Inhibition of B Cell Receptor-Mediated Apoptosis by IFN[1]

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IFNs are a family of cytokines that are involved in the regulation of immune and inflammatory responses. Clinical use of IFN-α/β encompasses treatment for a variety of diseases; however, prolonged exposure to IFN-α/β results in elevated levels of autoreactive Abs. In this study, we investigated the potential of IFNs to modulate apoptotic signals in B cells. We demonstrate that IFN-α or IFN-β inhibit Ag receptor-mediated apoptosis in a dose-dependent manner. Inhibition of phosphatidylinositol 3′ (PI3)-kinase did not abolish the effect of IFN, indicating that the antiapoptotic mechanism is PI3-kinase- and protein kinase B/Akt-independent. Instead, IFN-α and IFN-β, but not IFN-γ, significantly increase the levels of the survival protein Bcl-2, and to a lesser extent, Bcl-x_L expression. Thus, IFN-α/β-mediated inhibition of B cell Ag receptor-triggered apoptosis may offer a model for the process that leads to the escape of self-reactive B cells from negative selection and consequently results in autoantibody production. ***The Journal of Immunology,*** **1999, 162: 6317–6321.**

Interferons stand out among the members of the cytokine superfamily through their unique pleiotropy of biological effects, which encompass growth inhibition, antiviral defense, and immunomodulatory properties (1, 2). Consequently, IFNs find diverse clinical application in the treatment of tumors (particularly of hematologic and dermatologic malignancies), as well as in the therapy of viral infections (hepatitis C) or immunological disorders (multiple sclerosis, myasthenia gravis). Therapy with type I IFNs, IFN-α and IFN-β, is associated with a variety of undesirable side effects, one of the most common being the development of autoreactive Abs (3–9). A similar autoimmune response has been observed in instances of systemic infectious disease, a condition that is also likely to result in a massive production of type I IFNs.

The rise of autoreactive Abs is ordinarily prevented through the elimination of B cells recognizing self Ag at various stages during their development in the bone marrow and in peripheral lymphoid organs (10, 11). B cell Ag receptor (BCR)[3]-mediated programmed cell death in germinal centers can be triggered by Ag binding in the absence of interaction with CD40 ligand-bearing $CD4^+$ Th cells (11). These findings can be reproduced in vitro with immature B cell lines by initiating apoptosis via cross-linking of the surface Ig with anti-IgM μ-chain Abs, and by the prevention of apoptotic cell death through simultaneous activation of CD40 signaling (12, 13). The abrogation of the apoptotic program after CD40 ligation is likely mediated through the induction of the survival factors and protooncogenes Bcl-x_L and Bcl-2 (13). Indeed, transgenic mice that express Bcl-2 in a disregulated manner display substantial production of autoreactive Abs, presumably due to impaired negative selection of self Ag-directed B cells (14–16). Based on these observations, we decided to investigate whether IFNs could promote B cell survival by way of a similar mechanism. Using the EBV-negative germinal center B cell line Ramos, we demonstrate that type I IFNs not only cause up-regulation of Bcl-2 and, to a much lesser extent, Bcl-x_L expression, but are, in fact, also able to prevent BCR-mediated apoptosis.

Materials and Methods

Cells and reagents

Ramos cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin (Irvine Scientific, Santa Ana, CA). Goat $F(ab')_2$ anti-human IgM (μ-chain-specific) was obtained from Southern Biotechnology Associates (Birmingham, AL). mAb to human Bcl-2 was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal Ab to human Bcl-x_L and murine mAb to human PTP1D were ordered from Transduction Laboratories (Lexington, KY). Phospho-specific Akt and STAT1 Abs were obtained from New England Biolabs (Beverly, MA). Merocyanin 540 (MC540) and propidium iodine were purchased from Sigma (St. Louis, MO), and genistein, wortmannin, and rapamycin were obtained from Calbiochem (La Jolla, CA). IFN-α, IFN-β, and IFN-γ were generous gifts from Hoffman LaRoche (Nutley, NJ), Chiron (Emeryville, CA), and Genentech (South San Francisco, CA), respectively.

Induction and detection of apoptosis

All experiments were performed in RPMI 1640 with 10% FCS, L-glutamine, penicillin, and streptomycin. Ramos cells (5×10^5 cells/ml) were cultured with anti-IgM mAb (10 μg/ml) in the presence or absence of IFN-α, IFN-β, or IFN-γ in 24-well culture plates for 24 h. After treatment, cells were harvested and diluted in ice-cold PBS, pelleted, and resuspended in FACS staining buffer (1× PBS, 1% FCS, 0.1%NaN_3). Cells were stained with MC540 and immediately analyzed by flow cytometry (FACScalibur System; Becton Dickinson, Mountain View, CA; excitation 488 nm, detection 560–600 nm).

Western blot analysis

Following treatment, cells were washed with PBS and lysed with lysis buffer (0.5 ml) containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium-vanadate, and 1 mM PMSF. Lysates were centrifuged at 15,000 × *g* for 15 min at 4°C, and protein concentration was determined by Bradford (Bio-Rad, Richmond, CA). Equivalent concentrations of protein were boiled in SDS sample buffer containing 2-ME and resolved by 7.5% SDS-PAGE (Bio-Rad) and electrotransferred onto Immobilon-P membranes (Millipore, Bedford, MA). After transfer onto poly(vinylidene difluoride) membrane, proteins were detected with anti-Bcl-2, anti-Bcl-X_L, anti-PTP1D, anti-phospho-Akt, and anti-phospho-STAT1. Blots were developed with HRP-conjugated secondary Abs and enhanced chemiluminescence (Amersham, Arlington Heights, IL).

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[3] Abbreviations used in this paper: BCR, B cell Ag receptor; MC540, merocyanin 540; PI3, phosphatidylinositol 3′.

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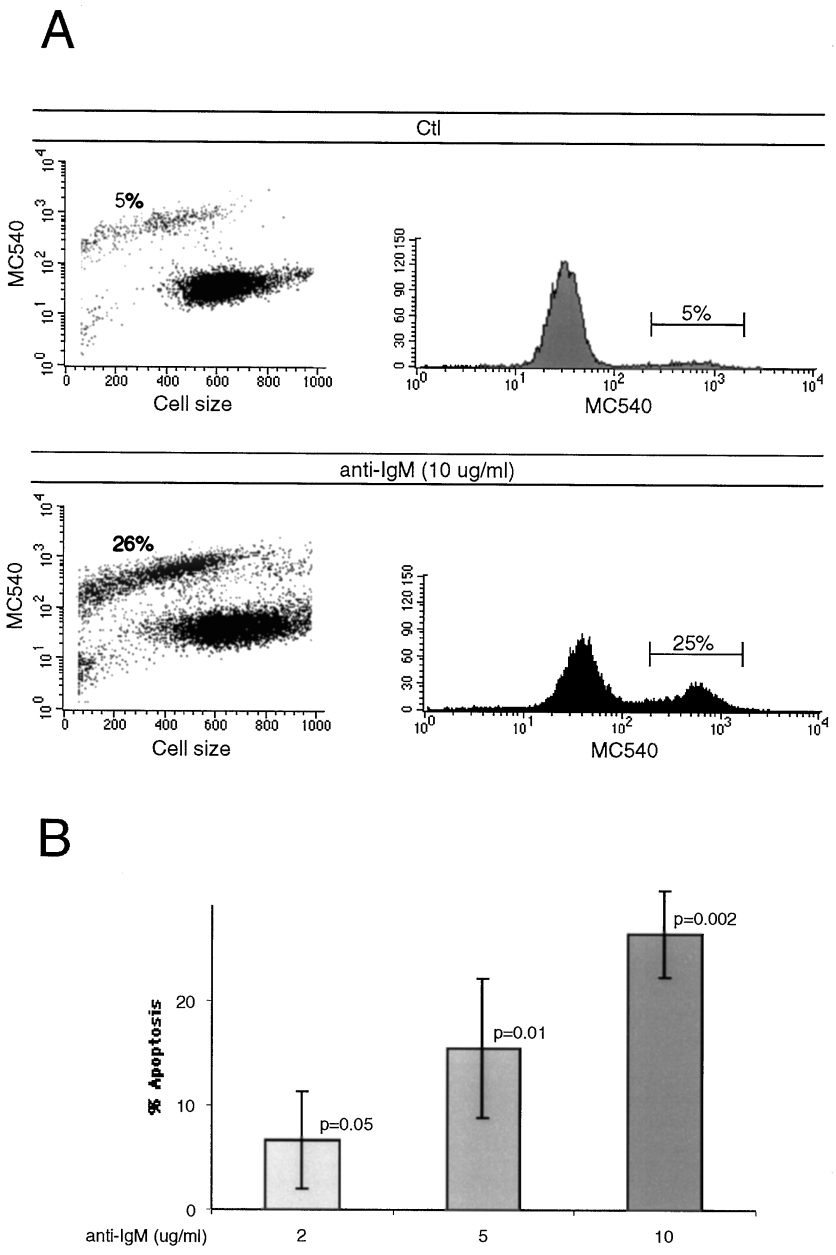

FIGURE 1. Ag receptor-mediated induction of apoptosis. *A*, Ramos cells were left untreated (*top panel*) or treated with 10 μg/ml anti-IgM Ab for 24 h (*bottom panel*). Apoptotic cells were detected by flow cytometry after staining with MC540. Dot plots (*left panels*) show cell size vs MC540 staining. Histogram plots (*right panels*) demonstrate the increase in MC540 staining after anti-IgM treatment. *B*, Cells were treated with increasing concentrations of anti-IgM Ab for 24 h and analyzed by flow cytometry as described above. *p* values are derived from a one-tailed, paired Student's *t* test.

Results

The EBV-negative Burkitt lymphoma cells Ramos, whose phenotype is representative of germinal center B cells, have been reported to undergo anti-Ig-induced programmed cell death (17). To evaluate the extent of apoptosis initiated by cross-linking of the Ag receptor, Ramos cells were incubated for 24 h without (Fig. 1*A*, *upper panel*) or with (*lower panel*) anti-IgM Ab. Several other studies resorted to incubations for up to 48 h; however, we observed only minor differences in the rate of apoptosis between 24 and 48 h (data not shown). Therefore, we restricted our analysis to 24 h intervals to avoid interference with the antiproliferative effects of IFN. The rate of apoptosis was determined by flow cytometry after staining cells with merocyanin, a fluorescent dye that detects changes in the organization of the membrane lipid bilayer associated with early apoptotic events (18). In agreement with earlier reports, anti-IgM-mediated cross-linking triggered programmed cell death in ~25% of the stimulated cells (12, 17). Similar results were obtained when propidium iodine staining was employed to determine the extent of apoptosis (data not shown). To establish a basic correlation between the level of cross-linking and the magnitude of apoptosis, cells were treated with increasing amounts of anti-IgM Ab and assayed for merocyanin binding. The dose-dependent induction of apoptosis after a 24-h stimulation reached a peak at a concentration of 10 μg/ml anti-IgM Ab (Fig. 1*B*).

To explore a potential modulatory function of IFNs on the apoptotic process, Ramos cells were incubated for 24 h with 10 μg/ml of anti-IgM Ab in the presence or absence of IFN-α (10,000 U/ml). As shown in Fig. 2*A*, addition of IFN-α during IgM cross-linking resulted in a 50% reduction of the rate of anti-IgM-induced apoptosis (compare *top right* and *bottom panels*). The observed inhibition of Ag receptor-mediated programmed cell death correlated directly with the concentration of IFN-α (Fig. 2*B*, *left*). Next, we wanted to test whether the ability to prevent apoptosis was restricted to IFN-α, or if IFN-β and IFN-γ were also able to interfere with the progression of BCR-induced cell death. Ramos cells were triggered to undergo apoptosis via BCR cross-linking in

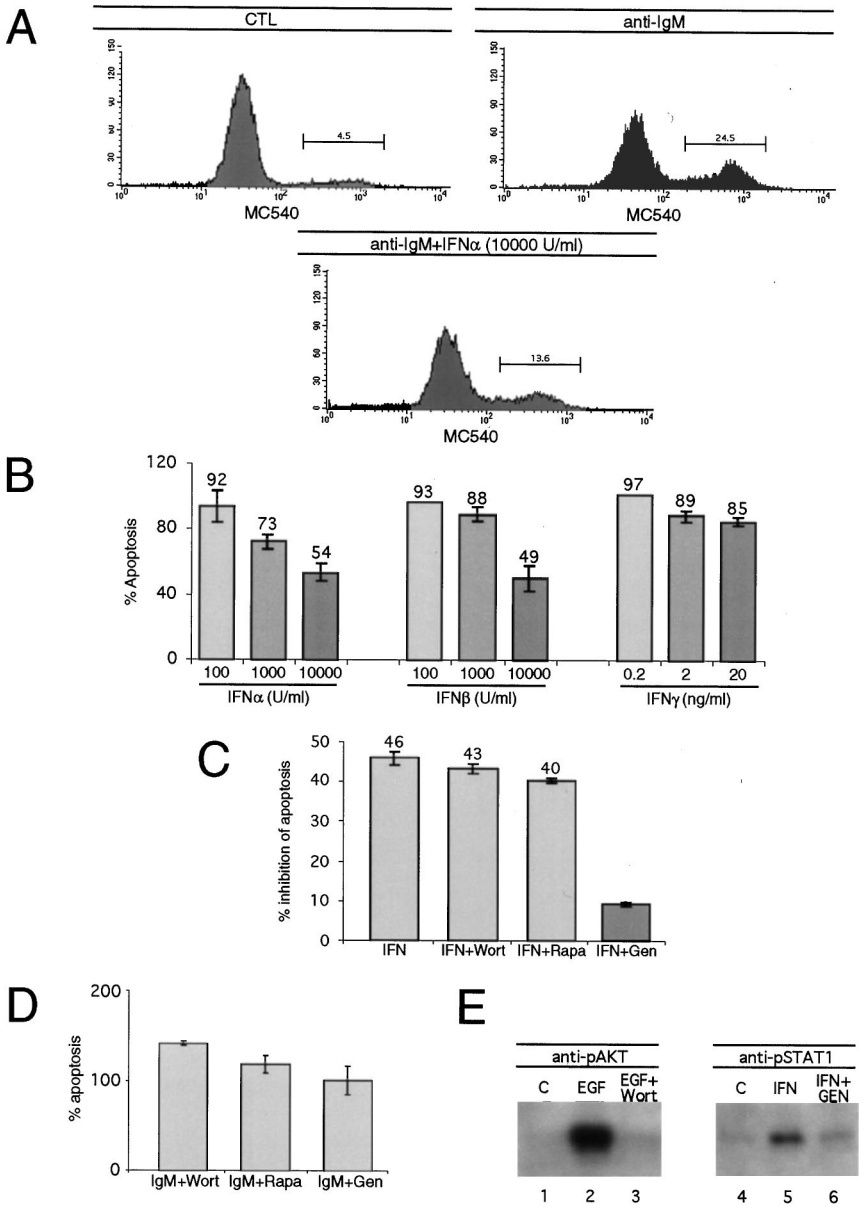

FIGURE 2. Inhibition of BCR-mediated apoptosis by type I IFNs. *A*, Ramos cells were left untreated (*top left panel*), or treated with 10 μg/ml anti-IgM Ab in the absence (*top right panel*) or presence (*bottom panel*) of IFN-α (10,000 U/ml) and the rate of apoptosis determined after 24 h. *B*, Cells were treated with anti-IgM Ab in the presence of increasing amounts of IFN-α, IFN-β, or IFN-γ for 24 h. Anti-IgM-induced apoptosis was set to 100%. *C*, Cells were exposed to either 500 nM wortmannin, 100 ng/ml rapamycin, or 25 μg/ml genistein for 60 min before stimulation with anti-IgM and IFN-α (10,000 U/ml) for 24 h. Apoptosis was analyzed as outlined above, and results are depicted as the percentage of IFN-mediated rescue from apoptosis. *D*, Ramos cells were stimulated with 10 μg/ml anti-IgM in the presence of 500 nM wortmannin, 100 ng/ml rapamycin, or 25 μg/ml genistein. Anti-IgM-induced apoptosis was set to 100%. *E*, Primary human foreskin fibroblasts were treated with 5 ng/ml epidermal growth factor (*lanes 2* and *3*) in the absence (*lane 2*) or presence (*lane 3*) of 500 nM wortmannin, and activation of Akt was analyzed by blotting with a phospho-Akt-specific Ab. Ramos cells were treated with IFN-α (*lanes 5* and *6*) in the absence (*lane 5*) or presence (*lane 6*) of 25 μg/ml genistein, and activation of STAT1 was analyzed by blotting with a phospho-STAT1-specific Ab.

the presence of increasing concentrations of IFN-β or IFN-γ, and the rate of apoptosis was determined after 24 h. IFN-β, which utilizes the same cell surface receptor as IFN-α, was able to prevent apoptosis to a similar extent as IFN-α (Fig. 2*B*, *center*). In contrast, IFN-γ affected the apoptotic process only marginally (Fig. 2*B*, *right*).

The receptor for type I IFNs has been shown to indirectly associate with phosphatidylinositol 3′ (PI3)-kinase via STAT3 (19) and to activate its enzymatic activity (20, 21). The PI3-kinase product PIP3 was identified as an effector molecule of the protooncogene Akt/protein kinase B (22), a Ser/Thr kinase that exerts its oncogenic potential through the prevention of apoptosis (23). Therefore, we decided to test whether the PI3-kinase inhibitors wortmannin or rapamycin could abrogate the antiapoptotic effect of IFN-α. Interestingly, inhibition of PI3-kinase activity by wortmannin or rapamycin (Fig. 2*C*) had no obvious effect on IFN-α's ability to prevent BCR-mediated apoptosis. The complete inhibition of Akt activation by epidermal growth factor in primary human foreskin fibroblasts demonstrates that wortmannin at the applied concentration is highly effective as a PI3-kinase inhibitor (Fig. 2*E*, *lanes 2* vs *3*).

The inhibitory effect of IFN-α was not altered when it was added 1 h before or 1 h after stimulation with anti-IgM Ab (data not shown). This indicated that IFN-α acts by a mechanism distinct from interference with early apoptotic signaling events and suggested hindrance of the apoptotic program based on gene induction. It has been extensively documented that IFN-mediated gene transcription requires the activation of members of the STAT family of transcription factors, which in turn depends on their tyrosine phosphorylation and dimerization (24–28). Therefore, we pretreated cells with the tyrosine kinase-specific inhibitor genistein before addition of IFN-α and initiation of apoptosis by anti-IgM Abs. As shown in Fig. 2*C*, genistein was indeed able to prevent IFN-α from modulating the apoptotic response, thereby strengthening the argument for IFN acting on a transcriptional level. Effectiveness of the genistein pretreatment is also evident from the complete inhibition of STAT1 tyrosine phosphorylation in response to IFN-α (Fig. 2*E*, *lanes 5* vs *6*). Neither inhibitors alone

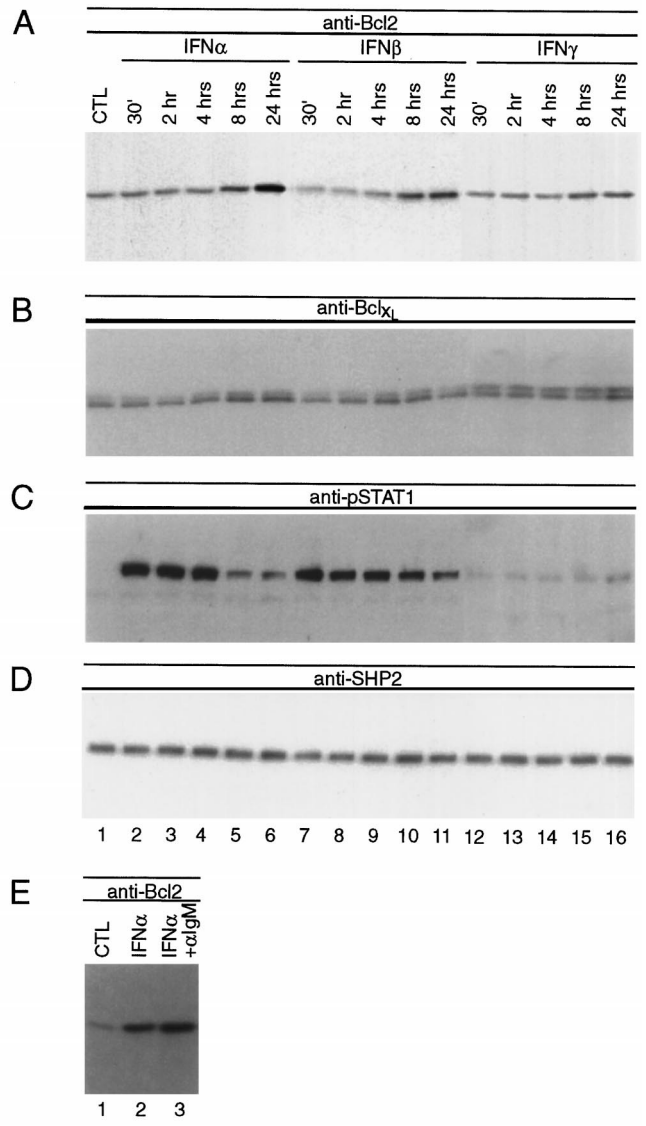

FIGURE 3. Effect of IFNs on levels of Bcl-2 and Bcl-x_L. Cells were treated with 10,000 U/ml IFN-α or IFN-β, or with 20 ng/ml IFN-γ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE, and blots were analyzed for levels of Bcl-2 (*A*), Bcl-x_L (*B*), and phospho-STAT1 (*C*). Reprobing with anti-SHP2 (*D*) was performed to demonstrate equal loading. *E*, Ramos cells were stimulated for 24 h with IFN-α (*lanes 2* and *3*) in the absence (*lane 2*) or presence (*lane 3*) of 10 μg/ml anti-IgM Ab, and Bcl-2 levels analyzed by Western blot analyses.

displayed an inhibitory effect on the anti-IgM-induced apoptosis per se (Fig. 2*D*).

In vivo, germinal center B cells are prevented from undergoing apoptosis after Ag recognition by receiving additional signals through CD40 via interaction with CD40 ligand-bearing Th cells (12, 13). Activation of CD40 has been shown to result in the transcriptional up-regulation of the antiapoptotic protooncogenes Bcl-2 and Bcl-x_L (29). Furthermore, both Bcl proteins have been recognized for their ability to prevent anti-IgM-induced cell death (17). Therefore, we decided to analyze whether stimulation of Ramos cells with IFN-α/β or IFN-γ would increase the expression levels of either Bcl protein. Exposure of cells to IFN-α or IFN-β for various times resulted in substantially increased amounts of Bcl-2, whereas IFN-γ treatment had only a marginal effect on the expression levels of this survival factor (Fig. 3*A*). Although the regulation of Bcl-x_L expression by IFNs seems to follow the pattern set by Bcl-2, the observed changes in protein levels were only marginal (Fig. 3*B*). The levels of STAT1 tyrosine phosphorylation induced by the different IFNs correlates directly with the extent of up-regulation of the Bcl proteins (Fig. 3*C*). To ensure that the demonstrated up-regulation of Bcl-2 and Bcl-x_L was specific for those proteins, and to verify equality of loading of the samples, the blot shown in Fig. 3*A* was stripped and reprobed for the unrelated tyrosine phosphatase SHP-2, revealing identical amounts of the protein in each sample (Fig. 3*D*). Addition of anti-IgM Ab to Ramos cells during stimulation with IFN-α (Fig. 3*E*, *lane 3*) or IFN-β (data not shown) did not alter IFN-mediated up-regulation of Bcl-2.

Discussion

IFNs are widely recognized for their antiviral and antiproliferative effects, and these properties are exploited through the clinical application of IFNs in the therapy of viral infections or malignant diseases (1, 2). An undesirable side effect of IFN therapy that occurs with recognized frequency is the development of autoreactive Abs (3–9), a complication that frequently forces the interruption of the treatment.

A critical outcome of B cell development is the selection of cells expressing functional Ag receptors lacking autoreactivity. Maturing B cells undergo positive selection in the bone marrow to ensure the presence of a functional B cell receptor. At later stages of lymphocyte development, autoreactive B cells are then eliminated from the mature B cell population by means of Ag receptor-mediated programmed cell death. Interference with this negative selection process, i.e., through the ectopic expression of the protooncogene Bcl-2, results in the abrogation of the apoptosis protocol and the escape of autoreactive B cells into the periphery with the subsequent production of self-directed Abs (14–16).

Recent studies have resulted in an increased appreciation of immunomodulatory responses elicited by IFNs that are distinct from their ability to interfere with cell cycle progression and viral replication (30). These biological functions of IFNs include their capability to manipulate the events that mediate programmed cell death. Interestingly, most of the investigations demonstrate the ability of IFNs to initiate apoptosis in a variety of cell types (31), presumably through the transcriptional up-regulation of components of the Fas-initiated apoptotic signaling cascade (32). However, a few studies also illustrate the potential of IFNs to act as negative regulators of programmed cell death, even though the underlying mechanism remained unidentified (33, 34).

Based on this information, we decided to investigate whether IFN treatment could lead to autoantibody production through interference with the apoptosis-based negative selection process of self-directed B cells. The Burkitt lymphoma line Ramos, which in its developmental stage resembles germinal center B cells, yields a robust apoptotic response after the cross-linking of cell surface IgM. As such, these cells provided an ideal model to investigate the putative modulatory actions of IFNs.

In the present study, we show that type I IFNs are indeed able to drastically reduce the level of apoptosis initiated through Ag receptor cross-linking. The type I IFN receptor has been shown to associate with the lipid kinase PI3K (19), whose phosphorylation product is a known activator of the antiapoptotic serine/threonine kinase Akt (PKB) (22, 23, 35). Consequently, it seemed likely that the prevention of IgM-mediated apoptosis by IFN-α/β involves the activation of Akt. However, our results clearly show that the prevention of apoptosis by IFN-α/β occurs independently of PI3K and subsequent Akt activity, since inhibition of the lipid kinase did not abrogate the antiapoptotic properties of IFN-α/β. Furthermore,

we were unable to observe an IFN-mediated increase of Akt kinase activity (unpublished observations). Additional experiments elucidating the kinetic requirements for IFN-α/β to prevent apoptosis suggested that the underlying mechanism depended on IFN-induced gene transcription. Abrogation of the antiapoptotic properties of IFN-α/β in response to the tyrosine kinase inhibitor genistein further supported the notion of survival signal originating from a transcriptional response mediated through the Jak/STAT signaling cascade.

Previous studies had shown that CD40 ligation is able to prevent programmed cell death in Ramos B cells (13), and that increased expression levels of the protooncogene Bcl-2 are the basis for this event. The results presented here demonstrate that the survival factor Bcl-2 is substantially up-regulated in response to type I IFNs, whereas the related factor Bcl-x_L is only slightly affected. Interestingly, IFN-γ, which only marginally influenced BCR-mediated apoptosis, also failed to induce a significant change in Bcl-2 expression levels. These observations were found to further correlate directly with the level of STAT1 tyrosine phosphorylation induced in response to the individual IFNs.

In summary, our results demonstrate that type I IFNs, in contrast to IFN-γ, are able to prevent Ag receptor-mediated programmed cell death. This abrogation of apoptosis is PI3-kinase-independent, but displays a direct correlation with the IFN-α/β-induced up-regulation of the antiapoptotic survival factors Bcl-2 and, to a lesser extent, Bcl-x_L. As inappropriate expression of Bcl-2/Bcl-x_L is a recognized cause for the development of self-directed Abs by limiting apoptotic deletion of autoreactive B cells, our results provide an attractive hypothesis by which the evolvement of autoimmune responses in patients undergoing IFN therapy could be explained.

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