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IFN- α Is a Survival Factor for Human Myeloma Cells and Reduces Dexamethasone-Induced Apoptosis¹

Martine Ferlin-Bezombes,* Michel Jourdan,* Janny Liautard,* Jean Brochier,* Jean-François Rossi,[†] and Bernard Klein^{2*‡}

IFN- α is used as a maintenance therapy in patients with multiple myeloma, but its benefit is a matter of controversy. In vitro studies show that IFN- α can both stimulate and inhibit myeloma cell proliferation. We have tested the effect of IFN- α on the survival of myeloma cell lines and primary plasma cells. IFN- α significantly reduced the apoptosis induced by removal of IL-6 in four IL-6-dependent myeloma cell lines. It also reduced the level of apoptosis induced by dexamethasone in these cell lines as well as in purified primary myeloma cells from seven patients. IFN- α promoted the survival of myeloma cells, which, following removal of IL-6, were blocked in G1 and died. However, unlike IL-6, IFN- α -treated cells remained mainly blocked in the G1 phase of the cycle. While the effects of IL-6 are mediated through stimulation of its gp130 receptor subunit, the IFN- α -induced survival of myeloma cells was independent of gp130 transducer activation (as demonstrated using a neutralizing anti-gp130 Ab). However, the signal transduction cascades activated by these two cytokines share at least some common elements, since stimulation with either IFN- α or IL-6 resulted in STAT3 phosphorylation. These results indicate that IFN- α promotes the survival, but not the proliferation, of myeloma cells, preventing the apoptosis induced by removal of IL-6 or addition of dexamethasone. This survival factor activity may explain the conflicting reports on the effects of IFN- α on myeloma cell proliferation. *The Journal of Immunology*, 1998, 161: 2692–2699.

The benefit of IFN- α as a maintenance therapy in patients with multiple myeloma (MM)³ is a matter of debate. As recently reviewed by the European Myeloma Research group (1), this cytokine had a beneficial effect in three randomized studies, whereas in three other protocols, it had no effect. Preliminary reports suggested that IFN- α could be of benefit as a maintenance treatment following high dose chemotherapy or in combination with corticosteroids (2, 3). The controversy concerning the in vivo use of IFN- α is further emphasized by in vitro studies showing that under various conditions IFN- α can either stimulate or inhibit myeloma cell proliferation (4–8). IL-6 and more generally the cytokines, which activate the gp130 receptor subunit, are the main survival and growth factors of malignant plasma cells (9). In particular, myeloma cell lines whose survival and growth are dependent upon addition of exogenous IL-6 can be reproducibly obtained from patients with terminal disease (10, 11). As IFN- α shares some common transduction pathways with IL-6 (12–15), we compared its effect on the survival of myeloma cells with that of IL-6.

Using both IL-6-dependent myeloma cell lines (11) and primary myeloma cells, we demonstrate that IFN- α is a survival factor for malignant plasma cells. IFN- α reduced the apoptosis induced by removal of IL-6 in all four myeloma cell lines studied. It also inhibited dexamethasone (DEX)-induced apoptosis of the myeloma cell lines and of primary myeloma cells.

Materials and Methods

Patients

Tumor samples were obtained from seven patients (no. 1–7) with MM (median age, 57 yr) after written informed consent was received. According to the Durie-Salmon classification, three patients were stage IIIB, and four were stage IIIA. Two patients had IgG1 MM, one had IgGk MM, three had Bence-Jones 1 MM, and one had Bence-Jones k MM.

Reagents

Recombinant IL-6 was provided by Dr. Ytier (Ares Sero, Geneva, Switzerland). Recombinant IFN- α 2b was provided by Shering-Plough (Levallois-Perret, France). DEX was purchased from Sigma (St. Louis, MO) and was dissolved in ethanol at a concentration of 10^{-2} M, filtered, and further diluted in RPMI 1640. The neutralizing (A1) and nonneutralizing (G4) mAbs to gp130 have been previously described (16). A1 has been shown to neutralize the activity of IL-6, leukemia inhibitory factor, oncostatin M, and ciliary neurotropic factor (16). An anti-IFN- α -neutralizing sheep polyclonal Ab was the gift of Dr. G. Uze (Centre National de la Recherche Scientifique, Montpellier, France). Rabbit phospho-specific STAT1 and STAT3 Abs were purchased from New England Biolabs (Beverly, MA), mouse anti-STAT1 and STAT3 were obtained from Transduction Laboratories (Lexington, KY). Abs to Bcl-2 (124) were purchased from Dako (Carpinteria, CA), anti-Bcl-X_L (S-18) and anti-Bax (SC-493) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), peroxidase-conjugated goat anti-mouse Abs were purchased from Bio-Rad (Hercules, CA), and peroxidase-conjugated goat anti-rabbit Abs and control purified murine IgG1 were obtained from Sigma.

Isolation of primary myeloma cells

Patients' myeloma cells were purified using the anti-myeloma cell MI15 mAb and Dynal magnetic beads (Dynal M450, Dynal, Oslo, Norway) coated with sheep anti-mouse IgG as previously described in detail (17). The MI15 mAb recognizes syndecan-1, which is present only on myeloma

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³ Abbreviations used in this paper: MM, multiple myeloma; DEX, dexamethasone; HMCL, human myeloma cell lines; PI, propidium iodide; TBS-T, 138 mM NaCl, 3 mM KCl, 25 mM Tris-HCl (pH 7.4), and 0.1% Tween-20; MAP, mitogen-activated protein.

cells (18, 19). This procedure yielded cell populations comprised of >95% viable myeloma cells. Purified myeloma cells were resuspended in RPMI 1640 medium supplemented with 10% FCS.

Human myeloma cell lines (HMCL)

Four HMCL were studied: XG-1, XG-2, XG-4, and XG-6. All had cytoplasmic Ig, expressed plasma cell Ags (CD38 and syndecan-1), and lacked the usual B cell Ags (CD19 and CD20). Their growth was completely dependent on addition of exogenous IL-6. Detailed characteristics of these lines have been reported previously (11). U266 and RPMI 8226 HMCL were purchased from American Type Culture Collection (Manassas, VA).

Cell culture

Cells were grown in RPMI 1640 medium with 10% FCS and/or cytokines. To investigate the effects of cytokines (IFN- α , IL-6) and DEX on apoptosis, cells were washed once with culture medium, incubated for 5 h at 37°C in culture medium alone, and then washed twice to remove rIL-6. They were then cultured at a cell concentration of 3×10^5 cells/ml either without exogenous cytokine or with IFN- α (100 U/ml), IL-6 (3 ng/ml), or IFN- α (100 U/ml) and IL-6 (3 ng/ml). The same culture conditions were used in the presence or the absence of DEX (10^{-7} M). In some culture groups, cells were cultured with 150 μ g of an anti-gp130-neutralizing (A1) or nonneutralizing (G4) IgG1 mAb or control murine IgG1.

Proliferation and growth assay of myeloma cell lines

To investigate the effects of IFN- α and/or IL-6 in the presence or the absence of neutralizing or nonneutralizing anti-gp130 transducer mAb on the proliferation of HMCL, cells were washed to remove bound rIL-6. They were cultured for 5 h with culture medium, washed again, and incubated in 96-well flat-bottom microplates for 5 days with either culture medium alone or the different cytokines and/or mAbs. Tritiated thymidine (0.5 μ Ci/mM; CEA, Saclay, France) was added for the last 8 h of culture, and incorporation was determined on a beta scintillation counter.

Assays for detection of apoptotic cells

Apoptosis was assessed by propidium iodide (PI) or annexin V staining. To detect apoptotic cells by PI staining, cells were washed twice with PBS,

resuspended in 70% ethanol, and incubated at 4°C overnight. After two washes with PBS, cells were resuspended in 940 μ l of PBS, 10 μ l of RNase A (10 mg/ml; Boehringer Mannheim, Meylan, France), and 50 μ l of PI (1 mg/ml; Sigma) and incubated in the dark at room temperature for 30 min at 37°C before flow cytometric analysis on a FACScan apparatus (Becton Dickinson, San Jose, CA). Apoptotic cells were also detected using annexin V-fluos (Boehringer Mannheim), which has a high affinity for phosphatidylserine present in the outer membrane of apoptotic cells (20). Cells were washed twice in PBS and resuspended in 100 μ l of labeling solution containing 2 μ l of annexin and 2 μ l of PI (50 μ g/ml) in HEPES buffer (HEPES/NaOH, pH 7.4; 140 mM NaCl; and 5 mM CaCl₂) for 20 min at room temperature. After two washes with HEPES buffer, the fluorescence of FL1-H (annexin V-fluos) and FL2-H (PI) was analyzed on a FACScan flow cytometer (Becton Dickinson).

Cell stimulation and Western blot analysis

To assay for IFN- α - or IL-6-mediated signal transduction in myeloma cells, XG cells were washed twice and cultured (2×10^6 cells/ml) without IL-6 for 12 h in RPMI 1640 and 1% FCS at 37°C. Cells were then stimulated with IFN- α (500 U/ml) or IL-6 (25 ng/ml) for 15 min at 37°C and immediately lysed at 4°C in 10 mM Tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 1% Triton X-100, 5 mM ZnCl₂, 100 mM Na₃VO₄, 1 mM DTT, 20 mM β -glycerophosphate, 20 mM *p*-nitrophenolphosphate, 2.5 mg/ml aprotinin, 2.5 mg/ml leupeptin, 0.5 mM PMSF, 0.5 mM benzamide, 5 mg/ml pepstatin, and 50 mM okadaic acid. After centrifugation at $14,000 \times g$, the supernatant was resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Membranes were blocked for 1 h at room temperature in 138 mM NaCl, 3 mM KCl, 25 mM Tris-HCl (pH 7.4), and 0.1% Tween-20 (TBS-T) containing 5% BSA. They were then incubated for 1 h at room temperature with primary Ab (phospho-specific STAT1 or STAT3 at a 1/1000 dilution), and the primary Abs were visualized with a peroxidase-conjugated goat anti-rabbit Ab (Sigma) and enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). The membranes were stripped by two 30-min incubations in a solution containing 100 mM glycine (pH 2.2), 0.1% Nonidet P-40, and 1% SDS. They were reprobbed with anti-STAT1 or anti-STAT3 Abs (at a 1/2000 dilution in 1% BSA TBS-T), and the Abs were visualized with peroxidase-conjugated

FIGURE 1. IFN- α is a survival factor for myeloma cells. XG-4 HMCL were cultured for 72 h at a concentration of 3×10^5 cells/ml in culture medium supplemented with 10% FCS either without exogenous cytokine or with IL-6 (3 ng/ml), IFN- α (100 U/ml), or IFN- α (100 U/ml) and IL-6 (3 ng/ml). **A**, Apoptotic cells were detected by PI staining. The percentages of apoptotic cells (DNA content less than that in G1 cells) and of cells in different phases of the cell cycle were determined using ModFitLT software. **B**, Apoptosis was also assessed by labeling with FITC-annexin V and PI. Early apoptotic cells were stained by FITC-annexin V alone (FL-1 H), whereas late apoptotic cells were stained by both FITC-annexin V and PI (FL-2 H).

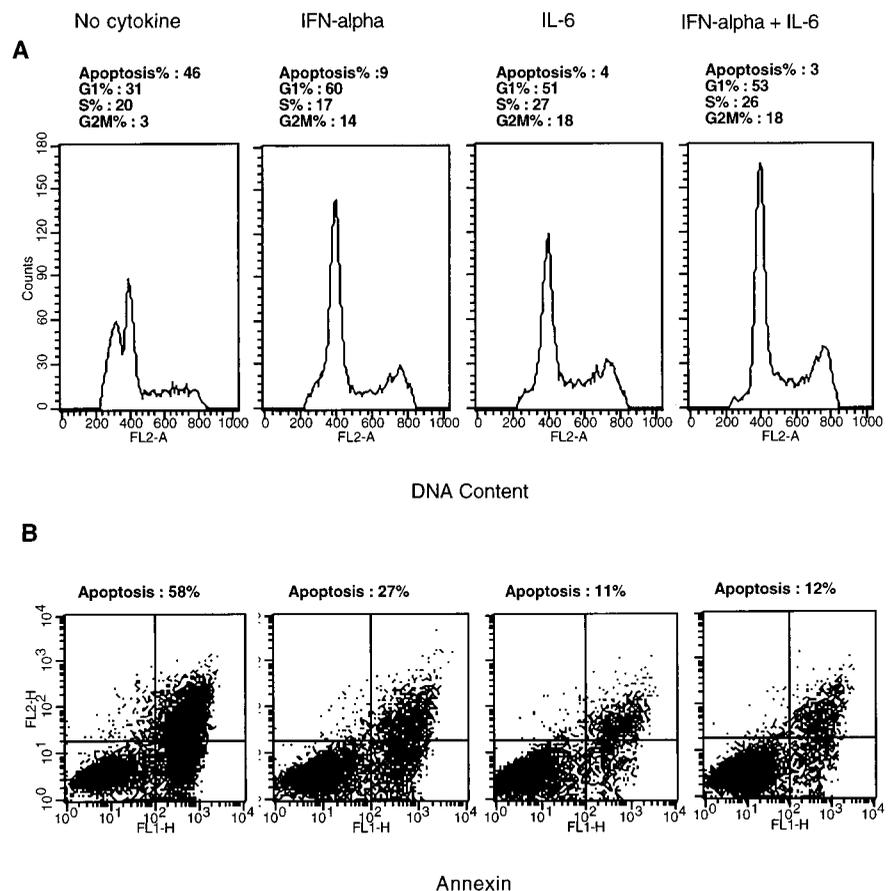


Table I. IFN- α is a survival factor^a

| Expt. | Apoptosis (%) | | | |
|-------|---------------|---------------|------|----------------------|
| | No cytokine | IFN- α | IL-6 | IFN- α + IL-6 |
| 1 | 31 | 14 | 7 | 8 |
| 2 | 34 | 17 | 6 | 7 |
| 3 | 36 | 8 | 8 | 7 |
| 4 | 25 | 12 | 4 | 6 |
| 5 | 46 | 9 | 4 | 3 |

^a XG-4 HMCL were extensively washed to remove exogenous IL-6. They were then cultured in medium supplemented with 10% FCS without exogenous cytokine or in the presence of IL-6 (3 ng/ml), IFN- α (100 U/ml), or IFN- α (100 U/ml) and IL-6 (3 ng/ml). The percentage of apoptotic cells was determined after 72 h of culture by analysis of PI-stained cells on a FACScan apparatus. Results of five separate experiments are shown. Percent apoptosis in each group was compared using a paired *t* test. No cytokine/IFN- α , $p = 0.007$; no cytokine/IL-6, $p = 0.022$; IL-6/IFN- α + IL-6, not significant.

goat anti-mouse Abs followed by enhanced chemiluminescence. In separate experiments, other membranes were probed with Abs to Bcl-2, Bcl-X_L, Bax, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that were all used at a 1/1000 dilution in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween-20/5% milk. Peroxidase-conjugated secondary Abs were then used at a 1/1000 dilution, and reactivity was revealed by enhanced chemiluminescence and was quantified using the Intelligent Quantifier (Bio Image, Ann Arbor, MI).

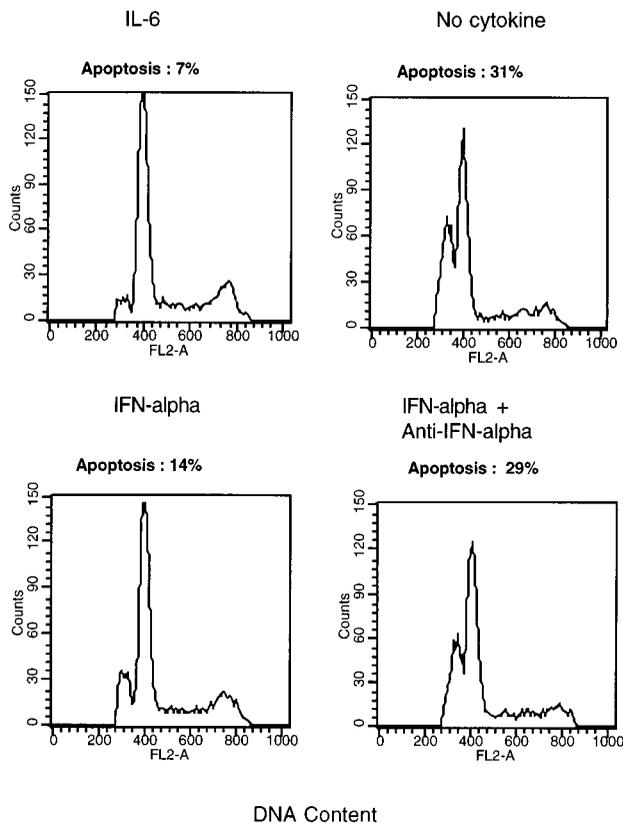


FIGURE 2. IFN- α -induced survival is inhibited by an anti-IFN- α antibody. XG-4 HMCL were cultured for 72 h at a concentration of 3×10^5 cells/ml in medium supplemented with 10% FCS either without exogenous cytokine or with IL-6 (3 ng/ml), IFN- α (100 U/ml), or IFN- α (100 U/ml) and an anti-IFN- α Ab. IFN- α was preincubated with 10 μ g/ml of the anti-IFN- α Ab for 2 h before addition to the culture. Apoptotic cells were detected by FACScan after staining with PI and were analyzed with ModFitLT software.

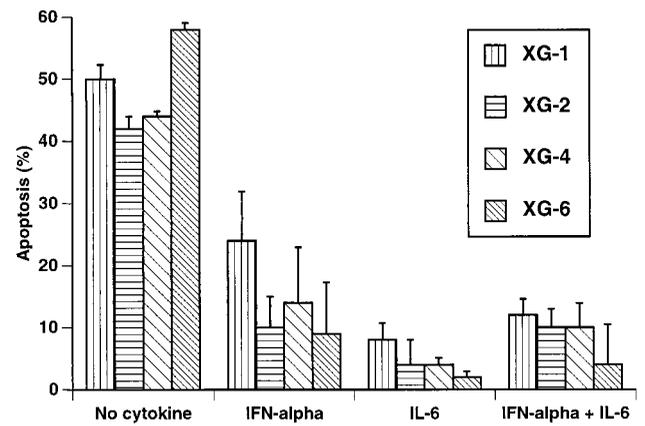


FIGURE 3. Effect of IFN- α on survival of IL-6-dependent myeloma cells lines XG-1, XG-2, XG-4, and XG-6. HMCL were extensively washed to remove exogenous IL-6. They were cultured in medium supplemented with 10% FCS either without exogenous cytokine or with IL-6 (3 ng/ml), IFN- α (100 U/ml), or IFN- α (100 U/ml) and IL-6 (3 ng/ml). The percent apoptosis was determined for the four HMCL after 72 h of culture. Apoptotic cells were detected by FACScan analysis after staining with PI. Results are the mean \pm SD of five experiments for each HMCL. The results of each of the five experiments are shown in Table I for the XG-4 HMCL.

Statistical analysis

For a given cell line, each experiment was repeated five times to compare apoptosis in different culture groups, and significance was assessed by paired *t* test.

Results

IFN- α is a survival factor for cytokine-dependent myeloma cell lines

To investigate the effect of IFN- α on myeloma cell survival, we used cell lines whose survival and proliferation are dependent on addition of exogenous IL-6. As cells necrotized by day 4 after IL-6 removal, apoptosis was studied on day 3 in the following experiments. Apoptosis was evaluated by PI staining of DNA content and additionally by combined annexin V and PI staining (see *Materials and Methods*). The two methodologies yielded similar results, although apoptotic cells were detected earlier with annexin V, as illustrated in Figure 1 for XG-4 HMCL. Upon removal of IL-6, approximately 50% of the myeloma cells died by apoptosis within 3 days, and IFN- α significantly reduced the percentage of apoptosis in five separate experiments (Fig. 1 and Table I). This effect was specific to IFN- α , as it was inhibited by neutralizing Abs to IFN- α (Fig. 2). Similar results were found for all four IL-6 dependent cell lines in five separate experiments as illustrated in Figure 3. The survival effect of IFN- α was maximal at a concentration of 100 U/ml and did not change with 1,000 or 10,000 U/ml. Results for the XG-1 HMCL are shown in Figure 4. IL-6 induced maximal survival at a concentration of 1 ng/ml. For the same XG HMCL, the survival activity of IFN- α was generally weaker than that of IL-6 (Table I and Fig. 3), but in some experiments, IFN- α was as potent as IL-6 (Table I and Fig. 4A). In addition, the survival activities of IFN- α and IL-6 were additive when suboptimal concentrations of IL-6 were used (Fig. 4B).

IFN- α reduces DEX-induced apoptosis in myeloma cell lines

The effect of IFN- α on myeloma cell survival led us to assess whether this cytokine could reduce apoptosis induced by DEX. DEX increased the level of apoptosis in myeloma cells cultured for 24 or 48 h without cytokines (Figs. 5 and 6). Both IL-6 and IFN- α

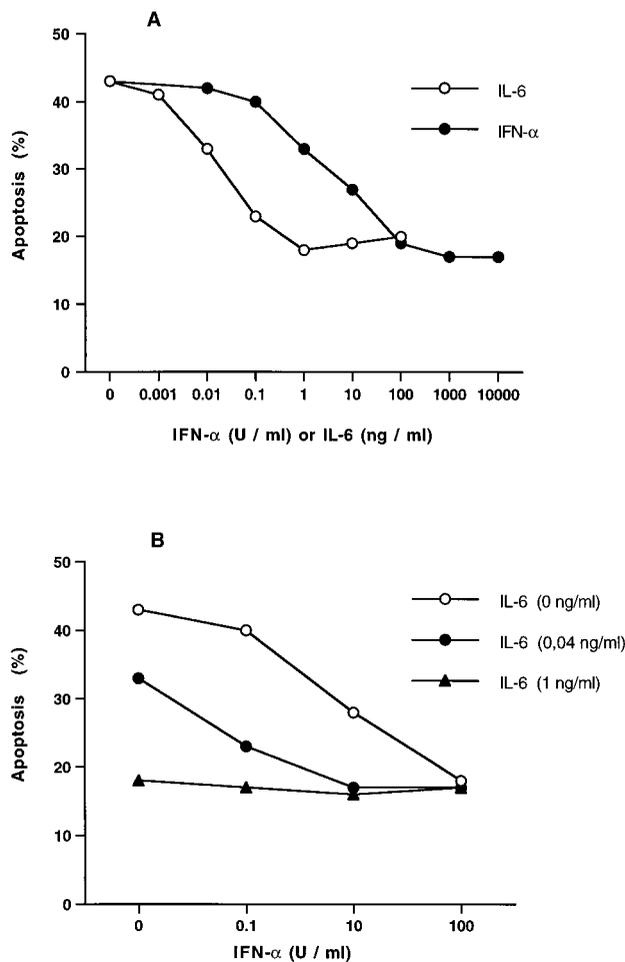


FIGURE 4. Dose effect of IFN- α on myeloma cell survival. XG-1 cells were extensively washed to remove exogenous IL-6. They were cultured at 3×10^5 cells/ml in medium supplemented with 10% FCS either without exogenous cytokine or with various concentrations of IFN- α or IL-6 (A) or with IFN- α (100 U/ml) and various concentrations of IL-6 (B). Apoptotic cells were detected by analysis of FITC-annexin-stained cells on a FACScan apparatus. Data are representative of one of three separate experiments.

significantly reduced the level of DEX-induced apoptosis, especially for XG-2 and XG-6 HMCL, but IL-6 was generally more efficient than IFN- α (Figs. 5 and 6). Although IFN- α induced a survival activity on IL-6-dependent HMCL, this phenomenon was not observed in autonomously growing HMCL, such as U266 or RPMI 8226. It was not possible to assess the effect of IFN- α on DEX-induced apoptosis in the IL-6-independent lines, since, in contrast to a previous report (21), we did not find that DEX induced apoptosis in either U266 or in RPMI 8226 (results not shown).

IFN- α reduces DEX-induced apoptosis in primary myeloma cells

We next investigated the effect of IFN- α on the survival and DEX-induced apoptosis of primary plasma cells purified from seven myeloma patients. Following purification, 95% of the cell populations were viable myeloma cells. Detailed results obtained with myeloma cells from one patient are shown in Figure 7 and summaries of the results with myeloma cells from the seven patients are presented in Table II. DEX induced a strong apoptosis in purified primary myeloma cells, while IL-6 significantly inhibited this ef-

fect. For six of the seven patients, IFN- α also significantly reduced DEX-induced apoptosis (Fig. 7 and Table II).

IFN- α is a gp130-independent myeloma cell survival factor

In a previous report, we showed that a weak proliferation of the XG-1 myeloma cell line in the presence of IFN- α was due to autocrine production of IL-6 (6). As IL-6 and other cytokines that activate signaling cascades through gp130 are the major survival factors for myeloma cells (9, 22, 23), we investigated whether the anti-apoptotic effect of IFN- α was mediated through the autocrine production of gp130 cytokine in the XG-1 and XG-6 cell lines. A high concentration (150 μ g/ml) of a neutralizing (A1) anti-gp130 mAb was used to block gp130 activation, and a nonneutralizing anti-gp130 mAb (G4) was used as a control. The anti-apoptotic effect of IL-6 was completely inhibited by the neutralizing A1 anti-gp130 mAb, but was unaffected by the nonneutralizing G4 mAb (Figs. 8, A and B). In contrast, the anti-apoptotic effect of IFN- α was not affected by the anti-gp130-neutralizing mAb (Fig. 8, A and B). These data indicate that the myeloma cell survival activity of IFN- α was independent of gp130 transducer activation. The neutralizing anti-gp130 mAb inhibited the weak proliferation obtained in the presence of IFN- α by approximately 50%, suggesting that it was mediated through an autocrine gp130 cytokine (Fig. 8, A and B). To further study the effect of IFN- α on proliferation, we took advantage of a property of the XG-2 HMCL to accumulate in the G1 phase of the cell cycle upon removal of IL-6 (Fig. 9). In the other myeloma cell lines, although removal of IL-6 induced apoptosis, only a partial blockage of the cell cycle was observed (Fig. 1). IFN- α was a survival factor for XG-2 cells, but unlike IL-6, IFN- α -treated cells were mainly blocked in G1 (Fig. 9). This blockage was reversible, since addition of IL-6 together with IFN- α resulted in their entry into the cell cycle.

To better investigate some signal transduction cascades that were activated by IFN- α in these myeloma cells, we assessed whether the transcriptional activators, STAT1 and STAT3, were stimulated following treatment with this cytokine. It has been previously shown that stimulation of gp130 results in phosphorylation and activation of both STAT1 and STAT3 (24). Additionally, IFN- α has been found to activate STAT1 and STAT3 in myeloma cells (7). After culture under conditions where cells were deprived of IL-6 for 16 h, addition of IFN- α induced a phosphorylation of the STAT1 and STAT3 activators in the XG-2 HMCL, but only STAT3 activation was detected in the XG-1 HMCL (Fig. 10). IL-6 also induced phosphorylation of STAT1 and STAT3 in XG-2 HMCL and of STAT3 in XG-1 HMCL (Fig. 10).

Discussion

In this study, we demonstrate that IFN- α is a survival factor for IL-6-dependent myeloma cell lines that are induced to apoptosis upon removal of IL-6. IFN- α also blocked the apoptosis induced by DEX in these cell lines and in primary cells from myeloma patients. For the different cell lines, we found that a maximal survival activity was obtained with 100 U/ml of IFN- α and did not change at concentrations up to 10,000 U/ml.

It was important to elucidate whether the survival factor activity of IFN- α was dependent upon a low production of IL-6 or another gp130-activating cytokine. Indeed, IL-6 or the gp130 transducer-activating cytokines are the main survival factors for myeloma cells (25). In addition, we have previously shown that a weak proliferation of the XG-1 myeloma cell line in the presence of IFN- α was due to an autocrine production of IL-6 (6). In the presence of a high concentration of an anti-gp130 mAb that completely inhibited the biologic activities of IL-6 and other gp130 activating

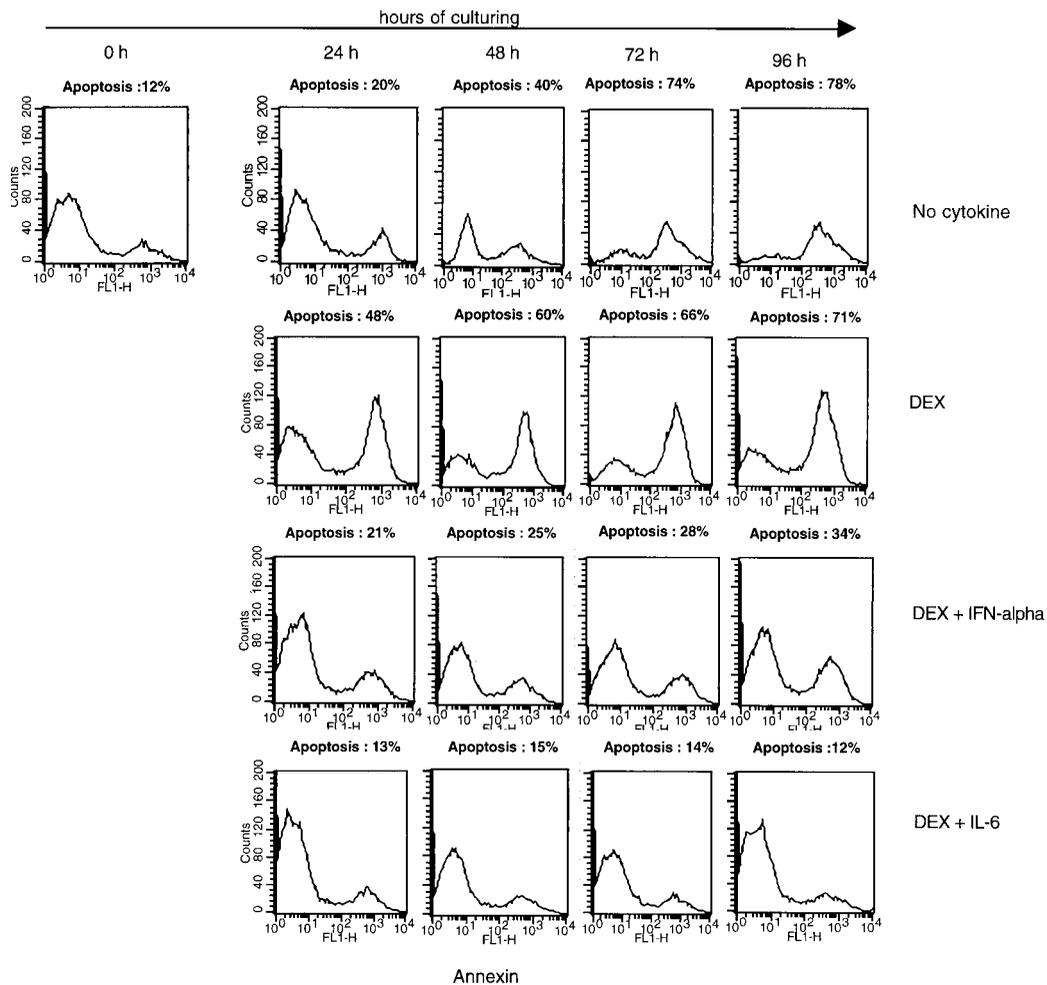


FIGURE 5. DEX-induced apoptosis in the XG-6 HMCL is inhibited by IFN- α . XG-6 cells were cultured at a concentration of 3×10^5 cells/ml in medium supplemented with 10% FCS and DEX (10^{-7} M) in the absence or the presence of IFN- α (100 U/ml) or IL-6 (3 ng/ml). Cells were recovered after 24, 48, 72, and 96 h of culture. Apoptotic cells were detected by analysis of FITC-annexin-stained cells on a FACScan apparatus.

cytokines (16, 22), IFN- α -induced survival was not affected. In contrast, this neutralizing anti-gp130 mAb inhibited the weak proliferation obtained in the presence of IFN- α by approximately 50%. It also completely inhibited the survival and growth factor activity of IL-6. These data strongly suggest that the observed IFN- α -induced survival is independent of gp130 transducer activation and that IFN- α is not a myeloma cell proliferation factor. This is further supported by data obtained with the XG-2 HMCL. In the presence of IL-6, XG-2 myeloma cells survived and highly proliferated. Upon removal of IL-6, XG-2 cells were blocked in the G1 phase and then died. IFN- α could promote the survival of these cells, but they remained mainly blocked in the G1 phase of the cell cycle. These data are in agreement with a recent report showing that IFN- α blocked Fas-induced apoptosis in autonomously growing lymphoblastoid and myeloma cell lines (26). The mechanism regulating this process in myeloma cells has not yet been determined, and we failed to find reproducible changes in the levels of the Bcl-2 or Bcl-X_L antiapoptotic or Bax proapoptotic proteins following addition of IFN- α (results not shown).

We have now identified two cytokines, IFN- α and IL-6, that are myeloma cell survival factors. The transduction pathways induced by gp130 transducer activation have become better characterized. Dimerization of gp130 by IL-6 induces phosphorylation of tyrosine residues on JAK kinases that are preassociated with gp130 (13, 24). This allows the recruitment of STAT1 and STAT3, and

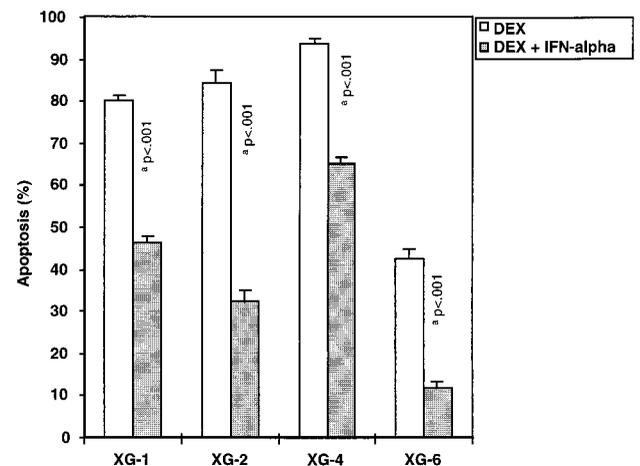


FIGURE 6. DEX-induced apoptosis is inhibited by IFN- α in IL-6-dependent myeloma cell lines. XG-1, XG-2, XG-4, and XG-6 HMCL were cultured at a concentration of 3×10^5 cells/ml in culture medium supplemented with 10% FCS and DEX (10^{-7} M) in the absence or the presence of IFN- α (100 U/ml). XG-1, XG-4, and XG-6 cells were cultured for 2 days, while XG-2 were cultured for 3 days, conditions that resulted in a maximum increase in apoptosis with DEX. Apoptotic cells were detected on a FACScan apparatus after staining with FITC-annexin. Results are the mean \pm SD of five experiments. *p* values (a) determined by paired *t* tests are shown.

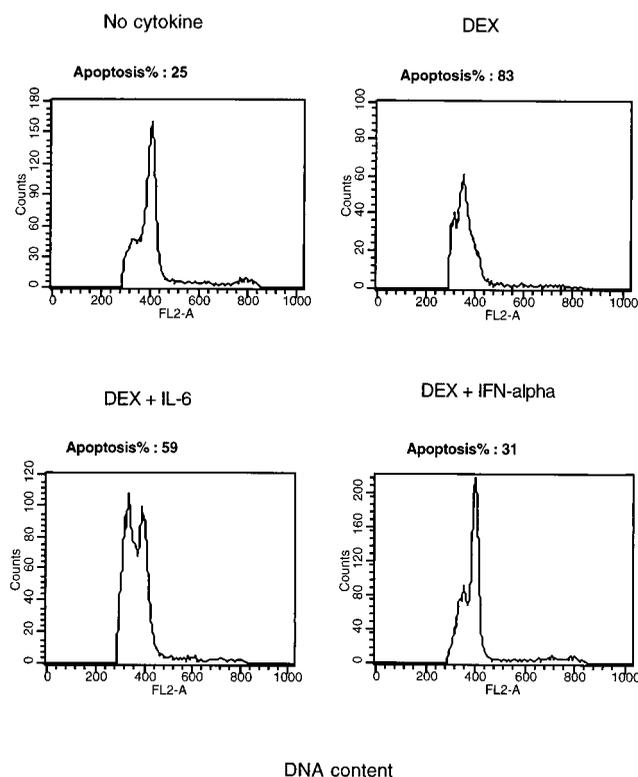


FIGURE 7. Effect of IFN- α on DEX-induced apoptosis in primary plasma cells. Myeloma cells from patient 1 were purified using the anti-syndecan-1 MI15 mAb as described in *Materials and Methods*. After purification, cells were extensively washed to remove exogenous IL-6 and were cultured in medium supplemented with 10% FCS in the absence or the presence of DEX (10^{-7} M), IL-6 (3 ng/ml), and IFN- α (100 U/ml). Apoptotic cells were detected after 72 h of culture on a FACScan apparatus by staining with PI.

phosphorylation of STAT results in further activation of genes by STAT homodimers or heterodimers. Activation of gp130 also stimulates the Ras/MAP kinase pathway through binding of SHP-2 or shc to gp130 phosphotyrosines (8). In BAF-BO3 murine cells transfected with various truncated cDNAs of the human gp130 transducer, IL-6-induced survival was shown to be dependent on STAT3 activation, whereas proliferation required both the activation of STAT3 and the Ras/MAP kinase pathway (27). The involvement of the Ras/MAP kinase pathway in myeloma cell pro-

Table II. IFN- α inhibited DEX-induced apoptosis in primary plasma cells from seven patients with myeloma^a

| Patient | Apoptosis (%) | | | | | |
|---------|---------------|---------------|-------------|-----|------------|---------------------|
| | IL-6 | IFN- α | No cytokine | DEX | DEX + IL-6 | DEX + IFN- α |
| 1 | 9 | 4 | 25 | 83 | 59 | 31 |
| 2 | 0.5 | 0.5 | 5 | 21 | 6 | 2 |
| 3 | 3 | 5 | 5 | 23 | 15 | 6 |
| 4 | 8 | 16 | 15 | 28 | 9 | 28 |
| 5 | 5 | 5 | 6 | 60 | 28 | 29 |
| 6 | 5 | 5 | 11 | 24 | 17 | 5 |
| 7 | 4 | 15 | 16 | 20 | 9 | 7 |

^a Purified myeloma cells from seven patients were cultured for 3 days at a concentration of 3×10^5 cells/ml with either no cytokine, 3 ng/ml of IL-6, or 100 U/ml of IFN- α in the absence or presence of DEX. Apoptotic cells were detected on a FACScan after staining with PI. Percent apoptosis in each group was compared using a paired *t* test. No cytokine/DEX, *p* = 0.021; DEX/DEX + IL-6, *p* = 0.004; DEX/DEX + IFN- α , *p* = 0.009.

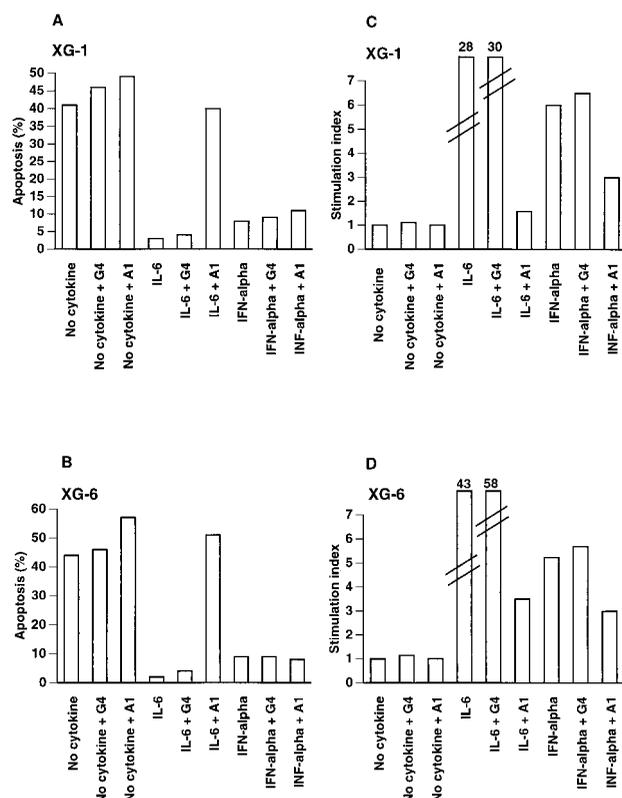


FIGURE 8. The anti-gp130 transducer-neutralizing Ab inhibits the effects of IFN- α on myeloma cell proliferation, but not survival. XG-1 (A and C) and XG-6 (B and D) cells were extensively washed to remove exogenous IL-6. They were then cultured for 3 days in culture medium supplemented with 10% FCS in the absence or the presence of the G4 (nonneutralizing) or A1 (neutralizing) anti-gp130 or control murine IgG1 mAbs (150 μ g/ml) with IL-6 (3 ng/ml), IFN- α (100 U/ml), or no cytokine. Apoptotic cells were assessed on a FACScan apparatus by staining with FITC-annexin V. The stimulation indexes were the ratios between the mean [³H]TdR incorporations obtained with an optimal concentration of IFN- α (100 U/ml) or IL-6 (3 ng/ml) in the presence or the absence of the mAbs. The mean [³H]TdR incorporations obtained with the control medium (culture medium with 10% FCS) were 1500 and 800 cpm for XG-1 and XG-6 HMCL.

liferation is further suggested by the inhibition of myeloma cell proliferation by MAP kinase antisense (28). As we now find that STAT3 was also phosphorylated by IFN- α in myeloma cells, this suggests that activation of STAT3 by either IL-6 or IFN- α may be involved in inhibiting apoptosis in myeloma cells.

This survival activity of IFN- α might help to explain the conflicting results concerning the effects of IFN on myeloma cell proliferation. Several reports have shown that IFN- α can both stimulate and inhibit the proliferation of primary myeloma cells (4, 29, 30) or cell lines (6, 31). In a recent study, Jelinek failed to find differences in the levels of STAT or IFN response factor (IRF) transcription factors in myeloma cell lines stimulated or inhibited by IFN- α (7). For U266 myeloma cells, the partial inhibitory effect of IFN on the proliferation was associated with a partial blockage of IL-6-induced gp130-linked SHP-2 activation and further MAP kinase activation (8). In our IL-6-dependent myeloma cell lines, we have previously shown by RT-PCR that there may be a weak production of the autocrine gp130 cytokines, IL-6 and/or oncostatin M (17), and IFN- α increases IL-6 gene expression in XG-1 cells (6). These autocrine gp130 cytokines may be sufficient to trigger the proliferation of myeloma cells but not their survival,

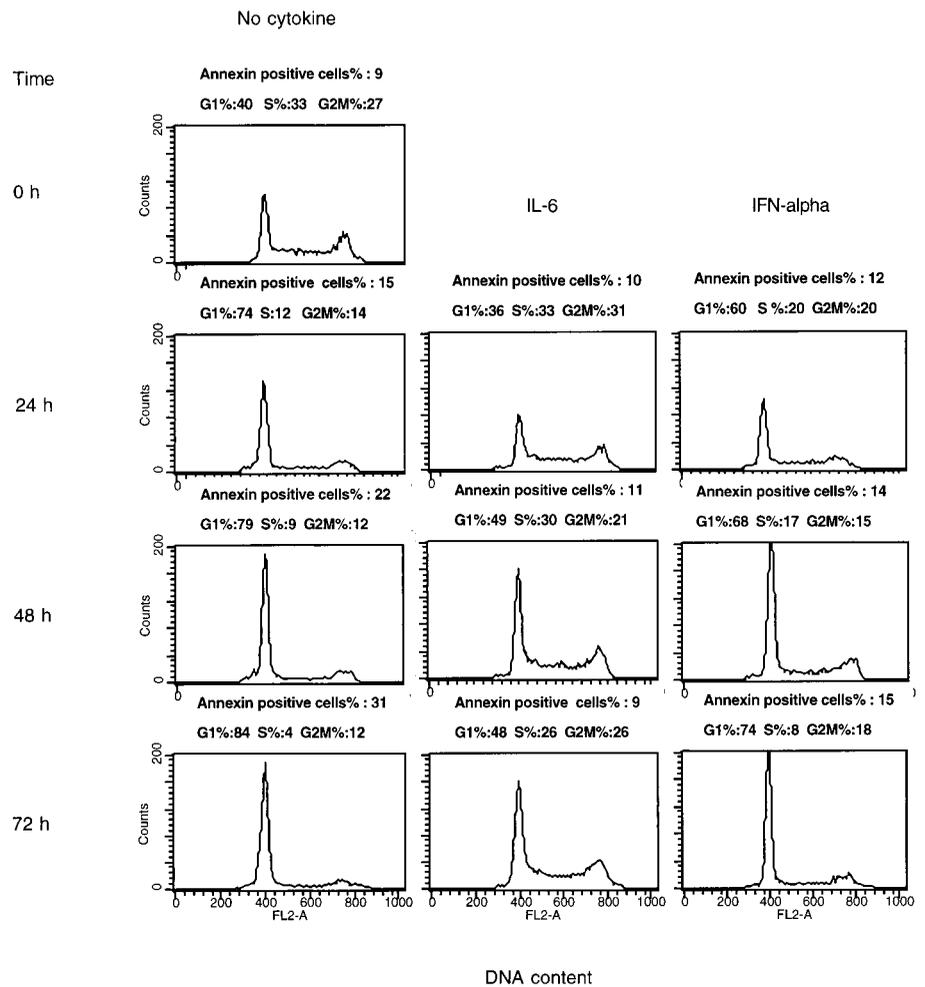
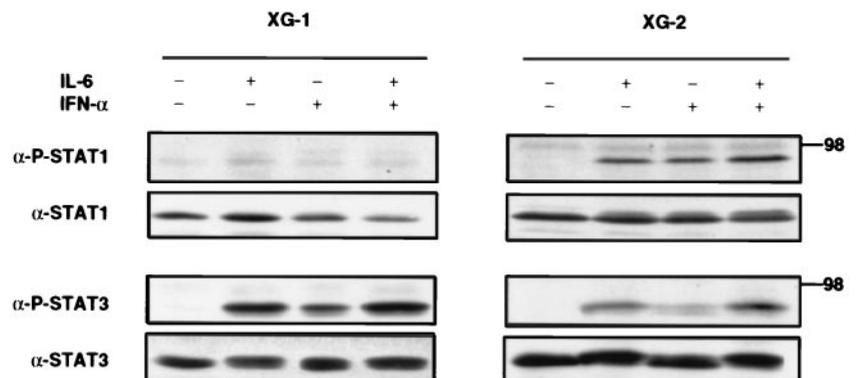


FIGURE 9. IFN- α is a survival factor, but does not increase entry into the cell cycle. XG-2 HMCL were extensively washed to remove exogenous IL-6 and were cultured for 72 h at a concentration of 3×10^5 cells/ml in culture medium supplemented with 10% FCS without exogenous cytokine or in the presence of IL-6 (3 ng/ml) or IFN- α (100 U/ml). The percentages of cells in different phases of the cell cycle were determined after PI staining using ModFitLT software. To better show the blockage of cells in G1, these percentages were determined on viable cells only. Early apoptotic cells were stained with FITC-annexin V.

since in most cell lines, removal of exogenous cytokine did not block entry of cells into the cycle even though the cells progressively died. Moreover, gp130 cytokines did not significantly contribute to the IFN- α -induced survival, as this activity is not inhibited by anti-gp130 Ab. IFN- α might partially inhibit the proliferation that could be induced by autocrine gp130 cytokines, since we have found that it can reduce the proliferation induced by exogenous IL-6 by approximately 50%. These results add to our understanding of the mechanism of action of IFN- α and emphasize that the survival of myeloma cells may be triggered by factors that are not members of gp130 cytokine family.

These results might be relevant for clinical studies, as IFN- α is used in the treatment of patients with multiple myeloma. The initial study by Mandelli *et al.* showed that the use of this cytokine as a maintenance treatment increased the plateau phase and overall survival of myeloma patients (32). However, further studies demonstrate that the effects of IFN- α are controversial (1, 33). IFN- α is also commonly used after high dose chemotherapy and autologous hemopoietic stem cell transplantation for multiple myeloma, but its efficacy, as assessed by randomized trials, has not been established (34). As IFN- α probably has pleiotropic effects in vivo (35), it might activate an anti-tumoral response in some patients.

FIGURE 10. STAT phosphorylation induced by IFN- α or IL-6 in myeloma cell lines. XG-1 and XG-2 cells were washed twice and starved for 16 h in RPMI 1640 with 1% FCS at 37°C. Cells were then either left unstimulated or were stimulated with IFN- α (500 U/ml), IL-6 (25 ng/ml), IFN- α (500 U/ml), and IL-6 (25 ng/ml) added for 15 min at 37°C. Lysates of unstimulated or stimulated cells (1×10^6 cells) were subjected to SDS-PAGE electrophoresis and immunoblotted with phospho-specific STAT1 or STAT3 Abs (α -P-STAT1 and α -P-STAT3). Blots were then stripped and reprobed with anti-STAT1 or anti-STAT3 Abs (α -STAT1 and α -STAT3) to ensure that equivalent levels of proteins were present in each lane.



However, as our data demonstrate that IFN- α can prevent apoptosis of myeloma cells in vitro, it may also be a myeloma cell survival factor in some patients. Such adverse effects might help to explain why IFN- α therapy was found to be associated with the development of plasma cell leukemia in two patients (36, 37).

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