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Elisabetta Dondi, Gaël Roué, Victor J. Yuste, Santos A. Susin and Sandra Pellegrini

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A Dual Role of IFN- α in the Balance between Proliferation and Death of Human CD4⁺ T Lymphocytes during Primary Response¹

Elisabetta Dondi,^{2*} Gaël Roué,[†] Victor J. Yuste,[†] Santos A. Susin,^{3†} and Sandra Pellegrini^{3,4*}

Type I IFNs (IFN- $\alpha\beta$) enhance immune responses, notably T cell-mediated responses, in part by promoting the functional activities of dendritic cells. In this study, we analyzed the direct impact of IFN- α on proliferative and apoptotic signals upon in vitro activation of human naive CD4⁺ T lymphocytes. We demonstrate that IFN- α protects T cells from the intrinsic mitochondrial-dependent apoptosis early upon TCR/CD28 activation. IFN- α acts by delaying entry of cells into the G₁ phase of the cell cycle, as well as by increasing Bcl-2 and limiting Bax activation. Later, upon activation, T cells that were exposed to IFN- α showed increased levels of surface Fas associated with partially processed caspase-8, a key component of the extrinsic apoptotic pathway. Caspase-8 processing was augmented furthermore by Fas ligation. Overall, these findings support a model whereby IFN- α favors an enhanced clonal expansion, yet it sensitizes cells to the Ag-induced cell death occurring at the end of an immune response. These observations point to a complex role of type I IFN in regulating the magnitude of proliferation and survival of naive CD4⁺ T cells during primary response and underline how crucial could be the timing of exposure to this cytokine. *The Journal of Immunology*, 2004, 173: 3740–3747.

The type I IFNs (IFN- $\alpha\beta$) are important mediators of the innate immune response for their potent and direct antiviral activity and their stimulatory effects on NK cells and macrophages. Recently, these cytokines have been reconsidered as critical actors in the generation of the adaptive immune responses toward virus infection and tumor growth. Evidence obtained in different model systems has highlighted their ability to support proliferation, functional activity, and survival of certain T cell subsets (1, 2). These activities of IFN are, at least in part, mediated by the dendritic cells (DCs).⁵ A promoting effect of type I IFN in maturation, differentiation, and functional activity of DCs has been indeed reported (reviewed in Ref. 3). Upon infection, DC development from monocytes is induced by type I IFN produced by specific cell types, such as macrophages and endothelial cells. Moreover, infected DCs, and particularly plasmacytoid-like cells (pDCs), produce large amounts of type I IFN that contributes to their maturation in an autocrine and paracrine fashion (4–6). The

secretion of type I IFN is indeed sustained in lymph nodes, where pDCs represent the principal source (7). Hence, in lymph nodes, naive T cells may encounter cognate Ags, undergo clonal expansion, and acquire effector functions while being exposed to type I IFN.

To investigate the impact of this cytokine on T cell responses, we set up an experimental model system that allows us to analyze the direct effects of IFN- α on human naive CD4⁺ T cells purified from umbilical cord blood (UCB). In this model, we previously found that IFN- α exerts an anti-proliferative effect that is strictly dependent on the activation state of the cells (8). Here, we sought to analyze whether IFN- α may intervene at the onset of the primary T CD4⁺ response by skewing the balance between proliferation and death. For this, we have characterized the apoptotic programs initiated in TCR/CD28-stimulated naive CD4⁺ T cells and, in parallel, we have investigated whether IFN- α affects them.

Apoptosis occurs via two major execution programs: the extrinsic pathway triggered by the ligation of membrane death receptors, and the intrinsic pathway that integrates the different cell stress signals at the mitochondrial level. In the extrinsic pathway, the stimulation of death receptors (e.g., Fas or TNF-R) provokes the processing and the activation of caspase-8, which, in turn, cleaves procaspase-3 and thereby initiates a proteolytic cascade. In the intrinsic pathway, disruption of the mitochondrial transmembrane potential ($\Delta\psi_m$) controls the release of several proapoptotic proteins (9, 10). The Bcl-2 family of proteins are pivotal intermediates in cell death signaling through the regulation of the $\Delta\psi_m$. Indeed, some Bcl-2 family members, such as Bcl-2 and Bcl-x_L, promote cell survival by inhibiting the $\Delta\psi_m$ loss, while other members of this family, such as Bax and Bak, participate actively in the disruption of the mitochondrial potential (11). The extrinsic and the intrinsic pathways can be connected through the caspase-mediated activation of the proapoptotic protein Bid (12).

In this study, we provide the evidence that the TCR/CD28-mediated stimulation of naive T CD4⁺ cells activates the mitochondrial-dependent apoptotic pathway. In this context, IFN- α protects cells from death through the up-regulation of the anti-apoptotic

*Unité de Signalisation des Cytokines, and [†]Apoptose et Système Immunitaire, Institut Pasteur, Paris, France

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² Current Address: Unité Institut National de la Santé et de la Recherche Médicale U567, Département d'Hématologie, Institut Cochin, Paris, France.

³ S.A.S. and S.P. share senior coauthorship of this manuscript.

⁴ Address correspondence and reprint requests to Dr. Sandra Pellegrini, Unité de Signalisation des Cytokines, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France. E-mail address: pellegrini@pasteur.fr

⁵ Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid-like cells; UCB, umbilical cord blood; $\Delta\psi_m$, mitochondrial transmembrane potential; PS, phosphatidylserine; 7-AAD, 7-aminoactinomycin D; ROS, reactive oxygen specie; FasL, Fas ligand.

protein Bcl-2 and the down-regulation of the proapoptotic protein, Bax. Interestingly, IFN- α sensitizes cells toward the extrinsic apoptotic pathway as it anticipates partial activation of caspase-8 and induces the up-regulation of the death receptor Fas. Overall, our data reveal contrasting protective and proapoptotic effects of IFN- α that ultimately may participate to the homeostatic control of lymphocytes.

Materials and Methods

Purification and stimulation of naive CD4⁺ T cells

Human neonatal leukocytes were isolated from freshly collected heparinized neonatal blood by Ficoll-Paque density gradient centrifugation. CD4⁺ T cells were purified by negative selection using a Pan T isolation kit (Miltenyi Biotec, Auburn, CA). The purity of the CD4⁺CD45RA⁺ T cells was >98% as determined by flow cytometry. Naive CD4⁺ T cells were incubated for 20 h in the presence or absence of 1 nM IFN- α 2 (recombinant IFN- α 2, a gift of D. Gewert, Wellcome Foundation, Beckenham, Kent, U.K.). This concentration, equivalent to 4000 IU/ml was estimated to be optimal in inducing B lymphocyte survival (13). Nontreated cells were activated in the presence or absence of 1 nM IFN- α 2 (see scheme in Fig. 1). Naive CD4⁺ cells were stimulated with 1 μ g/ml soluble anti-CD3 (clone CLB-T3/4.E; CLB, Sanquin Reagents, Amsterdam, The Netherlands) and anti-CD28 Abs (clone CD28.2; BD Pharmingen, San Diego, CA). At 48 or 72 h postactivation, cells were harvested and analyzed. To monitor Fas-induced apoptosis, cells activated or not in the presence of IFN were harvested 48 and 72 h postactivation and left untreated (control) or incubated with 50 ng/ml agonistic anti-Fas mAb (clone CH11; Upstate Biotechnology, Lake Placid, NY) for 16 h at 37°C.

T cell proliferation and cell cycle assays

Cells were stained with 2.5 μ M CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C before activation with anti-CD3/CD28. At 48 h postactivation, cells were analyzed for CFSE fluorescence intensity as a function of cell division on a FACSCalibur (BD Biosciences, San Jose, CA). Naive cells and activated cells, harvested at 18, 24, and 48 h postactivation, were stained with acridine orange (20 μ g/ml; Polysciences, Warrington, PA) in a citrate phosphate buffer, as described (14), and immediately analyzed by flow cytometry. For propidium iodide analysis, 48 h postactivation, cells were fixed in 70% ethanol, incubated with 100 μ g/ml RNase A and 20 μ g/ml propidium iodide (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, and analyzed by FACS. The percentage of cells in each stage of the cell cycle was determined by using the ModFit software (Verity Software House, Topsham, ME).

Cytofluorometric determination of apoptosis

Aberrant phosphatidylserine (PS) exposure on the cell membrane was measured by staining cells with an annexin V-APC conjugate (2 μ M; 15 min at 4°C; 660 nm; BD Biosciences). Lysosome permeabilization was assessed using a fluorescent acidotropic probe (50 nM; 15 min at 37°C; LysoTracker; Molecular Probes). To measure the $\Delta\psi_m$, cells were incubated at 37°C for 15 min with the cationic lipophilic fluorochrome 3,3'-dihexyloxycarbocyanine iodide (40 nM; 525 nm). Hydroethidine (2 μ M; 15 min at 37°C; 600 nm; Molecular Probes) was used to measure superoxide anion generation. Alteration in plasma membrane integrity was evaluated following 7-aminoactinomycin D (7-AAD; Sigma-Aldrich) incorporation; cells were incubated with 20 μ g/ml 7-AAD for 20 min at 4°C and then washed and resuspended in the presence of 20 μ g/ml actinomycin D (Sigma-Aldrich). The intracellular expression of Bcl-2 family members was analyzed on 48 h-activated cells fixed in 1% paraformaldehyde. Bcl-2 was detected by incubating cells for 20 min at room temperature in 0.1% saponin buffer with PE-conjugated anti-Bcl-2 mAb (clone 6C8; BD Pharmingen). Bax was stained with an anti-Bax mAb directed against the conformationally active form of the protein (clone 6A7; BD Pharmingen) and revealed with a goat anti-mouse IgG conjugated to FITC (Caltag Laboratories, Burlingame, CA). Surface expression of Fas was measured by staining with allophycocyanin-conjugated anti-CD95 mAb (clone DX2; BD Pharmingen).

Western blot analysis

Cells were lysed in 50–100 μ l of buffer containing 150 mM NaCl, 0.5% deoxycholic acid, 0.03% SDS, 1% Nonidet-P-40, 3 μ g/ml aprotinin, 3 μ g/ml pepstatin, 3 μ g/ml leupeptin, and 1 mM PMSF. Total proteins were separated on SDS-polyacrylamide gels (14% for caspase-8 and -9; 10–20% gradient gels for caspase-3 and DFF45) and transferred to polyvinylidene

fluoride membranes (Immobilon-P; Millipore, Bedford, MA). Blots were incubated with the following Abs: anti-caspase-8 mAb (clone 1C12; Cell Signaling Technology, Beverly, MA), anti-caspase-9 rabbit antiserum (Cell Signaling Technology), anti-caspase-3 rabbit antiserum (BD Pharmingen), anti-DFF45 mAb (clone 6B8; MBL, Nagoya, Japan), and finally, with secondary peroxidase-conjugated anti-mouse or anti-rabbit Abs.

Results

IFN- α affects the balance between cell cycle entry and cell death

To study whether the exposure of naive CD4⁺ T cells to type I IFN affects the establishment of the primary response, we analyzed the effects of IFN- α 2 on the balance between proliferation and apoptosis of T cells after TCR/CD28 triggering. IFN- α was added to CD45RA⁺CD4⁺ T cells, purified from UCB leukocytes before or at the moment of activation (scheme in Fig. 1). Forty-eight hours postactivation, the analysis of the CFSE fluorescence dilution showed that the IFN- α pretreatment delayed the progression through cell divisions (Fig. 2A; Ref. 8). To analyze at which phase of the cell cycle lymphocytes were delayed, control cells and cells preincubated with IFN- α were activated with anti-CD3/CD28 and analyzed at different times by staining with the metachromatic acridine orange fluorochrome, which differentially stains DNA and RNA. With this approach, we could distinguish the three main phases of the cell cycle ($G_{0/1}$ vs S vs G_2/M) based on differences in cellular DNA content, and also discriminate G_1 subcompartments differing in their RNA content (14). Resting G_0 naive cells are characterized by a minimal content of RNA (Fig. 2B, upper plot), which progressively augments when cells progress into G_1 upon activation (left plots). We observed a reduced accumulation of RNA in cells that had been preincubated with IFN- α , i.e., a reduced percentage of cells in G_1 (right plots). A similar trend was obtained with cells from three independent donors. Thus, the IFN-induced delayed progression through cell divisions is due to a slower G_0 to G_1 transition. This effect is reduced when cells were exposed to IFN only prior to their activation (data not shown).

The fate of Ag-specific T cells and the magnitude of their responses is determined by the fine integration of signals that commit cells to proliferation and signals that initiate cell death (15). We asked whether environmental type I IFN could affect this decision-making process in our experimental model. For this, we first measured the percentage of dead cells having undergone chromatinolysis at 48 h postactivation by propidium iodide staining and flow cytometric analysis (Fig. 2C). For both IFN-treated populations, a 50% reduction in the number of cells present in the sub G_1

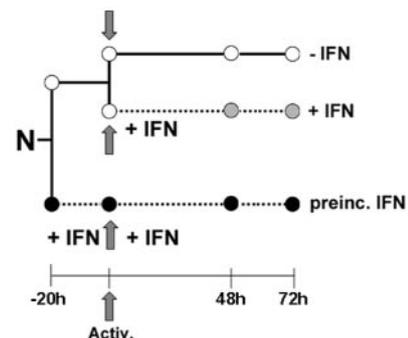


FIGURE 1. Activation scheme used in this study. Purified naive CD4⁺ T cells were preincubated (dashed line) or not (solid line) for 20 h with 1 nM IFN- α 2, then activated with mAbs to CD3/CD28. IFN- α was constantly maintained in the medium of preincubated cells. Untreated cells were activated in the presence (dashed line) or absence (solid line) of IFN- α . At 48 or 72 h after activation, cells were harvested and analyzed.

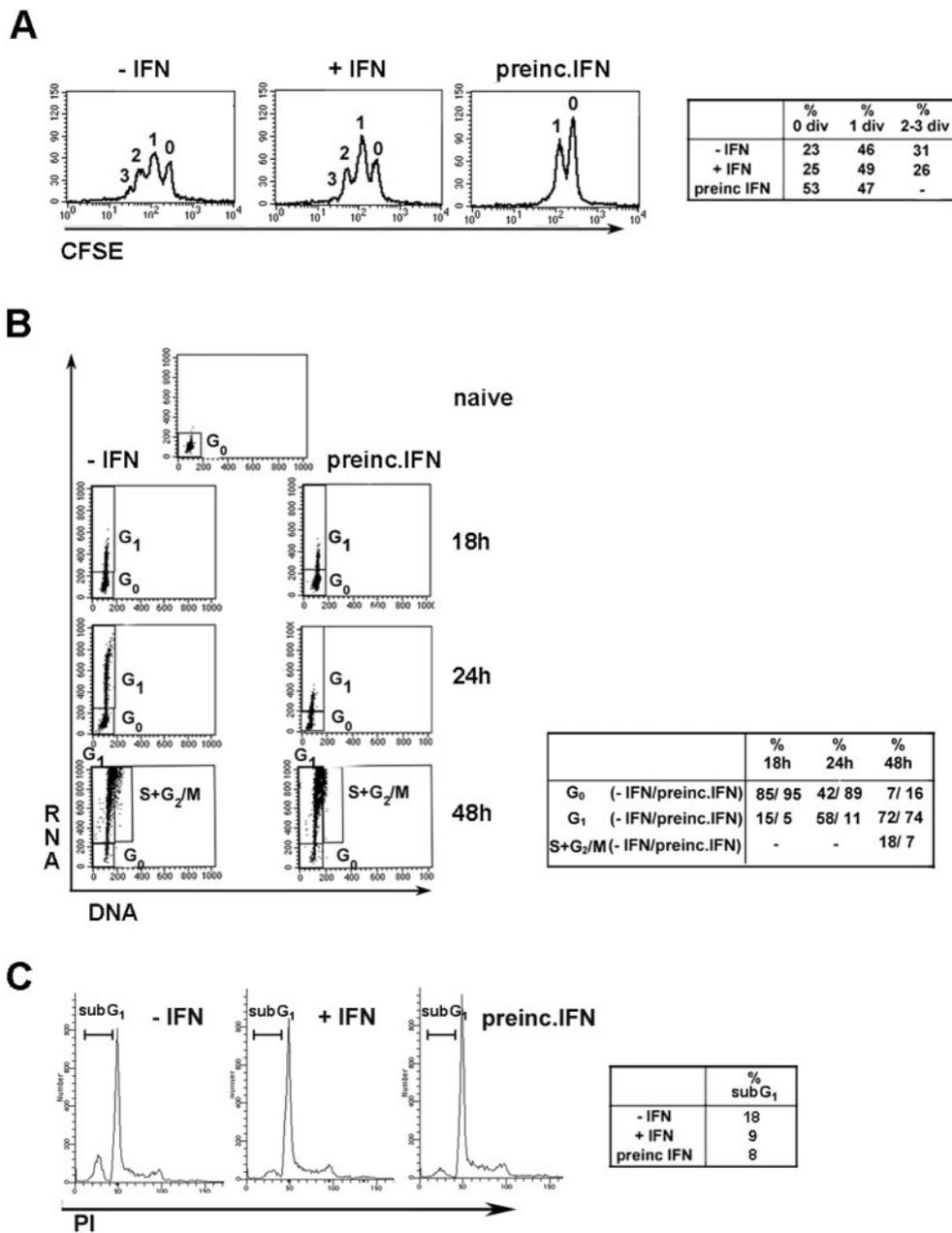


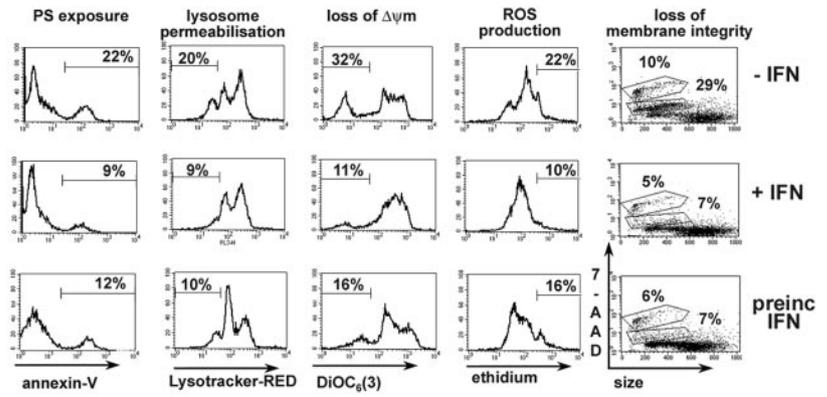
FIGURE 2. Effects of IFN- α on cell cycle entry and survival of human naive T cells. **A**, Effect of IFN- α on cell proliferation. Purified naive CD4⁺ T cells, treated as described in Fig. 1, were stained with CFSE before activation. The dilution of CFSE intensity was analyzed by FACS 48 h after TCR triggering. Numbers in the table indicate the percentages of cells that entered each discrete cell division. Data represent one of seven comparable experiments performed with cells from different donors. **B**, Effect of IFN- α on cell cycle progression. Cells, untreated or preincubated with IFN- α , were harvested at the indicated times after CD3/CD28 stimulation, and their DNA and RNA content was analyzed by FACS after acridine orange staining. RNA content increases as cells enter the G₁ phase. The table shows the percentages in the G₀, G₁, and S plus G₂/M phases for untreated vs IFN-pretreated cells. **C**, Effect of IFN- α on apoptosis. Forty-eight hours postactivation, cells were harvested, fixed in ethanol, DNA stained with propidium iodide, and analyzed for cell cycle position by FACS. The table shows the percentage of cells in the subG₁ compartment, characterized by <2 N DNA.

compartment (<2 N DNA content) was observed, pointing to an inhibitory effect of IFN- α on cell death.

To better characterize the cell death profile in each of the three cell populations, we analyzed well-described markers of apoptosis. As shown in Fig. 3 (*upper plots*), ~20–30% of the control cells showed the characteristic biochemical hallmarks of mitochondrial-dependent apoptosis: PS exposure, reduction of the lysosomal stability, fall in the $\Delta\psi_m$, superoxide anion generation, reactive oxygen species (ROS), and cell viability loss. In both cell populations exposed to IFN- α , we observed a 50% reduction in the number of

cells positive for all the apoptotic markers described above (Fig. 3, *middle and bottom plots*). The range of apoptosis observed in cells derived from independent donors was rather broad and this most likely reflected the variability intrinsic to human primary cell systems (16). Despite this, apoptosis was consistently reduced in cells exposed to IFN- α . Overall, these results demonstrate that TCR/CD28 stimulation of naive T CD4⁺ cells induces mitochondrial-dependent events of apoptosis and that exposure to IFN- α , before or at the moment of activation, protects cells from this type of death.

FIGURE 3. Effect of IFN- α on mitochondrial-dependent apoptosis of T cells. Cells treated as described in Fig. 1 were harvested 48 h postactivation and analyzed for hallmarks of apoptosis. Aberrant surface exposure of PS was monitored by annexin V-APC conjugate. Lysosome permeabilization was assessed using a LysoTracker-RED (Molecular Probes). Loss of $\Delta\psi_m$ was measured by 3,3'-dihexyloxacarbocyanine iodide (DiOC₆) staining. ROS production was determined by staining with ethidium, a substance oxidized by ROS to become ethidium. Loss of membrane integrity was detected by 7-AAD staining which allows discrimination between early and late apoptotic cells. Numbers indicate the percentage of cells displaying characteristic features of apoptosis.



IFN- α protects from mitochondrial-dependent apoptosis through the regulation of the Bcl-2/Bax ratio

One of the earliest events in mitochondrial-dependent apoptosis is mitochondrial membrane permeabilization, which is mainly controlled by the Bcl-2 family of proteins. The relative amount of death agonists (i.e., Bax and Bak) and antagonists (i.e., Bcl-2 and Bcl-x_L), all members of the Bcl-2 family, controls cellular susceptibility to apoptosis (17). We asked whether IFN- α protected cells from mitochondrial-dependent apoptosis by regulating the ratio and/or the activity of these four Bcl-2 family members. As shown in Fig. 4A, a higher percentage of cells expressing elevated Bcl-2 levels and a lower percentage of cells expressing the active form of

Bax were detected in the two IFN-treated populations. In contrast, the expression of Bak and Bcl-x_L remained unchanged (data not shown). These results suggest that IFN- α protects T cells from mitochondrial-dependent cell death by modulating the ratio of the apoptotic/anti-apoptotic proteins, Bcl-2 and Bax.

To confirm the protective role of IFN- α , we analyzed its effect on cells challenged with an exogenous inducer of mitochondrial-dependent cell death (Fig. 4B). UCB-derived CD4⁺ T cells were stimulated in the presence of the topoisomerase II inhibitor, etoposide, whose activity is mainly regulated by the Bcl-2 levels (18). As shown in Fig. 4B, while 85% of the cells treated with 50 μ M etoposide displayed signs of apoptosis, the percentage of apoptotic

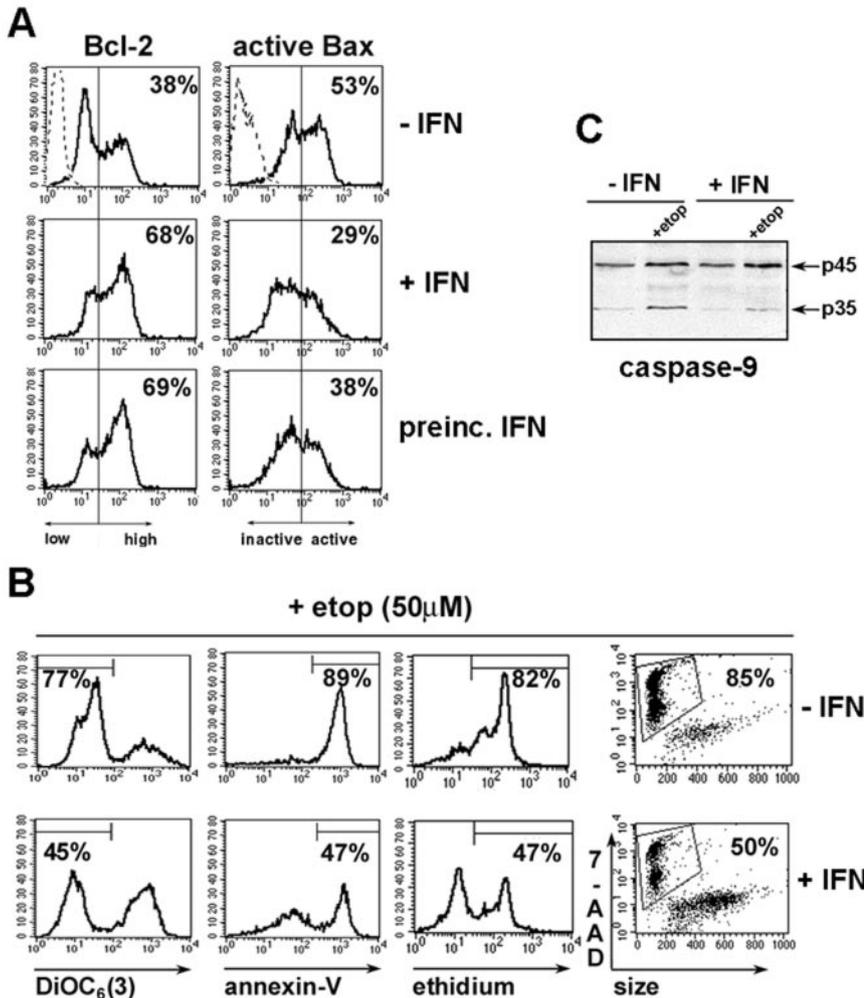


FIGURE 4. A, IFN- α modulates the expression of Bcl-2 family members. Forty-eight hours postactivation, cells were fixed, permeabilized, and stained with an anti-Bcl-2 mAb or with an anti-Bax mAb which recognizes only the active form of Bax. Shown are the percentages of cells displaying high or low expression of Bcl-2 and expressing the active or the inactive form of Bax. Dotted lines represent the expression of Bcl-2 and Bax in naive cells. B, IFN- α protects cells from etoposide-induced apoptosis. CD4⁺ T cells were activated in the presence of 50 μ M etoposide, in the absence (-IFN) or presence of IFN- α . Forty-eight hours postactivation, cells were harvested and analyzed for markers of apoptosis as described in Fig. 2. C, Whole cell lysates from aliquots of the same samples were separated on SDS-PAGE and Western blot analysis was performed using an anti-caspase-9 Ab.

cells decreased to <50% when IFN- α was present, as confirmed by the assessment of four different apoptotic markers (Fig. 4B). Consistently, IFN-treated cells displayed a reduced level of the activated form of caspase-9 (Fig. 4C). The activation of caspase-9 depends mainly on cytochrome *c* release occurring after mitochondrial damage. Thus, the new balance between Bcl-2 and Bax induced by IFN- α is likely to inhibit the mitochondrial injury.

IFN- α induces partial caspase-8 processing and surface Fas in activated cells

To further characterize apoptotic mechanisms in early activated CD4⁺ T cells, we analyzed the activation profile of components of the extrinsic apoptotic pathway and potential changes occurring in IFN- α treated cells. Thus, we monitored the processing of caspase-8 and -3 and DFF45, a caspase-3 substrate, in 48 h-activated cells. The caspase-8 p43/p41 cleavage products were not present in activated CD4⁺ T cells, where only the inactive p55 form was detected (Fig. 5A, 48 h, and C). Consequently, neither the activation of caspase-3 nor the cleavage of DFF45-inhibitor of caspase-activated DNase were observed. In contrast, initial processing of caspase-8 to the p43/p41 forms and of caspase-3 to the p20 form were evident in the two populations of cells treated with IFN- α . Notably, such processing was more evident in cells exposed to IFN- α at the moment of activation as compared with cells preincubated with IFN- α . This difference may be due to the fact that this latter population is characterized by a lower percentage of cells in G₁ (Fig. 2B) and that activation of caspase-3 is known to occur when T cells enter into G₁ (19). Procaspase-3 cleavage to the p20 form was not effective in processing DFF45, as revealed by the absence of the p12 form (Fig. 5A, 48 h). It is important to note that the IFN-induced partial caspase-8 and -3 processing occurred only in activated cells, because we did not detect any cleavage product in naive cells treated with IFN- α for 48 h (data not shown).

The caspase-8, -3, and DFF45 cleavage profiles were also assessed in cells activated for 72 h. In contrast to the results obtained at 48 h, an initial cleavage of caspase-8 was detectable in control cells analyzed at 72 h postactivation (Fig. 5A, 72 h). Moreover, the treatment with IFN- α strongly enhanced caspase-8 cleavage, lead-

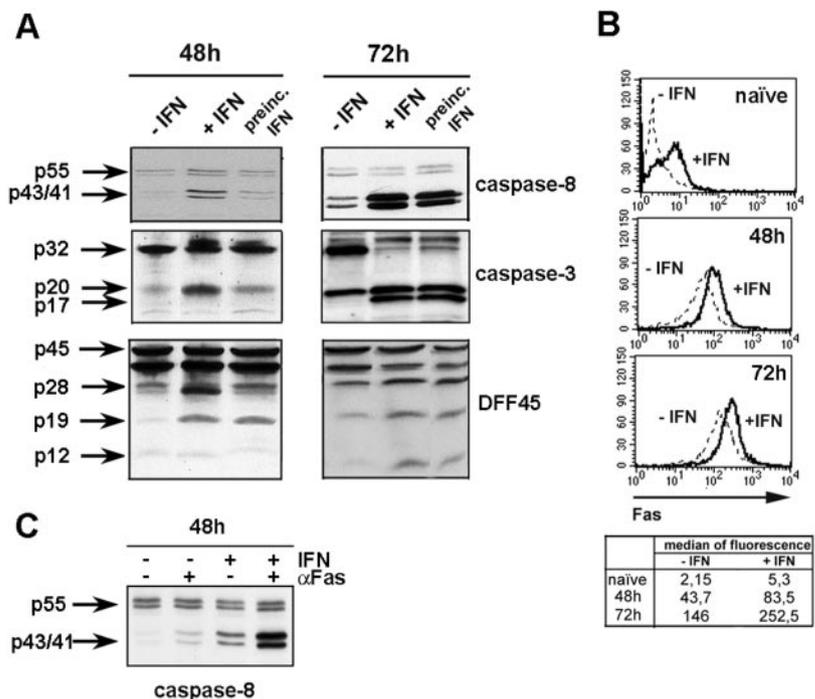
ing to the processing of caspase-3 to the active p17 form and the degradation of DFF45 to the caspase-3-dependent p12 form. IFN-treated cell populations displayed an equivalent level of processed caspases, which correlated with the comparable rate of proliferation detectable at this time (8). These results demonstrate that a prolonged stimulation of CD4⁺ T cells initiates the caspase-8-dependent cell death pathway. Importantly, IFN- α appears to sensitize cells to this apoptotic cascade.

Activation of caspase-8 is initiated by its recruitment to the death receptor Fas via the Fas-associated death domain protein or FADD. Therefore, we asked whether IFN- α participates to the activation of caspase-8 via a control over the Fas/Fas ligand (FasL) system. For this, we monitored the surface expression of Fas in nonactivated cells and in cells activated in the presence or absence of IFN- α . As seen in Fig. 5B, TCR/CD28 engagement strongly induced Fas surface expression. Notably, regardless of the state of activation of the cells, IFN- α led to a 2-fold increase in the level of Fas. Because Fas expression per se is not a measure of the susceptibility of T lymphocytes to Fas-mediated apoptosis (20), we tested the cellular sensitivity to Fas cross-linking. CD4⁺ T cells were activated for 48 h in the presence and absence of IFN- α and were then further incubated for 16 h with an agonistic anti-CD95 mAb. Although cells at this early time after activation were not yet competent to die via Fas, as assessed by measuring apoptotic hallmarks (Ref. 21 and data not shown), a clear increase in the level of the caspase-8 p43/41 forms was observed only in cells exposed to IFN- α (Fig. 5C), suggesting indeed an increased responsiveness. Thus, upon prolonged TCR/CD28 triggering, IFN- α may ultimately sensitize cells to the extrinsic pathway of programmed cell death.

Discussion

During their lifespan, T lymphocytes are subject to cell death checkpoints that ensure their proper development and maintain peripheral homeostasis. When naive T cells engage a high affinity MHC/foreign ligand, they are activated and clonally expand to initiate an immune response. The majority of this activated pool is then deleted either by death due to cytokine

FIGURE 5. IFN- α induces partial caspase and substrate cleavage. **A**, Cells treated or not with IFN- α (as in Fig. 1) were harvested at 48 or 72 h postactivation, lysed and analyzed for caspase-8, -3, or DFF45 processing. Whole cell lysates (50 μ g for caspase-8 and 100 μ g for caspase-3 and DFF45 analysis) were separated by SDS-PAGE and analyzed by Western blot using specific Abs. The proenzymes and cleaved subunits are indicated on the left. Results are representative of four independent experiments performed with UCB-derived CD4⁺ T cells from different donors. **B**, Surface levels of Fas were monitored by FACS on naive CD4⁺ T cells, untreated or treated for 20 h with IFN- α (upper plot) and on cells activated for 48 or 72 h in the presence or absence of IFN- α . Table shows the median fluorescence intensity obtained in one of three independent experiments. **C**, Cells were activated for 48 h in the presence or absence of IFN- α , incubated for 16 h with anti-Fas or control Abs, lysed and analyzed by Western blot for the processing status of caspase-8.



deprivation or by activation-induced cell death, requiring TCR restimulation and Fas/FasL binding. Death signaling in T lymphocytes is a complex process where several redundant apoptotic pathways can be induced in parallel, according to the state of activation of the cells. This redundancy is required both to maintain the size of the lymphocyte compartment and to ensure that activated, and thus potentially dangerous, T cells are eliminated when no longer required (22).

Several cytokines are crucial for the regulation of homeostasis of murine naive and memory T lymphocytes, and they appear to exert different effects in various cell subsets (23). A recent study reported that exposure to IL-2 and IL-7 of human naive CD4⁺ T cells from UCB or peripheral blood, increased their susceptibility to Fas-induced cell death. In contrast, the same treatment did not affect Fas-sensitivity of memory CD4⁺ T cells (16). Here, we aimed to study the impact of IFN- α on the onset of the primary T cell response and, for this, we have analyzed its direct effects on the fate of CD4⁺ T cells derived from UCB, the only population considered as homogeneously and truly "naive" (24). Importantly, in naive cells derived from neonatal cord blood or from adult peripheral blood, the transcriptional activity induced by IFN is comparable (E. Dondi, unpublished observation). Activation was obtained by TCR/CD28 triggering, in the absence of exogenous IL-2 or other costimulatory signals, in view of measuring solely IFN-induced effects. In the absence of IFN- α and at 48 h poststimulation, we could observe the typical features of mitochondrial-dependent cell death occurring in the absence of caspase-8 processing. The presence of IFN- α in the culture, before and/or at the time of activation, consistently reduced the percentage of cells displaying such apoptotic hallmarks. Notably, IFN- α increased the percentage of cells expressing high levels of Bcl-2 and reduced the percentage of cells expressing the active form of Bax, but it did not modulate Bcl-x_L expression or Bak activation. Moreover, the effect of IFN- α was extended to mitochondrial-dependent apoptosis induced by etoposide, whose activity is Bcl-2 dependent. These findings strongly suggest that IFN- α plays a direct role in protecting UCB CD4⁺ T cells from TCR-induced death by increasing Bcl-2 which, in turn, protects from mitochondrial damage. This direct anti-apoptotic activity of IFN is effective once T cell activation has taken place, because the basal Bcl-2 expression was not modulated by IFN in naive cells (data not shown). Accordingly, in cells that were treated with IFN only before activation, only a modest Bcl-2 increase was observed correlating with a reduced protection from apoptosis (data not shown). Because the Bcl-2 level is increased early after activation in untreated cells (Fig. 4A), IFN likely synergizes with TCR/CD28 signals to further augment its expression.

Modulation of the expression of Bcl-2 family members during early T cell activation has been previously investigated. In murine T cells, Bcl-2 and Bax levels have been reported to be increased upon *in vitro* Con A-induced activation (25). Conversely, *in vivo*, a comparable Bcl-2 expression was detected in naive and early Ag-specific CD4⁺ and CD8⁺ effector cells (26, 27). The effect of IFN on the expression of Bcl-2 molecules has been investigated in activated peripheral human and murine T cells rescued from cytokine deprivation-induced apoptosis by this cytokine (28, 29). The level of Bcl-x_L, but not of Bcl-2, was shown to be up-regulated by IFN and exclusively in human cells. In clonal Th2 cells purified from patients with chronic hypereosinophilia, IFN- α prevented mitochondrial changes but it did not increase Bcl-2 and Bcl-x_L expression (30). Overall, these results underline the fine modulation of these proteins in specific T cell subsets according to the type and/or strength of stimulation.

Our data suggest an additional and indirect mode by which IFN restrains cell death. We know that cell cycle entry is consistently delayed upon exposure of naive cells to IFN- α prior and upon their activation (8) and this slower progression into cell division is due to a delayed G₀ to G₁ progression (Fig. 2B). Notably, the presence of IFN-only before activation is not sufficient to induce such an inhibition (data not shown). A balance between cell division and cell death is inherent to the maintenance of homeostasis and, indeed, it has been clearly established that the entry of T cells in the G₁ phase increases their sensitivity to apoptosis (19, 31). Moreover, many proteins that can induce apoptosis are also a component of the cell cycle (32). In this context, IFN- α may protect naive cells from apoptosis, at least in part, by delaying their entry in G₁, likely through the modulation of cell cycle effectors known to be critically involved in this transition (33).

Preliminary signs of activation of the extrinsic apoptotic pathway could be observed in 72 h-activated cells, in the absence of exposure to IFN- α . As opposed to this, it has been reported that, in polyclonally activated PBMC, the processing and the activity of caspase-8 and -3 and the selective cleavage of caspase substrates occur as early as 24 h upon activation in viable and proliferating cells (34–36). We believe that the difference between these and our data relates to the cell system used. T lymphocytes from UCB are truly CD45RA naive cells displaying a high threshold of activation and, consequently, detectable caspase processing in these cells is likely to require a stronger or longer stimulation than cells derived from peripheral blood, where some CD45RO cells convert back to CD45RA expression (37, 38).

Interestingly, and despite the protective effect of IFN, 48 h-activated cells exposed to IFN- α were found to contain partially cleaved caspase-8 and -3 forms and, accordingly, partially cleaved DFF45. It is known that caspase-3 activation to the p20 form does not necessarily lead to cell death, suggesting the existence of downstream checkpoints. Recently, it has been reported that mitochondria activation is required for full caspase-3 processing in granzyme B-induced cell death. Indeed, in Jurkat cells, pro-caspase-3 appears only partially processed by granzyme B, due to the binding of the inhibitor of apoptosis proteins. The release of proapoptotic mediators from mitochondria relies on membrane destabilization in response to truncated Bid and facilitates autoprocessing and full activation of caspase-3. Moreover, Bcl-2 overexpression suppresses the release of these proapoptotic molecules, resulting in cell survival despite partial caspase activation (39, 40). By analogy, in IFN-treated UCB CD4⁺ T cells, the release of proapoptotic molecules is unlikely because mitochondrial integrity is preserved by increased Bcl-2 (Fig. 4A) and Bid is not processed (data not shown).

Caspase activation has been proposed to be a physiological response to TCR triggering and to be involved in nonapoptotic functions at early steps of lymphocyte proliferation (41, 42). Evidence for a role of caspases in T cell proliferation was provided by a recent study showing that in patients with a defect in the caspase-8 function, not only lymphocyte apoptosis and homeostasis, but also T cell activation and proliferation are impaired (43). In view of these findings, we cannot exclude the possibility that the proactivated caspase forms present in IFN- α -treated cells play a positive role during T cell activation.

In our cell system, IFN- α -induced caspase-8 processing was augmented in response to Fas cross-linking, suggesting an enhanced responsiveness due to an increased expression of this receptor (Fig. 5, B and C). IFN-induced expression of Fas and FasL has been reported to occur in T cells purified from peripheral blood, in different types of malignant cells and, notably, in bone

marrow progenitor cells derived from chronic myelogenous leukemia patients (44–46). Several studies have shown that IFN- α exerts potent proapoptotic activities in a number of established cell lines and primary tumor cells (47–49). Induction of apoptosis indeed has been proposed as a major mechanism responsible not only for the antiviral activity of IFN- α (50) but also for its anti-tumor effects. A recent study showed that the IFN- α -induced apoptosis of malignant cells is dependent on both the activation of different caspases and the disruption of mitochondrial integrity (49). The expression of other genes involved in apoptotic pathways, such as caspase-4, -8, and TRAIL, has been found to be induced by IFN in tumor cell lines (45). More recently, an intact PI3K/mTOR pathway was shown to be necessary for the ability of IFN- α to induce apoptosis in a myeloma cell line (51). In contrast, type I IFN plays also an anti-apoptotic role, notably in immune cells. Particularly, IFN- $\alpha\beta$ were shown to inhibit both cytokine-deprivation (28, 29) and Fas-induced apoptosis (52) in activated T cells and to delay, in a PI3K-dependent manner, spontaneous apoptosis in neutrophils (53). Similarly, spontaneous apoptosis of primary B lymphocytes was shown to be inhibited by type I IFN in a PI3K-dependent way (54). On the contrary, IFN- $\alpha\beta$ -mediated inhibition of B cell Ag receptor-triggered apoptosis was described to occur in a PI3K-independent manner via increased Bcl-2 levels (13). Altogether, these results underline the significant cell type-specific qualitative and quantitative differences of the signals activated in response to type I IFN.

Our data demonstrate a dual role of IFN- α during the onset of the primary T cell response that depends on the activation state of the cells and suggest the following scenario. Early upon activation, naive CD4⁺ T cells are sensitive to mitochondrial-dependent apoptosis, which may limit their clonal expansion. As their activation program proceeds, activated cells become increasingly sensitive to Fas-induced death and they can undergo activation-induced cell death (15, 55). When migrating or resident naive cells are exposed to type I IFN, as for example in an inflamed lymph node where pDCs have been recruited, their delayed entry into the cell cycle is counterbalanced by increased resistance to the intrinsic apoptosis. As the T cell response proceeds and environmental IFN decreases, cells may lose mitochondrial protection while being already primed to execute the caspase-dependent process due to an increased Fas expression. Therefore, despite an early resistance to apoptosis favoring an enhanced clonal expansion, T cells activated in the presence of type I IFN may nevertheless become sensitive to activation-induced cell death, a crucial event for maintaining peripheral homeostasis. At the onset of the primary response, T cells are indeed exposed, in a stochastic fashion, to various levels of stimulation according to the amount of Ag, costimulatory molecules, and density of Ag-presenting DCs which control the relative probabilities of proliferation, differentiation, and death. Accordingly, type I IFN may contribute to the survival of cells with an optimal level of activation and to the elimination of over-activated cells. An increasing body of literature supports the rationale of using type I IFN as adjuvant in vaccination against cancer and infectious diseases. In this context, our findings highlight its positive role in shaping T cell-mediated responses.

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