Brief Definitive Report

Interferon γ Inhibits Apoptotic Cell Death in B Cell Chronic Lymphocytic Leukemia

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Summary

The malignant, $CD5^+$ B lymphocytes of B cell chronic lymphocytic leukemia (B-CLL) die by apoptosis in vitro. This is in contrast to the prolonged life span of the leukemic cells in vivo and likely reflects the lack of essential growth factors in the tissue culture medium. We found that interferon γ (IFN-γ) inhibits programmed cell death and promotes survival of B-CLL cells in culture. This effect may also be important in vivo: increased serum levels of IFN-γ, ranging from 60 to >2,200 pg/ml, were found in 7 of 10 B-CLL samples tested, whereas the sera of 10 healthy individuals did not contain detectable levels of this cytokine (<20 pg/ml). High levels of IFN-γ message were detected in RNA from T cell–depleted B-CLL peripheral blood samples by Northern blot analysis. Synthesis of IFN-γ by B-CLL lymphocytes was confirmed by in situ hybridization and flow cytometry. The majority of B-CLL cells (74–82%) expressed detectable levels of IFN-γ mRNA, and $CD19^+$ B-CLL cells were labeled with anti-IFN-γ monoclonal antibodies. These results show that IFN-γ inhibits programmed cell death in B-CLL cells and suggest that the malignant cells are able to synthesize this cytokine. By delaying apoptosis, IFN-γ may extend the life span of the malignant cells and thereby contribute to their clonal accumulation.

B cell chronic lymphocytic leukemia (B-CLL) is characterized by the slow and progressive accumulation of monoclonal, apparently mature, $CD5^+$ B lymphocytes (1). The majority of circulating cells appear to be nondividing (2), and it has been suggested that a prolonged life span is mainly responsible for the accumulation of the leukemic cells (3).

The absence of growth factors induces programmed cell death (apoptosis) in many cultured lymphohemopoietic cells. For example, growth factor–dependent cell lines and IL-2-dependent T lymphocytes initiate apoptosis after the appropriate factors are withdrawn (4). When B-CLL samples are placed in culture, apoptosis occurs despite the presence of FCS in the tissue culture medium (5). Thus, apoptosis in cultured B-CLL cells may be triggered by the absence of a survival-promoting factor (or factors), present in vivo. The identification of such a molecule would not only increase our understanding of pathogenesis in B-CLL, but might ultimately allow the development of targeted treatment.

The $CD5^+$ B cells that accumulate in B-CLL are rare in normal periperal blood (6). Whether normal or malignant human $CD5^+$ B cells respond to different survival or proliferation signals than $CD5^-$ B cells is not yet clear. However, it has been shown that administration of IFN-γ shortens the life span of the F_1 hybrids of New Zealand Black and New Zealand White mice (7) in which elevated levels of Ly-1^+ B cells, the murine equivalent of human $CD5^+$ B lymphocytes, parallel the progression of autoimmune disease (6). Furthermore, injection of anti-IFN-γ antibodies significantly prolonged survival of the animals compared with untreated controls (7).

These observations prompted us to examine the effects of IFN-γ on $CD5^+$ B cells from patients with B-CLL and to test the hypothesis that IFN-γ acts as a survival signal for these cells. This study provides evidence that IFN-γ inhibits programmed cell death in cultured B-CLL cells. We also present data suggesting that IFN-γ may promote survival of B-CLL cells in vivo and that this cytokine is produced by the malignant cells themselves.

Materials and Methods

Samples. Sterile heparinized peripheral blood samples were obtained from nine B-CLL patients and five normal individuals. Serum

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samples were obtained from 10 additional patients and 10 healthy volunteers. Diagnosis of B-CLL was unequivocal according to clinical, morphologic, and immunologic criteria. None of the patients had received chemotherapy for at least 3 mo before the samples were drawn; serum samples were obtained from previously untreated patients. Fresh and cryopreserved B-CLL cells were studied. Mononuclear cells were isolated by two consecutive centrifugations on Ficoll-Hypaque density gradient (Lymphoprep; Nyegard, Oslo, Norway). B-CLL cells were further purified by depletion of monocytes by adherence to plastic tissue culture dishes (Nunc, Roskilde, Denmark). For RNA analysis T cells were depleted (see below). B-CLL cell preparations contained ≥90% $CD19^+$ cells, ≥90% $CD5^+$ cells, <5% $CD2^+$ cells, and <1% $CD14^+$ cells as assessed by staining with mAbs (AMAC, Inc., Westbrook, ME). Trypan blue excluding cells exceeded 95% in all cases studied.

Cell Culture. Separated cells were cultured at a concentration of 1–2 × 10^6 cells/ml RPMI 1640 supplemented with 10% heat-inactivated FCS (batch 9MO57; Whittaker M.A. Bioproducts, Walkersville, MD) and antibiotics. The following recombinant cytokines were used at the final concentrations indicated: 100 U/ml IL-1 (Hoffmann-La Roche, Inc., Nutley, NJ), 1,000 U/ml IL-2, (Cetus Corp., Emeryville, CA), 50 ng/ml IL-6 (Sandoz Pharmaceuticals, East Hanover, NJ), 25 ng/ml IL-7 (R & D systems, Minneapolis, MN), and 100 U/ml IFN-γ (sp act, 3 × 10^7 U/mg; Genentech, South San Francisco, CA). 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma Chemical Co., St. Louis, MO), which has been shown to prevent apoptosis of B-CLL cells (8), was used at a final concentration of 2 × 10^{-8} M. After initial titration, IFN-γ was added daily at a final concentration of 100 U/ml.

Measurement of Soluble IFN-γ. Serum samples were analyzed for the presence of IFN-γ by an ELISA (Bethesda Research Laboratories, Gaithersburg, MD), with an IFN-γ detection limit of 20 pg/ml.

Analysis of Apoptosis. Apoptosis was studied by light microscopy, by DNA gel electrophoresis, and flow cytometry. Cytocentrifuge preparations were stained with Wright-Giemsa for light microscopy analysis of apoptosis. 2 × 10^6 cells were assayed for DNA fragmentation after 16–24 h of culture as described (9). For flow cytometric analysis, 10^6 cells were stained with CD19 mAbs (AMAC, Inc.) and analyzed by flow cytometry on day 0, as well as after 3, 5, and 7 d in culture as detailed elsewhere (9).

Determination of Cell Numbers. Trypan blue–excluding cells of three aliquots of every sample were counted in a standard hemocytometer. A previously published technique was used for counting $CD19^+$ cells from samples harvested on days 0, 1, 3, 5, 7, and 9 of culture by flow cytometry (9).

Ki-67 Staining. Cytospin preparations of cells from four patients cultured with and without the addition of 100 U/ml IFN-γ for 3 and 7 d were fixed with methanol for 30 min at 4°C and stained with Ki-67 antibody (Dako Corp., Carpinteria, CA) followed by incubation with FITC-labeled goat anti-mouse (GAM) Ig (Jackson Immunoresearch Laboratories, West Grove, PA). Slides were examined by fluorescence microscopy.

RNA Analysis. Cryopreserved B-CLL cells from three patients, T cell depleted by rosetting with sheep red blood cells (<2% CD2 cells), were used for Northern analysis of IFN-γ mRNA. Cells were cultured for 6 h in the presence or absence of 2 × 10^{-8} M TPA and 7.5 × 10^{-7} M calcium ionophore A23187 (Sigma Chemical Co.). 20 μg RNA was separated on 1% agarose/6% formaldehyde gels, transferred to nitrocellulose, and the filter hybridized to a ^{32}P-labeled 1.0-kb PstI IFN-γ cDNA fragment (gift of Dr. G. Ricca, Rorer Biotechnology, King of Prussia, PA) as described (10). The stripped filter was hybridized to a 2.0-kb PstI chicken β-actin probe. For in situ hybridization analysis, $CD2^+$ cells of two patients were further depleted with CD2-coated magnetic beads following the manufacturer's recommendations (Dynal, Great Neck, NY): cell preparations contained >99.5% $CD19^+$ cells after separation. After stimulation (see above), cytocentrifuge preparations were fixed in 4% paraformaldehyde and hybridized to a ^{35}S-labeled complementary IFN-γ RNA probe comprising positions 220–520 of the human IFN-γ cDNA as described (11). Sense RNA transcripts served as negative controls.

Detection of Intracellular IFN-γ. Cells from five patients were cultured in the presence of TPA and calcium ionophore for 4–24 h. After surface staining with CD19 mAb of IgM subclass (clone AB1), 10^6 cells were incubated with GAM IgM Abs conjugated to PE (Jackson Immunoresearch Laboratories). Cells were then fixed for 16 h with 0.5% paraformaldehyde at 4°C and permeabilized with 0.2% Tween 20 (Bio-Rad Laboratories, Richmond, CA) in PBS as described (12). After permeabilization cells were incubated with carefully titrated neutralizing anti–human IFN-γ mAbs of IgG1 subclass DC10 or GZ-4 (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 10 min at room temperature followed by one wash in PBS supplemented with 0.2% Tween 20 and one wash in PBS supplemented with 0.2% albumin and 0.2% sodium azide (PBSA). Isotype-matched unreactive Abs were used as control. Bound antibodies were visualized by incubation with FITC-labeled GAM IgG as above followed by fixation with 0.5 ml 0.5% paraformaldehyde.

Results

IFN-γ Inhibits Apoptosis of Cultured B-CLL Cells. Untreated B-CLL cells rapidly initiated apoptosis when placed in culture, as previously described (5). After 16 h, 5–20% of cells showed morphologic evidence of apoptosis such as nuclear fragmentation and loss of cell volume (Fig. 1). At the same time degradation of DNA into fragments of 180 bp and multiples was seen (Fig. 2). The forward and sideward light scattering changes of apoptotic cells (9; data not shown) also became apparent when cultured B-CLL cells were analyzed by flow cytometry. By day 5, only 56 ± 6.1% (mean ± SD) of the original cell number was recovered.

Addition of rIFN-γ to the tissue culture medium inhibited apoptosis (Figs. 1 and 2). In the presence of IFN-γ (100 U/ml), 94 ± 3.6% (mean ± SD) of the $CD19^+$ lymphocytes originally plated were recovered on day 5. IFN-γ was effective at a concentration of 25 U/ml with maximum inhibition achieved in the range of 100–1,000 U/ml (equivalent to ~3–30 ng/ml recombinant protein) and the effects of IFN-γ persisted for 7–9 d (data not shown).

Although IFN-γ prevented apoptosis, there was no evidence of cell proliferation as determined by staining with Ki-67, an antibody that recognizes a cell cycle–related antigen (13): <0.1% lymphocytes of four patients were labeled after 3 and 7 d of culture in the presence of 100 U/ml IFN-γ, confirming previous reports (14). In contrast to IFN-γ, IL-1, IL-2, IL-6, and IL-7 failed to prevent DNA degradation and to support survival (Fig. 2).

B-CLL Cells Synthesize IFN-γ. Next, we measured IFN-γ levels in sera from 10 B-CLL patients and in 10 healthy individuals. As illustrated in Fig. 3, 7 of 10 B-CLL sera contained IFN-γ. The amount of IFN-γ detected ranged from

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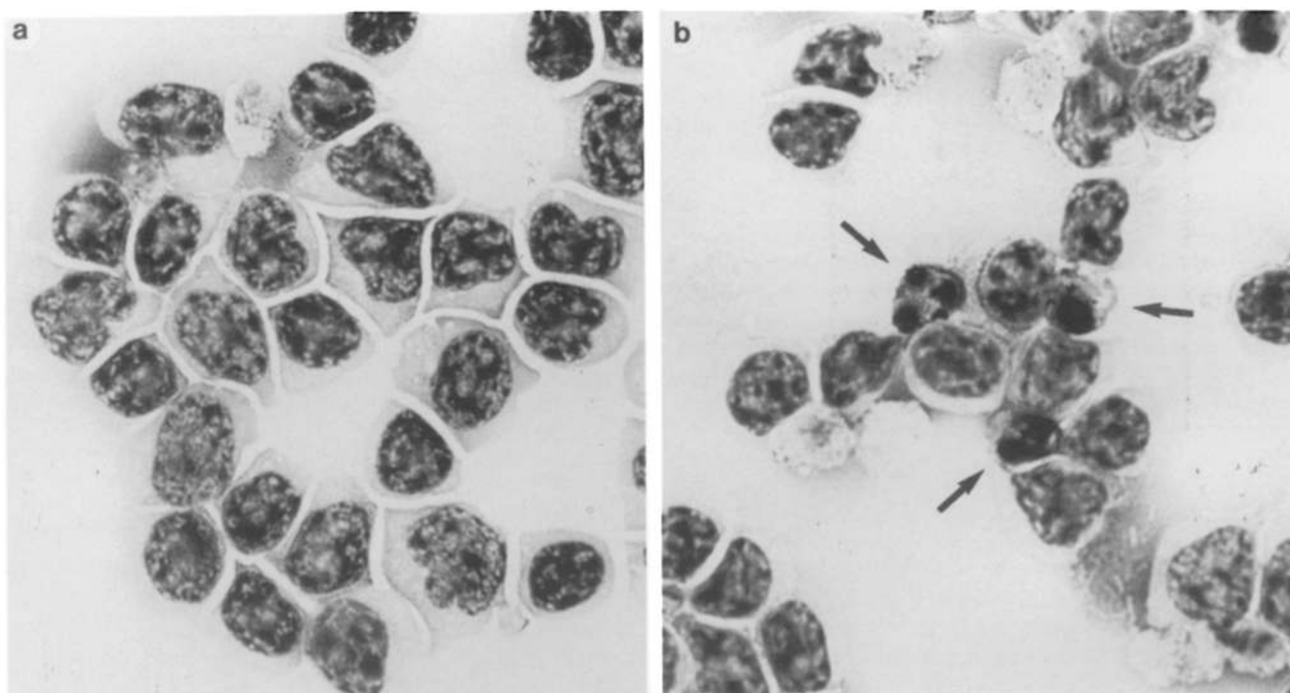

Figure 1. Apoptotic cell death of B-CLL cells is prevented by IFN-γ. B-CLL cells were cultured for 16 h and cytocentrifuge preparations stained with Wright-Giemsa. (*a*) B-CLL cells treated with 100 U/ml rIFN-γ. (*b*) Apoptotic B-CLL lymphocytes in untreated culture.

60 to >2,200 pg/ml. IFN-γ levels in the sera tested did not correlate with the white blood cell count. Five samples with detectable IFN-γ were from patients at stage 0 of the disease according to Rai et al. (15), whereas the other two positive samples were from patients at stages I and II, respectively. The remaining three patients with no detectable IFN-γ levels in the serum were at stage 0. None of the patients had clinical signs of infection. No IFN-γ (<20 pg/ml) was detectable in sera from 10 healthy volunteers.

IFN-γ synthesis by B-CLL cells in vitro was studied at the RNA and protein level. For Northern analysis, three samples with <2% $CD2^+$ lymphocytes were selected. B-CLL cells produced IFN-γ mRNA rapidly and at high levels upon activation with TPA and calcium ionophore (Fig. 4). By in situ hybridization using antisense RNA probes, the majority of B-CLL cells in two cases tested (74 and 82%) were found to synthesize IFN-γ transcripts (Fig. 5).

Using double-color immunofluorescence and flow cytometry, we found that in normal peripheral blood samples acti-

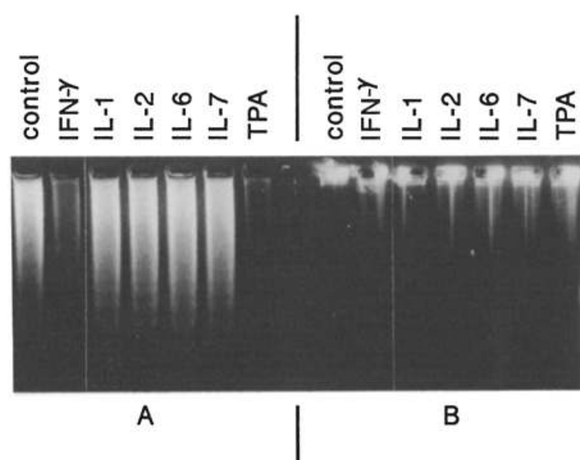

Figure 2. IFN-γ inhibits DNA fragmentation in cultured B-CLL cells. B-CLL cells were lysed after 16 h in culture in the absence (*control*) or presence of IFN-γ, IL-1, IL-2, IL-6, IL-7, and TPA as described in Materials and Methods. (*A*) Fragmented DNA. (*B*) Pellets containing intact DNA and cell debris, serving as control for equal loading.

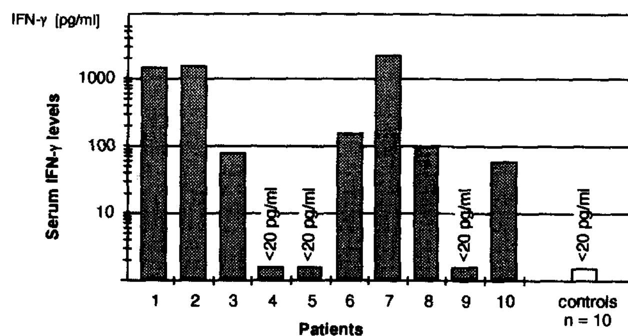

Figure 3. B-CLL sera contain IFN-γ. Serum samples from 10 B-CLL patients and 10 healthy volunteers were analyzed by ELISA for the presence of IFN-γ. Levels of IFN-γ were heterogeneous in patients ranging from 60 to >2,200 pg/ml. No IFN-γ was detectable in sera of healthy volunteers.

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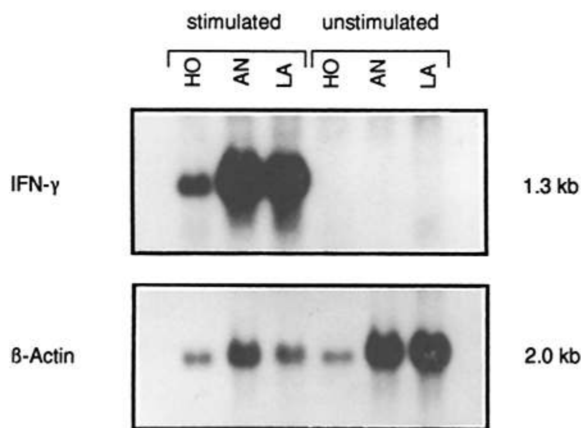

Figure 4. B-CLL cells synthesize IFN-γ mRNA. RNA from B-CLL cells was analyzed for expression of IFN-γ message after stimulation with TPA and calcium ionophore for 6 h. $CD2^+$ cells represented <2% of each sample tested. Filters were rehybridized with β-actin, indicating the relative amount of RNA loaded.

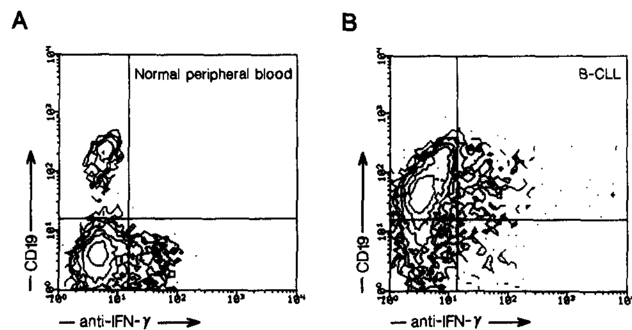

Figure 6. IFN-γ protein is synthesized by stimulated B-CLL cells. B-CLL lymphocytes and PBMC were stimulated with TPA and calcium ionophore for 24 h and stained simultaneously with CD19 and anti-IFN-γ mAbs as described in Materials and Methods. Preparations were analyzed by flow cytometry. (*A*) PBL: 8% $CD19^-$ cells are labeled with anti-IFN-γ mAb. (*B*) B-CLL cells: 2.5% of the lymphocytes are $CD19^+$/IFN-γ^+; 0.5% $CD19^-$ cells were also labeled with anti-IFN-γ mAb. A representative experiment is shown.

vated with TPA and calcium ionophore, IFN-γ was exclusively synthesized by 6–8% $CD19^-$ cells, presumably T lymphocytes (16) (Fig. 6). By contrast, in B-CLL samples most IFN-γ-producing cells were $CD19^+$ B lymphocytes: between 1 and 5% $CD19^+$ B-CLL cells synthesized high levels of this cytokine and a small proportion (≤0.5%) of $CD19^-$ lymphocytes were also labeled by the anti-IFN-γ mAb (Fig. 6). IFN-γ synthesis was observed 6 h after activation, with maximum levels detectable after 24 h.

Discussion

Alterations in the mechanisms leading to cell death may play an important role in the pathogenesis of cancer. Deviation from a programmed death mechanism could result in clonal accumulation of cells normally present at very low levels or representing a transitory cell type. Programmed cell death appears to be regulated by cytokines acting as survival signals (17). Thus, autocrine secretion or aberrant levels of certain cytokines could be responsible for delayed apoptosis and extended survival of malignant cells (18).

Our studies showed that IFN-γ can promote survival in malignant cells from patients with B-CLL. The potential in vivo relevance of this phenomenon is suggested by the detection of IFN-γ in the serum of 7 of 10 B-CLL cases tested. At the concentrations observed in vivo, this cytokine could indeed be responsible for prolonging the life span of the malignant cells.

IFN-γ may act directly on B-CLL cells or through the induction of, or in conjunction with, other factors. For example, it has been recently reported that IFN-γ increases expression of the CD23 antigen on the surface of B-CLL cells, and elevated levels of the soluble form of CD23, which prevents apoptosis of germinal center B cells (19), are also found

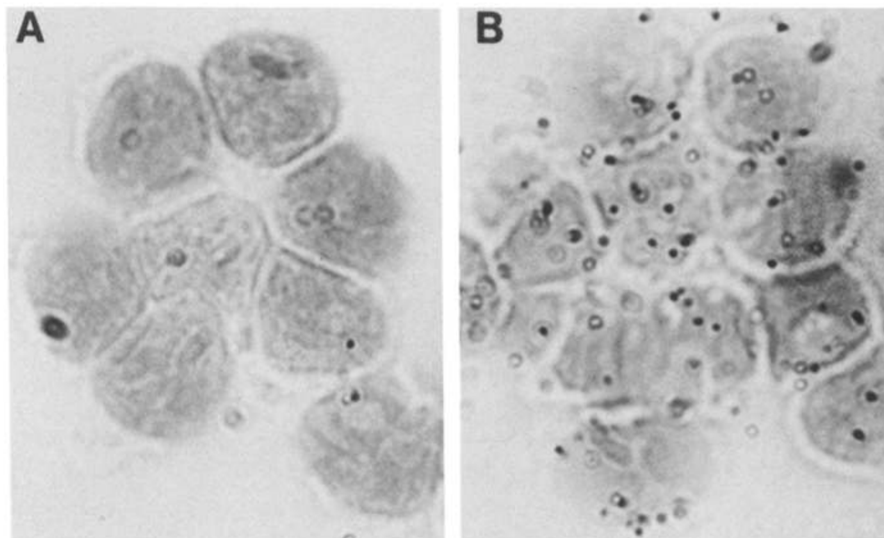

Figure 5. In situ hybridization analysis of IFN-γ synthesis: the majority of B-CLL (74 and 82%) cells synthesize IFN-γ transcripts. Samples contained >99.5% $CD19^+$ B lymphocytes. (*A*) B-CLL cells probed with IFN-γ sense transcripts (negative control). (*B*) B-CLL cells hybridized with a complementary IFN-γ RNA probe.

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in sera of B-CLL patients (20). IFN-γ is unlikely to induce proliferation of B-CLL cells, as cell numbers in IFN-γ-treated samples remained constant and we and others (14) did not find evidence for cell division of the leukemic cells after IFN-γ treatment. In vivo proliferation of B-CLL cells may be induced by other lymphokines such as TNF (21).

IFN-γ appears to be synthesized by the malignant, $CD5^+$ B cells of patients with CLL. We were able to detect IFN-γ mRNA by Northern blot analysis and in situ hybridization in the majority of these malignant B lymphocytes. Furthermore, analysis of IFN-γ protein synthesis using double-color immunofluorescence and flow cytometry showed that $CD19^+$ B-CLL lymphocytes contained high levels of this cytokine, suggesting an autocrine production of IFN-γ by the malignant B-CLL cells.

In conclusion, our results indicate that IFN-γ is a survival promoting factor for B-CLL cells and suggest that the malignant cells may contribute to the synthesis of the survival-promoting cytokine. Our observations may provide a rationale for novel therapeutic strategies based on inhibition of IFN-γ effects on B-CLL cells.

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