo2L/TRAIL released from IFN- α -stimulated blood monocytes would be a major mediator of the anti-myeloma effect of IFN- α in vivo. © 2005 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: Apoptosis; Multiple myeloma; Apo2L/TRAIL; Caspases; Interferon- α

1. Introduction

Interferon- α (IFN- α), a cytokine originally discovered by its anti-viral activity, is normally synthesized by macrophages and dendritic cells and is currently used in the therapy of some haematologic neoplasias such as hairy cell leukaemia [1] and chronic myelogenous leukemia [2]. IFN- α has been also used in multiple myeloma (MM) maintenance therapy for more than 25 years, but with conflicting results [3]. Clinical data suggest that only 5–10% of patients could really benefit from IFN- α therapy [4]. Unfortunately, there are no predictive factors that can identify these patients. A major difficulty to the rational use of IFN- α in myeloma therapy is the insufficient information available about its mechanism of cell growth suppression. In some MM cell lines and cells from patients IFN- α induces apoptosis [5,6], but the precise pathway involved remains unclear. Recently, it has been proposed that IFN- α -induced apoptosis in myeloma cells occurs through the up-regulation and release of Apo2L/TRAIL in an auto-crine fashion [5,7]. Since we were interested in the usefulness of both cytokines for selective myeloma therapy we have

comparatively analysed the apoptotic inducing ability of IFN- α and Apo2L/TRAIL in a number of MM cell lines. Our results indicate that the direct apoptotic effects of IFN- α and Apo2L/TRAIL on human MM cells occur through essentially different mechanisms. However, normal human monocytes stimulated with IFN- α secrete significant amounts of bioactive Apo2L/TRAIL suggesting that this death ligand is a major mediator of the IFN- α apoptotic potential in vivo.

2. Materials and methods

2.1. Materials

Recombinant human interferon- α (IFN- α , specific activity 1.8×10^8 U/mg) was from PeproTechEC (London, UK), recombinant human Apo2L/TRAIL (Apo2L/TRAIL.0) [8] and blocking anti-Apo2L/TRAIL antibody 5C2 were from Genentech (CA, USA), DR5-Fc (TRAILR2) chimera was from R&D Systems Vitro (Madrid, Spain), cytotoxic antihuman Fas antibody (CH-11) was from Upstate (NY, USA) and blocking anti-Fas antibody (SM1/23) from Bender (Barcelona, Spain). Peptide caspase inhibitors Z-VAD-fmk and Z-DEVD-fmk were from Bachem (Switzerland) and Z-IETD-fmk from BD Biosciences (Madrid, Spain).

2.2. Cell proliferation and toxicity assays

Human multiple myeloma (MM) cell lines MM.1S, RPMI 8226, NCI-H929, U266 (clone B1) and IM-9 were from the ATCC. All cells were cultured at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine and penicillin/streptomycin (hereafter, complete medium). Peripheral blood mononuclear cells (PBMC), obtained as described [9], were cultured in complete medium in polystyrene culture flasks at 37 °C for 2 h to favour monocyte adhesion. After vigorously washing with RPMI medium the remaining adherent cells, were considered as monocytes. Monocytes (13×10^6 cells in 10 ml) or MM cells were cultured for 48 h in the presence or absence of IFN- α (2000 U/ml) and culture supernatants used in bioassays. In proliferation assays, MM cells ($3\text{--}5 \times 10^5$ cells/ml) were treated in flat-bottom, 24-well (1 ml/well) or 96-well plates (100 μ l/well) with 2000 U/ml IFN- α , 100 ng/ml Apo2L/TRAIL or 100 ng/ml cytotoxic anti-Fas in complete medium for different times, as indicated. The effect of caspase inhibitors Z-DEVD-fmk, Z-IETD-fmk or ZVAD-fmk (all at 100 μ M) on IFN- α and Apo2L/TRAIL toxicity was evaluated. Inhibitors were added 1 h prior to treatment with IFN- α or Apo2L/TRAIL. In some experiments, cytotoxicity of Apo2L/TRAIL in culture media was neutralized by preincubation with 100 ng/ml blocking anti-Apo2L/TRAIL (5C2), or with 200 ng/ml of a DR5-Fc chimera, as validated in previous studies [9].

2.3. Flow cytometry analysis

Apoptosis was evaluated by the simultaneous determination of phosphatidylserine (PS) exposure and mitochondrial membrane potential ($\Delta\Psi_m$) in the same cells. Briefly, cells (2.5×10^5 in 200 μ l) were incubated with 2 nM DiOC₆(3) (Molecular Probes) at 37 °C for 10 min in binding buffer (140 mM NaCl, 2.5 mM CaCl₂, 10 mM HEPES/NaOH, and pH 7.4). Then, 0.5 μ g/ml annexin V-PE (Caltag)

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were added and incubated at room temperature for 15 min. Cell suspension was diluted to 1 ml with binding buffer and analysed in a flow cytometer (Epics XL-MCL, Beckman/Coulter). Changes in the levels of intracellular Apo2L/TRAIL during the IFN- α treatment were evaluated by flow cytometry. Cells (5×10^5) were cultured in complete medium (controls) or medium containing IFN- α for different times (12–48 h). Then, cells were washed and fixed with 1% paraformaldehyde in PBS (15 min, 4 °C) and incubated for 1 h at 20 °C with 1 μ g/ml anti-Apo2L/TRAIL (5C2, Genentech) in 50 μ l PBS containing 0.1% saponin. Cells were washed with PBS and then incubated for 30 min with a FITC-labelled anti-mouse IgG antibody (Caltag), diluted to 500 μ l and analysed by flow cytometry. Cell surface expression of IFN- α/β receptor was carried out by incubating cells (3×10^5) with 2 μ g/ml anti-IFNR2 antibody (MMHAR-2, Calbiochem) in 50 μ l PBS and 50 μ l human AB serum, at 20 °C for 30 min followed by incubation with an anti-IgG-FITC antibody and analysed by flow cytometry.

2.4. Western blot analysis

Caspase activation was analysed by Western blot by using a modification of the multiple blotting assay method, as described [10]. After the corresponding treatments cells were lysed in 50 mM Tris/HCl, pH 7.4 buffer containing 0.15 mM NaCl, 10% glycerol, 1 mM Na_3VO_4 , 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, 1 mM EDTA, 10 μ g/ml leupeptin, 1 mM PMSF, and 1% Triton X-100. Solubilized proteins from equal numbers of Trypan-blue negative cells (1×10^6 /lane) were resolved by SDS-15%PAGE, transferred to nitrocellulose membranes and incubated with primary antibodies diluted in TBS-T (10 mM Tris/HCl pH 8.0, 0.12 M, NaCl, 0.1% Tween-20, and 0.05% sodium azide), containing 5% skimmed milk, as described previously [11]. Primary antibodies anti human proteins used were: anti-caspase-8 (clone 5F7, Upstate), anti-procaspase-3 (clone 19) and anti-active caspase-3 (#557035), both from BD Biosciences and anti-Bid (AF860, R&D Systems). Membranes were washed with TBS-T and incubated with 0.2 μ g/ml of the corresponding phosphatase alkaline-labelled second-

ary antibody (Sigma) and revealed with BCIP/NBT, as described [11]. Control of protein loading was achieved by reprobing with anti β -actin (1/10000) (Sigma).

3. Results

3.1. Effect of IFN- α and Apo2L/TRAIL on myeloma cell proliferation

IM-9 and MM.1S cells were completely insensitive and RPMI 8226 cells showed a slight sensitivity to IFN- α (Fig. 1A). However, IFN- α exerted a significant apoptotic effect on U266 cells and NCI-H929 (around 30%). Analysis of surface IFN- α/β receptor expression by flow cytometry revealed that the low or no expression of IFN- α/β receptor in MM.1S and RPMI 8226 cells, respectively (Fig. 1B), could account for the reduced sensitivity of these cells to IFN- α . However, IM-9 cells, although refractory to IFN- α , express significant levels of IFN- α/β receptor (Fig. 1B). We also analysed the sensitivity of these cell lines to anti-Fas and to Apo2L/TRAIL. RPMI 8226 cells were the more sensitive to apoptotic effects of anti-Fas and Apo2L/TRAIL (DL_{50} : 2 ng/ml). U266 and IM-9 cells were resistant to Apo2L/TRAIL, but partially sensitive to anti-Fas (around 15% and 40% specific cell death, respectively). MM.1S showed a moderate susceptibility to both anti-Fas and Apo2L/TRAIL (18% and 34% apoptotic cells, respectively). Finally, H929 cells were sensitive to Apo2L/TRAIL and resistant to anti-Fas. In general, no correlation between the sensitivity to IFN- α and Apo2L/TRAIL was observed among MM cell lines.

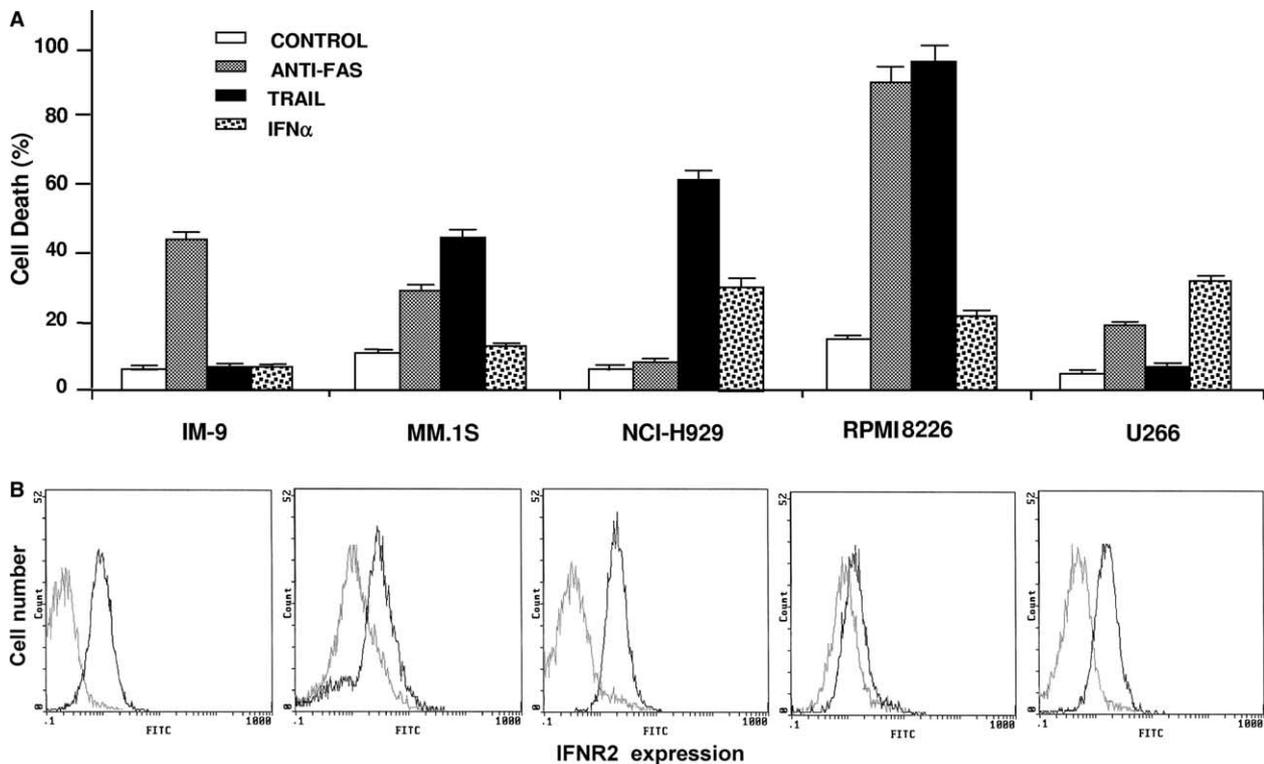


Fig. 1. Cytotoxicity of anti-Fas, Apo2L/TRAIL and IFN- α in myeloma cells. (A) Cells were incubated with anti-Fas (100 ng/ml), Apo2L/TRAIL (100 ng/ml) for 22 h or with IFN- α (2000 U/ml) for 48 h and apoptosis determined by annexin-PE binding and flow cytometry. (B) Cell surface expression of IFN- α/β receptor in myeloma cells. Cells were sequentially incubated with an anti-IFNR2 antibody and an anti-IgG1-FITC Ab (dark line) or with anti-human IgG1 Ab alone (light line) and analysed by flow cytometry.

We next evaluated the effect of the sequential treatment with IFN- α and Apo2L/TRAIL on the MM cell lines. Cells were treated for 22 h with Apo2L/TRAIL after a 48 h preincubation with IFN- α . IFN- α did not alter the sensitivity to Apo2L/TRAIL of most MM cell lines. In the case of H929 cells, IFN- α pre-treatment even reduced the toxicity of Apo2L/TRAIL (Fig. 2).

3.2. IFN- α induces release of Apo2L/TRAIL from normal monocytes but not from MM cells

No expression of Apo2L/TRAIL in plasma membrane was detected in any MM cell line, either in basal conditions or after IFN- α treatment (data not shown). However, MM cells expressed detectable amounts of intracellular Apo2L/TRAIL,

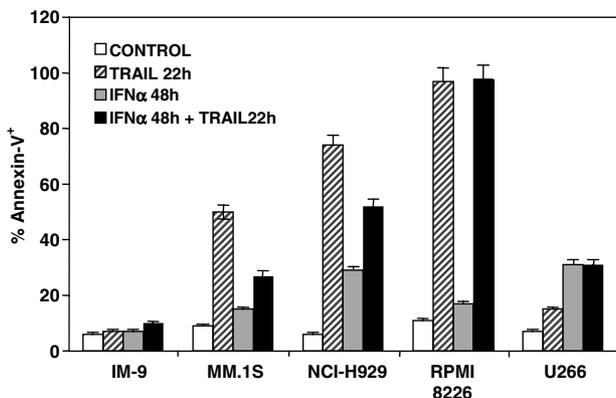


Fig. 2. Apoptosis induction in MM cell lines by sequential treatment with IFN- α and Apo2L/TRAIL. Cells (5×10^5 cells/ml) were cultured for 48 h in 24-well plates in the absence or presence of 2000 U/ml IFN- α . Then, 100 ng/ml Apo2L/TRAIL were added in some wells and cells incubated for a further 22 h. Apoptosis was determined by measuring annexin-PE binding by flow cytometry.

which transiently increased upon IFN- α treatment (Fig. 3A,B). No release of bioactive Apo2L/TRAIL to culture media could be detected in supernatants of IFN- α treated U266 or H929 cells with a sensitive bioassay [9] using RPMI 8226 cells as targets (data not shown). Also, no cell death above basal levels could be noticed in co-cultures of U266 and RPMI 8226 cells (not shown). Anyway, to rule out the possibility that the apoptotic effect of IFN- α in H929 cells (the only sensitive both to IFN- α and Apo2L/TRAIL) could be indirectly mediated by an autocrine loop involving Apo2L/TRAIL, H929 cells were co-incubated with IFN- α and with blocking anti- Apo2L/TRAIL antibodies (5C2) or with a DR5-Fc chimera. No reduction in IFN- α toxicity was observed in any case (Fig. 4A). However, normal human monocytes incubated with IFN- α under comparable conditions release to culture media bioactive Apo2L/TRAIL (Fig. 4B). Conditioned media from IFN- α treated monocytes was cytotoxic for RPMI 8226 (highly sensitive to TRAIL, insensitive to IFN- α) and to a much lesser extent for H929 cells (Fig. 4B). This conditioned media was not cytotoxic for U266 cells (resistant to Apo2L/TRAIL). Cytotoxicity was prevented by co-incubation with the blocking anti-Apo2L/TRAIL antibody 5C2, but not by the blocking anti-Fas mAb SM1/23 (Fig. 4B). These results indicate that IFN- α induces the release of Apo2L/TRAIL from monocytes, which in turn may cause apoptosis in myeloma cells.

3.3. Caspase activation induced by IFN- α or Apo2L/TRAIL.

Caspase 8 or 3 inhibitors (Z-IETD-fmk and Z-DEVD-fmk, respectively) or the pancaspase inhibitor Z-VAD-fmk fully prevented $\Delta\Psi_m$ loss, PS exposure and apoptosis induced by Apo2L/TRAIL in MM sensitive cells (Fig. 5A), according to the key role of these caspases in the extrinsic pathway. However, these inhibitors only have a partial or no protective effect on $\Delta\Psi_m$ loss, PS exposure and apoptosis induced by IFN- α (Fig. 5B). Replenishment of peptide caspase inhibitors each

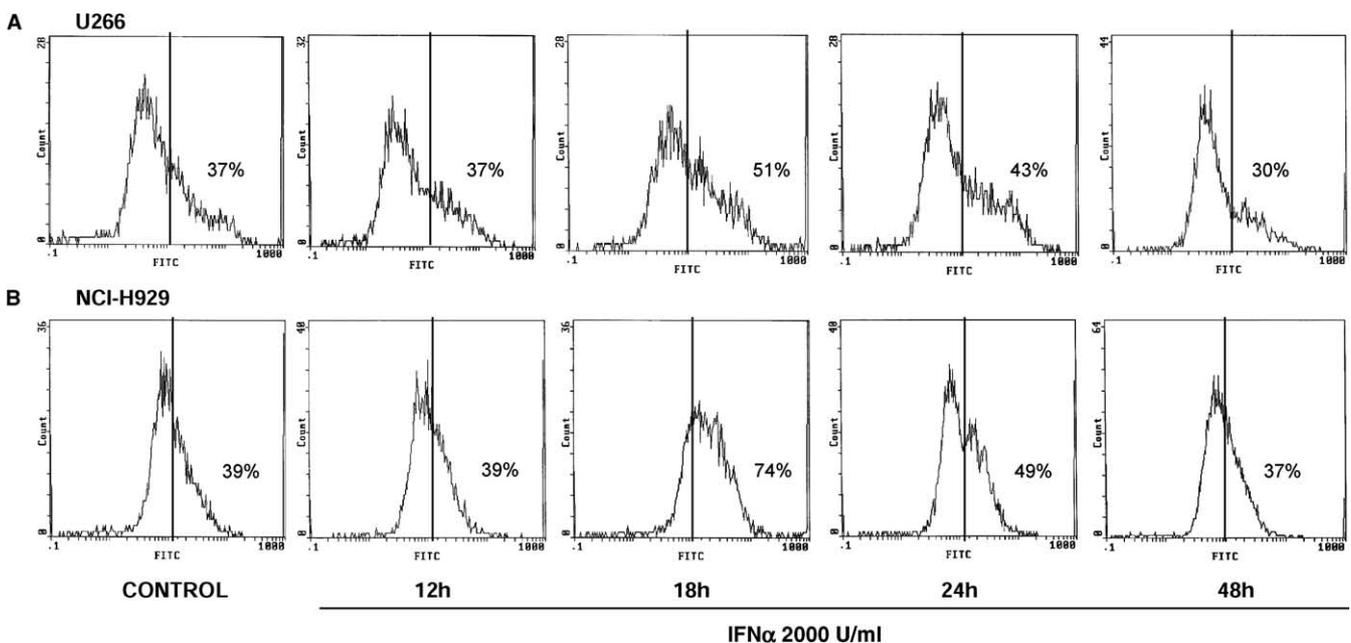


Fig. 3. Effect of IFN- α treatment on intracellular Apo2L/TRAIL levels. U266 (A) and H929 (B) cells were treated with IFN- α for different times, as indicated, permeabilised and immunostained for Apo2L/TRAIL, and analysed by flow cytometry.

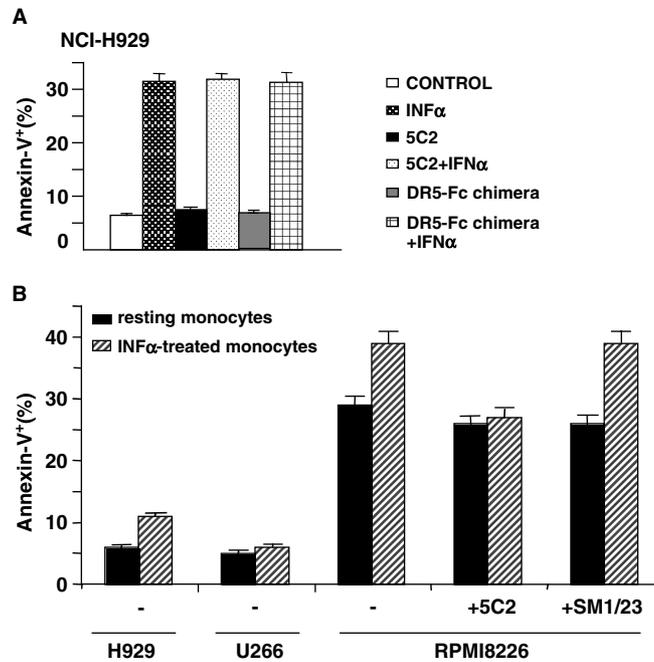


Fig. 4. Effect of neutralizing anti-TRAIL antibodies on IFN- α toxicity. (A) Toxicity of IFN- α (2000 U/ml) to H929 cells was analysed in the absence or presence of a blocking anti-TRAIL antibody (5C2) or a DR5-Fc chimera. After 48 h treatment, apoptosis was evaluated by annexin V-PE binding and flow cytometry. (B) Release of Apo2L/TRAIL by IFN- α treated human monocytes. Toxicity of culture supernatants of monocytes treated with IFN- α was bioassayed by using several myeloma cells as targets. Toxicity of the supernatants was analysed in the absence or presence of blocking anti-Apo2L/TRAIL (5C2) or anti-Fas (SM1/23) antibodies.

24 h did not improve survival of IFN-treated cells (not shown). Congruent with these results, caspase-8 and caspase-3, a substrate of caspase-8 in the death receptor pathway, became activated in sensitive H929 cells incubated with Apo2L/TRAIL. Caspase activation was characterized by a decrease in the amount of proenzyme and, in the case of caspase-3, the appearance of activation bands (Fig. 6). A reduction in the levels of Bid, a specific substrate of caspase-8, was also observed in Apo2L/TRAIL treated cells. However, no significant reduction in the levels of caspase-8, caspase-3 and Bid was detected in IFN- α treated cells (Fig. 6).

4. Discussion

IFN- α is used in maintenance therapy in patients with multiple myeloma, but its benefit is still a matter of controversy [3]. IFN- α can induce apoptosis in some MM cells [5], although most information relies in studies carried out with the U266 cell line. It has been reported that IFN- α induced apoptosis in this cell line is accompanied by caspase activation, $\Delta\Psi_m$ loss, release of cytochrome *c* [12] and Bak conformational change [13]. It has been also proposed that IFN- α induces apoptosis in myeloma cells by upregulating Apo2L/TRAIL expression [5]. However, the precise subcellular localization of Apo2L/TRAIL and its possible release was not analysed. In a recent work, and using different myeloma cell lines, it has been reported that IFN- α induces upregulation of PML gene expression and this correlated with Apo2L/TRAIL induction and release to culture media [7]. However, a measurement of the apoptosis directly induced by IFN- α and the released Apo2L/TRAIL on MM cell lines was not performed. Here

we show that IFN- α and Apo2L/TRAIL cause different effects on myeloma cells and in particular they induce apoptosis through essentially different mechanisms. U266 cells, the most sensitive to apoptotic effects of IFN- α were insensitive to Apo2L/TRAIL and to cytotoxic anti-Fas antibodies. Moreover, U266 treated with IFN- α for two days were not sensitized to Apo2L/TRAIL. Conversely, culture supernatants of U266 cells treated with IFN- α were not cytotoxic in bioassays on Jurkat and RPMI 8226, which are very sensitive to the apoptotic effects of FasL and Apo2L/TRAIL, respectively ([9] and this work). The intracellular apoptotic pathways elicited by IFN- α and Apo2L/TRAIL were also different since Z-IETD-fmk (an inhibitor of caspase-8) efficiently blocked apoptosis induced by Apo2L/TRAIL but not that induced by IFN- α . A previous work [5] indicated that a stable transformant, obtained after extensive selection and cloning, expressing a dominant-negative DR5 receptor was insensitive to both Apo2L/TRAIL and IFN α / β . However, our results using a DR5-Fc chimera indicate that this protein blocks apoptosis induced by Apo2L/TRAIL but not that induced by IFN- α (Fig. 4A). Since the expression of IFN receptor in the transformant was not assayed, the possibility that resistance to IFN could be due to reduced IFNR expression cannot be ruled out.

Therefore, IFN- α and Apo2L/TRAIL induce apoptosis through essentially independent mechanisms in myeloma cells and, in particular, Apo2L/TRAIL is not the direct mediator of the cytotoxic effects of IFN- α . However, these results do not exclude Apo2L/TRAIL as a relevant mediator of IFN- α anti-tumor effect in vivo. It has been reported that host CD4⁺ T and NK cells, neutrophils, and mainly monocytes increase gene expression and/or release of Apo2L/TRAIL upon IFN-

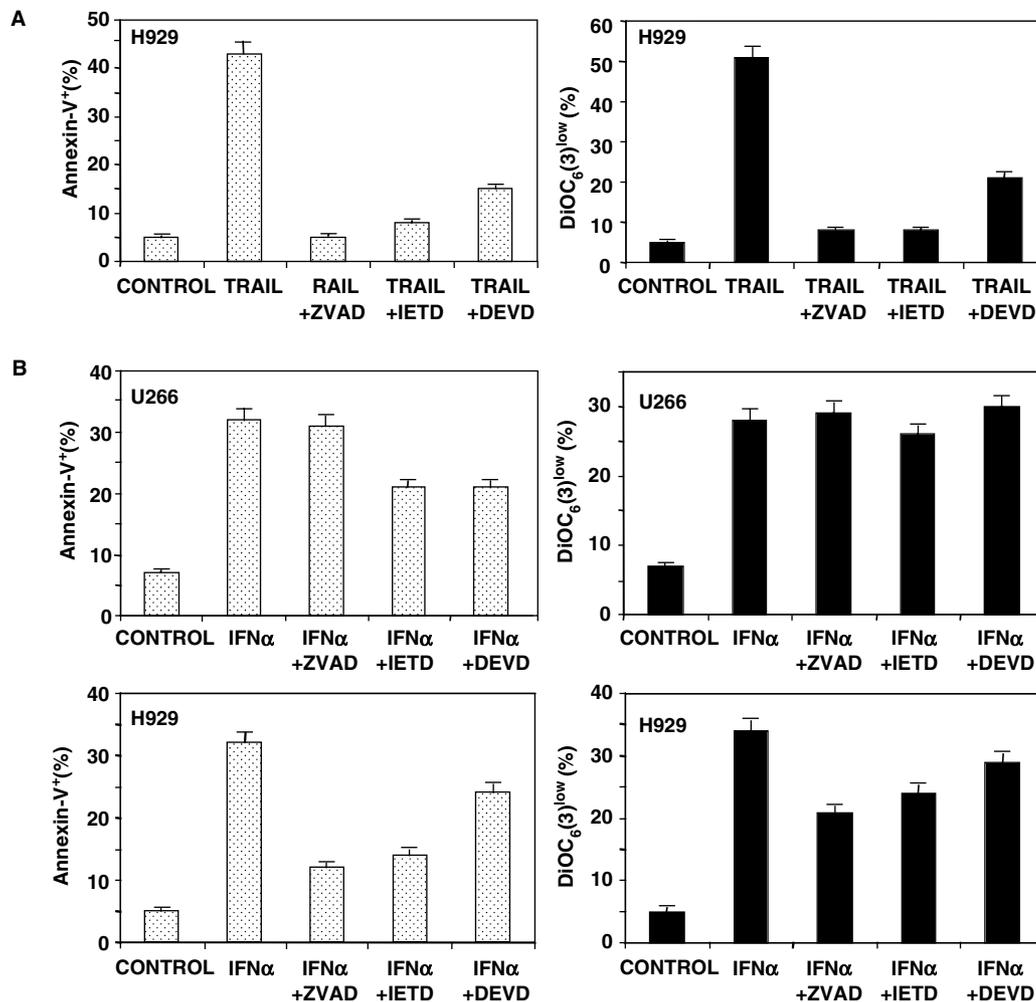


Fig. 5. Effect of caspase inhibitors on IFN- α and Apo2L/TRAIL-induced apoptosis. Toxicity of Apo2L/TRAIL on H929 cells (A) and IFN- α on U266 and NCI-H929 cells (B) was evaluated in the presence of Z-DEVD-fmk, Z-IETD-fmk or Z-VAD-fmk (all at 100 μ M). Inhibitors were added 1 h prior to treatment with IFN- α (48 h) or Apo2L/TRAIL (22 h). PS exposure and loss of $\Delta\Psi_m$ were analysed by flow cytometry.

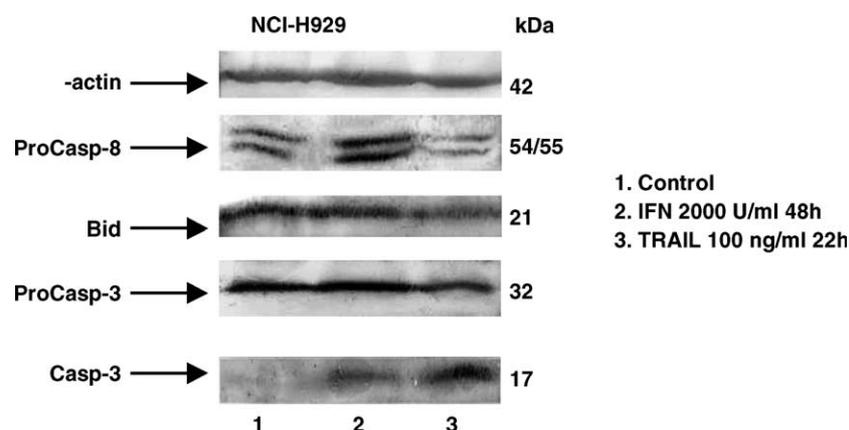


Fig. 6. Distinct pattern of caspase-8 and caspase-3 activation after IFN- α or Apo2L/TRAIL treatment. H929 cells were treated with IFN- α (2000 U/ml, 48 h) or Apo2L/TRAIL (100 ng/ml, 22 h) and caspase activation and Bid levels analysed by Western blotting with specific antibodies for procaspase-8, procaspase-3, active caspase-3 (17 kDa band) and Bid. β -Actin levels serve as control for equal protein loading.

α/β treatment [14–19]. According to this, our present results show that stimulation of monocytes with IFN- α induces the secretion of significant amounts of bioactive Apo2L/TRAIL,

which causes apoptosis in MM cells, even in some that are insensitive to IFN- α . Therefore, this effect would be more important in vivo than the moderate apoptotic effect directly

exerted by IFN- α on MM cell lines. If this was the case, then Apo2L/TRAIL would be a good candidate to replace IFN- α in maintenance/consolidation therapy in multiple myeloma patients.

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