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# Gamma-interferon induces apoptosis of the B lymphoma WEHI-279 cell line through a CD95/CD95L-independent mechanism

## Abstract

Gamma-interferon (IFN-gamma) a cytokine produced by CD4+ T helper type 1 cells, CD8+ T cells and natural killer (NK) cells, plays a central role in the development of humoral and cell-mediated immunity. IFN-gamma participates in the maturation and differentiation of B cells, but it has been previously reported that IFN-gamma may inhibit the early stages of B cell activation. We report that

the inhibition of the B lymphoma cell WEHI-279-proliferation induced by IFN-gamma, involves the induction of typical features of apoptosis (nuclear chromatin condensation and fragmentation, cell shrinkage, phosphatidyl-serine (PS) exposure and mitochondrial membrane potential (delta psim) loss). IFN-gamma-mediated B cell apoptosis was decreased by the addition of the T helper type 2 cytokine, IL-4. WEHI-279 cells express CD95 and undergo apoptosis after treatment with either an agonistic anti-CD95 Ab or with a soluble recombinant CD95L. However, incubation with CD95-Fc or TRAIL-R1-Fc fusion proteins, did not prevent IFN-gamma-mediated apoptosis, suggesting that IFN-gamma-mediated apoptosis occurs independently of CD95/CD95L and TRAIL-R/TRAIL interactions. IFN-gamma-mediated apoptosis is associated with caspase-3 activation that can be prevented by the addition of the broad caspase inhibitor zVAD-fmk. These data indicate that IFN-gamma may play a major role in the regulation of B cell apoptosis, and suggest the involvement of an alternative pathway which is independent of the death receptors.

## Full text

### INTRODUCTION

Gamma-interferon (IFN-gamma) has been shown to exert both stimulatory and inhibitory effects on polyclonal and antigen specific immune responses. These include the activation of macrophages to enhance phagocytosis and tumor killing, the activation of cytolytic T lymphocytes, and natural killer (NK) cells [1, 2], and the induction of the cell surface expression of major histocompatibility complex (MHC) class II molecules on monocytes [3, 4]. IFN-gamma also plays a major role in B-cell maturation and regulates immunoglobulin (Ig) isotypes switching [5-9] that can be antagonized by cytokines secreted by type 2 T helper (TH2) cells [10-15].

*In vitro*, impairment of proliferation of IL-7-dependent B cells and Burkitt's lymphoma RAMOS B cells induced by IFN-gamma has been reported to involve programmed cell death (PCD) or apoptosis [16-18]. *In vivo*, Staphylococcal toxic shock syndrome toxin-1 (TSST-1)-mediated B cell apoptosis has been reported to be IFN-gamma-dependent [19]. Altogether, these data suggest that IFN-gamma may play a major role in the control of B cell homeostasis and may be an effective mechanism for the removal of unwanted immune cells [20-26]. Apoptosis, is a physiological process representing one of the main phenotypes

of PCD, characterized by nuclear chromatin condensation and DNA fragmentation, mitochondrial membrane potential (delta psi m) loss, decrease in cell volume, and phosphatidyl-serine (PS) exposure. However, the mechanism by which IFN-gamma mediates B cell apoptosis remains to be elucidated.

CD95, a member of the tumor necrosis factor receptor (TNF-R) family (TNF-R1/R2, TRAIL-R1/R2/OPG), is a mediator of cell death [27]. The death domain of these receptors are responsible for recruiting adaptator molecules that in turn activate caspases. CD95/CD95 ligand (CD95L) interactions are responsible for controlling clone size after expansion, in responses to antigens, and are crucial in the regulation of immune homeostasis. *In vivo*, CD95 is abundantly expressed on the B cells in germinal centers and is involved in the activation-induced death of B cells [28]. These observations have suggested a major role for CD95/CD95L interaction in the control of B cell death.

Here, we have examined the effect of IFN-gamma on WEHI-279, a murine B lymphoma cell line [5, 29, 30] reported to be sensitive to IFN-gamma which induces an inhibition of both proliferation and differentiation of this cell line [31, 32]. However, the mechanisms involved remain unknown.

Our results indicate that IFN-gamma induces apoptosis of the B lymphoma WEHI-279 cell line, that can be delayed by the T helper 2 cytokine IL-4. While the WEHI-279 cells express CD95 on their surface and are especially susceptible to apoptosis after treatment with either soluble recombinant CD95L or agonistic anti-CD95 Abs, IFN-gamma induces an apoptotic process independent of CD95/CD95L interaction but dependent upon caspase-3 activation. Thus, IFN-gamma-mediated B cell death may represent an alternative pathway, independent of the death receptors that may have an important role in the control of B cell homeostasis.

## MATERIALS AND METHODS

### *Reagents and cytokines*

Fluorescent probes used were: YOPRO-1 nuclear dye, Calcein AM, DiOC6 (Molecular probes, Eugene, OR, USA), acridine orange, and propidium iodide nuclear dyes, Annexin-V-FITC (Beckman Coulter, Marseille, France). Cytokines used were recombinant interleukin-4 (rIL-4), recombinant interleukin-10 (rIL-10)

(Pharmingen, San Diego, CA, USA) and recombinant gamma-interferon (rIFN-gamma) (Genzyme, Cambridge, MA, USA). Purified and FITC conjugated anti-CD95 antibodies (J02) were purchased from Pharmingen. Soluble recombinant CD95L and, recombinant CD95-Fc and TRAIL-R1-Fc molecules were purchased from Alexis Corporation (San Diego, CA, USA). The competitive peptide inhibitor of caspases was the irreversible broad caspase inhibitor zVAD-fluoromethylketone (zVAD-fmk) (Bachem, Voisins-Le-Bretonneux, France). Protease inhibitors that do not inhibit caspases included leupeptin (Ac-LLR-CHO), Pepstatin A (Sigma, L'Isle d'Abeau, Chesnes, France) and zFA-fmk (Calbiochem, Meudon, France).

### *Cells and culture conditions*

The murine B lymphoma WEHI-279 cell line was obtained from the American Tissue and Cell Culture (ATCC, Bethesda, MD, USA). The WEHI-279 cell line expresses IgM on the cell surface but not IgG or IgA molecules (data not shown). Cells were maintained in RPMI 1640 (GIBCO, Courbevoie, France), supplemented with 5% heat-inactived fetal calf serum (Boehringer Mannheim, Meylan, France), 2 mM L-glutamine, 1 mM sodium pyruvate (GIBCO) and 5 **x** $10^{-5}$ M beta-2 mercaptoethanol (Merck, Fontenay-sous-Bois, France). Cells were maintained in a humidified, 5% $CO_2$ atmosphere at 37° C. The WEHI-279 cell line was cultured at $10^4$ cells per well in 96-well culture plates (BD Biosciences, Pont-de-Claix, France), or at $10^5$ cells per well in 24-well culture plates. In some experiments, cells were irradiated with X-rays (20 Gy) using a Philips RT, to induce apoptosis.

### *Proliferation assay*

Cells were cultured for three days and then labelled with [$^3$H] thymidine (1 muCi per well, ICN, Orsay, France) for 16 hours. [$^3$H]TdR incorporation was measured by liquid scintillation. To measure mitochondrial dehydrogenase enzyme activity, MTT (3, 4, 5 dimethylthiazol-2,5 diphenyltetrazolium bromide) (5 mg/ml, Sigma) was added to the culture at day three, and cells were incubated for an additional 3 hours. Converted dye was solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol) and optical density was determined at 570 nm.

### *Characterization and measurement of programmed cell death*

Different approaches were used to explore programmed cell death.

*Flow cytofluorimetric analysis:* the percentage of dying cells in culture was determined by flow cytofluorimetric analysis (Epics Profile, Coulter Coultronics) using the YOPRO-1 nuclear dye (10 muM), the calcein AM (5 muM), DiOC6 mitochondrial dye (5 nM) and the acridine orange nuclear dye (0.1 mug/ml).

Briefly, YOPRO-1 enters only non-viable cells, gives a green fluorescence, and is used to assess membrane permeability and cell viability. Dying cells show a bright fluorescence intensity [33].

Calcein AM was used to assess cell viability. It labels live cells, fluorescing green. Dying cells show a lower fluorescence intensity [34].

DiOC6 was used to assess mitochondrial membrane potential (delta psi m). It fluoresces green. Dying cells show a lower fluorescence intensity.

Acridine orange enters all cells, fluoresces green, and is used to assess nuclear condensation and fragmentation, a typical feature of apoptotic cells [35, 36].

Cells were gated under forward- and side-scatter parameters, dying cells presenting a characteristic distinct peak of fluorescence intensity as compared to living cells, and a forward scatter below the peak of living cells (shrunken cells).

*Spectrofluorimetric analysis:* live and dying cells were assessed by spectrofluorimetric analysis (Labsystems, Fluoskan II) of cells cultured in 96-well culture plates following staining with either calcein AM (5 muM, 20 mn) or YOPRO-1 nuclear dye (5 muM, 5 mn). Data are expressed as fluorescence intensity units (f.i.u.). This new method of quantification has been used to automate the assessment of cell death.

*DNA fragmentation:* qualitative analysis of DNA fragmentation was carried out following phenol chloroform extraction of DNA. DNA was electrophoresed into 2% agarose gels and stained by ethidium bromide.

*Trypan blue exclusion assay:* viable cells were counted after staining with trypan blue (Sigma). At least 400 cells were counted in duplicate cultures.

### *Western blottings*

Cells were incubated in SDS lysis buffer, boiled for 10 min and centrifuged for 15 min at room temperature. Protein determination was performed using the DC Protein Assay (Bio Rad, Marnes-La-Coquette, France). Equal amounts of proteins were boiled for 5 min in 2X Laemmli sample buffer with 2-beta ME, and run on a 4/20% polyacrylamide gel (Bio Rad). Proteins were then transferred to a PVDF membrane (Bio Rad), and immunoblotted with a rabbit polyclonal anti-caspase-3 (kindly provided by Dr. Vandenabeele, Ghent, Belgium), a mouse IgG2b anti-Tubulin (KMX-1, Boehringer Mannheim, Meylan, France), and then visualized using horseradish-peroxidase-conjugated secondary antibodies (Amersham, Orsay, France), followed by enhanced chemiluminescence (Amersham).

## RESULTS

### *IFN*-gamma *inhibits B-cell proliferation and induces cell death*

WEHI-279 cells were incubated for three days with different concentrations of IFN-gamma. As previously reported [32], incorporation of [$^3$H]TdR was decreased in a dose-dependent manner in cultures treated with IFN-gamma (Figure 1A). Cellular metabolic activity was determined by MTT assay (Figure 1B). In living cells, MTT is metabolized to a formazan salt by the mitochondrial dehydrogenase enzymes. We observed that after treatment with IFN-gamma, enzyme activity decreased in a dose-dependent manner, comparable to that observed for [$^3$H]TdR incorporation.

The decrease in B cell proliferation induced by IFN-gamma was associated with a loss of cell viability. Cell viability was assessed by spectrofluorimetric analysis based on the use of either calcein AM (Figure 1C), that measures esterase activity of living cells [34] and YOPRO-1 nuclear dye (Figure 1D), an impermanent nuclear dye that only penetrates dead cells [33]. Fluorescence intensity from cell esterase activity decreased in cells treated with IFN-gamma, whereas YOPRO-1 labelling increased under the same conditions. The use of these two different fluorescent dye reagents revealed that inhibition of B-cell proliferation was associated with cell death.

To determine that the loss of cell viability was associated with the induction of apoptosis, cells were analyzed by flow cytofluorimetry (Figure 2A). Acridine orange nuclear dye [35, 36] was used to assess nuclear chromatin

condensation and DNA fragmentation, a typical feature of apoptotic phenotype. Cells treated with IFN-gamma display 57% of sub-G0/G1 (Figure 2A), a percentage quite similar to that observed with calcein AM (59%) and YOPRO-1 (58%). DNA from IFN-gamma treated WEHI-279 cells showed a typical ladder of fragments at 180-200 intervals, following electrophoresis into agarose gels (Figure 2B). As a control, treatment with saponin (0.1%) (Lane 3), which leads in 10 min to 95% cell death as determined by trypan blue dye exclusion, did not induce DNA fragmentation as compared to gamma-irradiation (Lane 4). Our data suggest that IFN-gamma induced a typical apoptotic phenotype.

### *Regulation of IFN*-gamma-*mediated apoptosis*

We next investigated whether IFN-gamma-mediated apoptosis can be modulated by the Th2 cytokines, IL-4 and IL-10, cytokines that are reported to inhibit the effects of IFN-gamma on B cells [15] and to modulate surface Ig-crosslinking-mediated cell death [37, 38]. Two hundred pg/ml of IL-4 reduced both phosphatidyl-serine (PS) exposure and chromatin condensation, and DNA fragmentation induced by IFN-gamma (25 IU/ml) (Figure 3). These data also indicate that the cell cycle was preserved. Similarly, IL-4 delayed cell death mediated by IFN-gamma, as assessed by spectrofluorimetric analysis using the calcein AM dye reagent; IL-10 had almost no effect on cell death (data not show). To investigate whether new protein synthesis is required for apoptosis mediated by IFN-gamma in WEHI-279 cells, the effect of cycloheximide (CHX) was evaluated. The inibition of the new protein synthesis using CHX induced massive apoptosis (data not shown). This observation points towards the constitutive presence of an inhibitory factor that prevents cell death and allows continuous growth of these cells. Thus, the pretreatment of the cells with CHX did not allow the exploration of the role of *de novo* protein synthesis following IFN-gamma.

### *IFN*-gamma-*mediated apoptosis in a CD95/CD95L-independent pathway*

Death receptors play a major role in the control and homeostasis of the immune system [27]. We investigated the potential involvement of CD95/CD95L in the process of apoptosis mediated by IFN-gamma of the B lymphoma WEHI-279 cell line. Our data demonstrated that WEHI-279 cells display a marked expression of CD95 on the surface, as assessed by flow cytofluorimetric analysis using FITC-labelled antibody to CD95 (J02) (Figure 4A). The WEHI-279 cells undergo apoptosis following ligation of the CD95 molecule using agonistic anti-CD95

antibodies (Figure 4B and 4E) and soluble recombinant CD95L (Figure 4E). However, while IFN-gamma enhanced CD95 expression in a dose-dependent manner (Figure 4C), there is no synergic effect between IFN-gamma and CD95 ligation (Figure 4D), suggesting two distinct pathways. In an effort to understand the mechanism of IFN-gamma-mediated apoptosis, we used a recombinant CD95-Fc chimeric molecule that blocks the interaction between CD95 and CD95L. In the presence of 100 mug/ml of CD95-Fc, a dose 10-fold greater than that required to prevent apoptosis mediated by CD95L, IFN-gamma-induced apoptosis was not prevented (Figure 4E). Moreover, *in vitro* treatment with TRAIL-R1-Fc did not prevent IFN-gamma-mediated cell death (Figure 4E). Our data suggest that IFN-gamma-induced apoptosis is at least CD95L- and TRAIL-independent.

### *The role of cysteine proteases in cell death*

The activation of caspases, a family of cysteine proteases, is one of the main features involved in the apoptotic phenotype [39]. To study the potential involvement of caspases in IFN-gamma-induced apoptosis, we first assessed caspase activation by western blotting. We observed that both IFN-gamma and CD95 ligation mediated the cleavage of the 32 kDa proenzyme caspase-3 into active forms (Figure 5A). zVAD-fmk (100 muM) a broad irreversible caspase inhibitor, prevented both IFN-gamma- and CD95-ligation-mediated caspase-3 activation and apoptosis in a dose-dependent manner (Figure 5A and 5B). As shown in Figure 5C, all the features of the apoptotic phenotype (phosphatidyl-serine (PS) exposure, mitochondrial membrane potential (delta psi m) loss and chromatin condensation and DNA fragmentation) were prevented. Pepstatin A, an inhibitor of cathepsin D, previously reported to prevent cell death in HeLa cells, mediated by IFN-gamma [40], had no effect on cell death, suggesting that cathepsin D did not play a major role in IFN-gamma-mediated WEHI-279 cell death. Taken together, these data suggest that caspases are the main effectors involved in IFN-gamma-mediated B cell death.

## DISCUSSION

The WEHI-279 B lymphoma cell line is highly sensitive to IFN-gamma [8, 9, 31, 32]. We report that growth inhibition of this B cell line by IFN-gamma is mediated by the induction of typical apoptosis. This process involves cell shrinkage, mitochondrial membrane potential (delta psi m) loss, phosphatidyl-serine (PS)

exposure, nuclear chromatin condensation and DNA fragmentation. Alterations in mitochondrial function are also an important feature of apoptosis, and play a major role in the executionary phase of the apoptosis [41, 42]. Both MTT assay and DiOC6 labelling show that IFN-gamma induced a rapid decline in mitochondrial function, which may reflect an early step in the control of the executionary phase of the apoptotic machinery. Indeed, two pathways are known to be important for transducing a death signal to the mitochondria. The "extrinsic" pathway involves activation of death receptors, and thereby activates caspase and stimulates the apoptogenic activity of mitochondria. By contrast, the death receptor-independent apoptotic pathway is activated directly by death stimuli and induces the disregulation of the mitochondria.

Our data demonstrated that the WEHI-279 cells strongly express CD95, and undergo apoptosis in the presence of either agonistic anti-CD95 antibodies or recombinant CD95L. However, in the presence of recombinant chimeric CD95-Fc, which blocks the interaction between CD95 and CD95L, CD95Fc did not prevent IFN-gamma-mediated apoptosis. Moreover, the absence of a protective effect of TRAIL-R1-Fc may suggest that IFN-gamma-mediated apoptosis is a death-receptor-independent pathway. Watanabe *et al.* [28] have previously reported the presence of apoptotic cells in the germinal center of CD95-deficient mice (Lpr mice) after immunization. This report and our data indicate that IFN-gamma may play a major role in the control of B cell survival and may represent an alternative pathway to the death receptor.

Several novel genes, DAP genes (death associated proteins), have been reported to function as positive mediators of IFN-gamma-mediated cell death [40, 43-46]. One of these genes, DAP6 is identical to a known aspartic protease, cathepsin D, and its participation has been established in a variety of apoptotic systems [40]. However, we did not observe any preventive effect of pepstatin A, a cathepsin D inhibitor, on IFN-gamma-mediated WEHI-279 cell death. In contrast, the cell-permeable, irreversible peptide inhibitor of caspases, zVAD-fmk, prevented both CD95- and IFN-gamma-mediated caspase-3 activation and cell death. These observations support the idea that at least caspase-3 plays a major role in IFN-gamma-mediated cell death in B lymphoma WEHI-279 cells.

Apoptosis is a physiological and dynamic process that can be modulated. Cytokines play a major role in the cross-talk between B and T cells [47]. Thus, B cell selection and isotype switching may be dependent on the initial survival of B

cells, a process that can be regulated by cytokines in the local environment. IL-4 has been reported to have a direct effect on the modulation of B cell apoptosis, depending on the stage of differentiation and activation of the B-cell [37, 38]. Here, we have observed that IL-4, a cytokine secreted by CD4 Th 2 cells, delayed IFN-gamma-mediated WEHI-279 cell death and preserved the cell cycle. IL-4 can also prevent Ig-crosslinking-mediated apoptosis by increasing Bcl-2 expression [48], suggesting that the balance between genes of the Bcl-2 family may participate in the control of B cell apoptosis [49]. The effect of IFN-gamma on the expression of these genes merits further attention.

CONCLUSION

Altogether, our data suggest that IFN-gamma mediated apoptosis of the B lymphoma WEHI-279 cell line, through a caspase-dependent pathway, does not require CD95L and TRAIL. WEHI-279 cells may therefore, represent an attractive model to further explore the molecular mechanisms of apoptosis induced by IFN-gamma. Thus, our data support the possibility that IFN-gamma may play a major role in the regulation of B cell survival, and may represent an alternative pathway, independent of the death receptor, for the control of B cell homeostasis.

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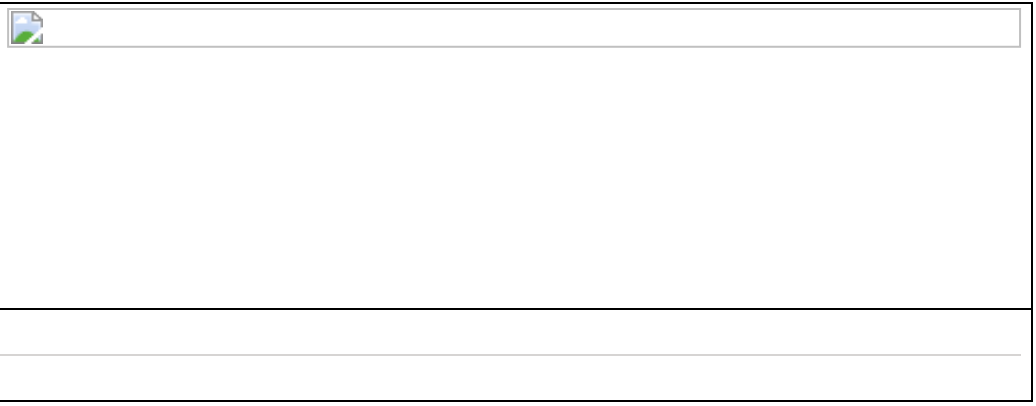
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## Figures

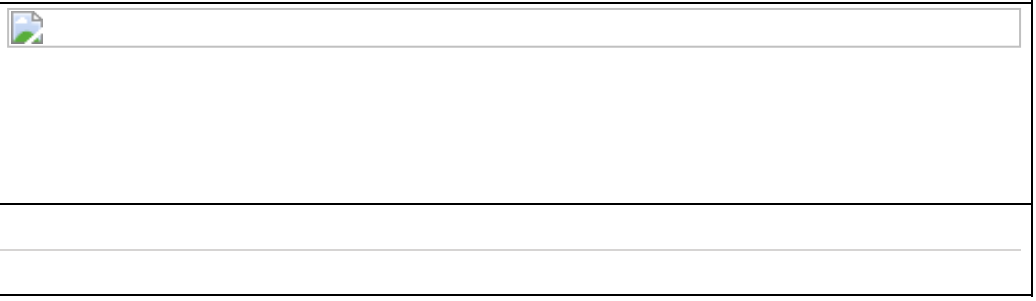




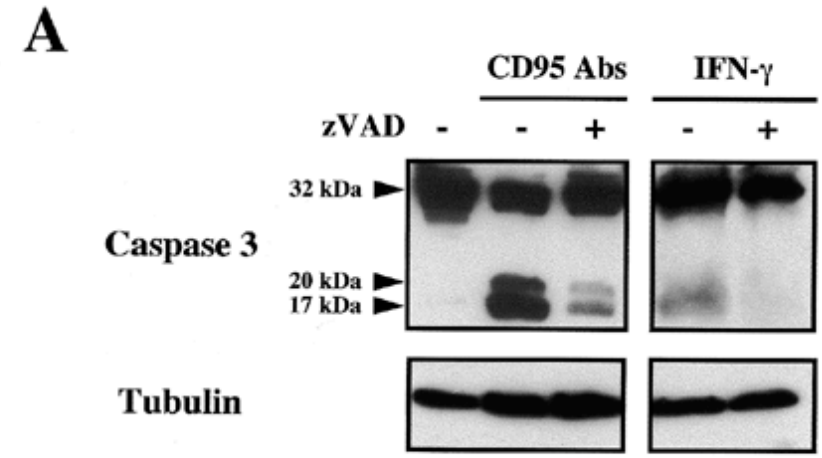


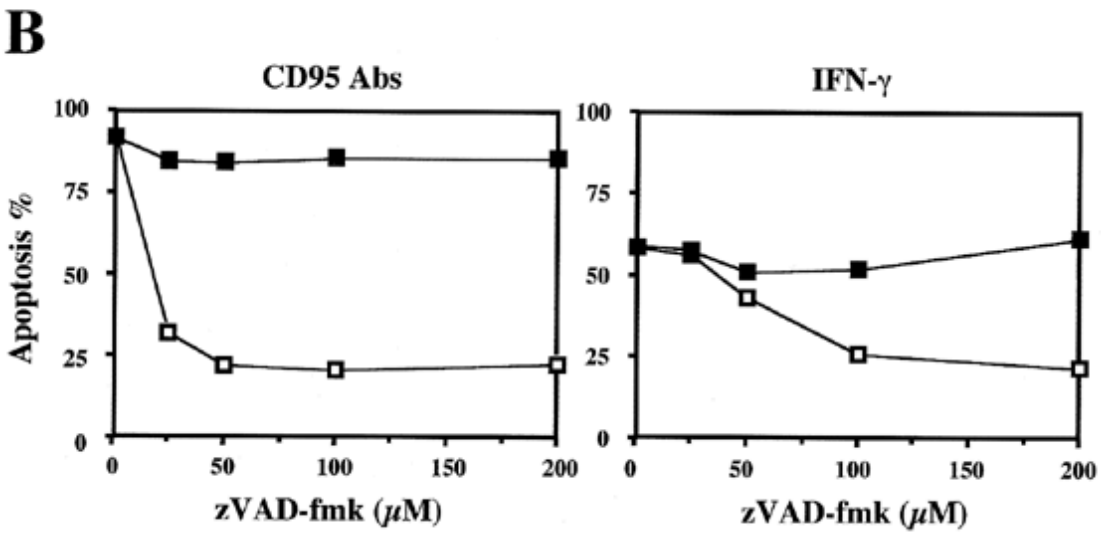









A
CD95 Abs
IFN-γ
zVAD - - + - +
32 kDa
Caspase 3
20 kDa
17 kDa
Tubulin


B
CD95 Abs
IFN-γ
Apoptosis %
100
75
50
25
0
0 50 100 150 200
zVAD-fmk (μM)

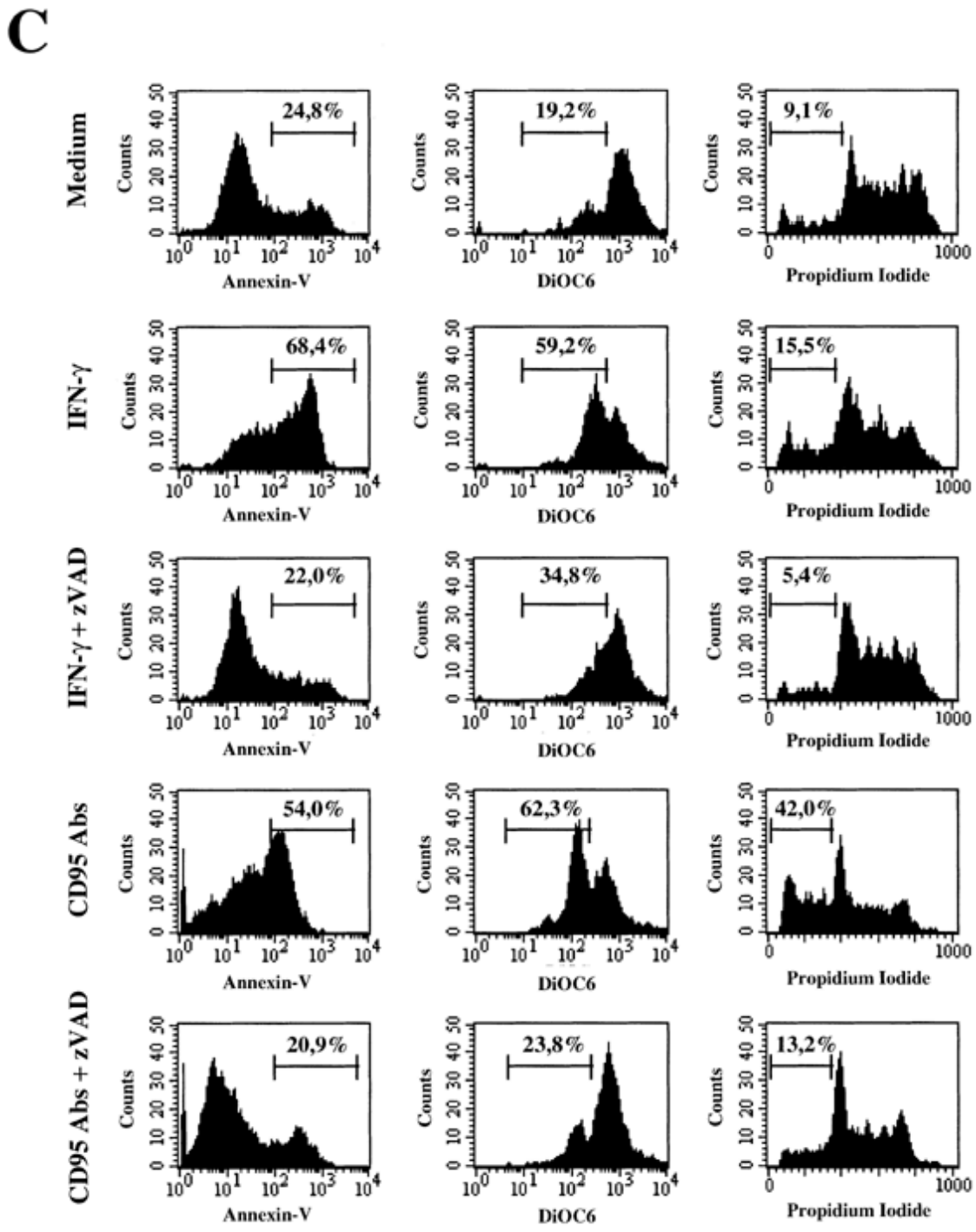



**Figure 5**

**Caspase inhibitor prevent caspase activation and cell death.**

**A:** extracts from WEHI-279 cells were analyzed for caspase-3 activation by western blotting. Cells were cultured in the absence (–) or presence (+) of CD95 Abs (10 ng/ml) or IFN-γ (100 IU/ml). Cells were pretreated with (+) or without (–) 100 μM of zVAD-fmk. Tubulin was used as the loading control. The experiment is representative of two independent experiments performed.

**B:** cells (5 x $10^4$) were incubated with either agonistic anti-CD95 antibody (J02, 20 ng/ml) or IFN-γ (100 IU/ml). The cells were pretreated with the indicated amounts of zVAD-fmk (□) for 30 min. Apoptosis was assessed by flow cytofluorimetry using Annexin-V-FITC. As the control, cells were treated with leupeptin (■) or zFA-fmk peptide or pepstatin, giving similar results, A (data not shown). The experiment is representative of three independent experiments performed.

**C**: Cells pretreated for 30 mn in the absence (medium) or presence of zVAD-fmk (100 μM), were further incubated in the absence or presence of anti-CD95 antibody (20 ng/ml) or IFN-γ (100 IU/ml). Cells were analyzed by flow cytometry using Annexin-V-FITC, DiOC6 and propidium iodide. The experiment is representative of three independent experiments performed.

## Authors

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human recombinant interleukin-1 beta, myelopoiesis, myelodepression, cancer chemotherapy.

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human recombinant interleukin-1 beta, leukopoiesis, myelodepression, cancer chemotherapy.

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