Monoclonal Antibody to the Interferon-Inducible Protein Leu-13 Triggers Aggregation and Inhibits Proliferation of Leukemic B Cells

By Sharon S. Evans, Deborah B. Lee, Tin Han, Thomas B. Tomasi, and Robert L. Evans

Interferon (IFN)-α inhibits DNA synthesis stimulated by low molecular weight B-cell growth factor (BCGF) in hairy cells in vitro, suggesting that the therapeutic efficacy of IFN-α in hairy cell leukemia (HCL) involves growth inhibition of malignant B cells. Evidence that the 16-Kd cell surface protein Leu-13 mediates an antiproliferative signal in T lymphocytes and is IFN-inducible in endothelial cells prompted us to examine the expression and functional role of this molecule in leukemic B cells. Leu-13 density, determined by flow cytometry, was upregulated in vitro and in vivo by IFN-α on malignant B cells from patients with HCL, chronic lymphocytic leukemia, and prolymphocytic leukemia. Monoclonal anti-Leu-13 triggered homotypic aggregation of leukemic B cells via an adhesion pathway that was not inhibited by antibodies to leukocyte function associated antigen-1 (LFA-1) or intercellular adhesion molecule-1 (ICAM-1). Moreover, anti-Leu-13 potentiated the inhibitory effects of IFN-α on BCGF-stimulated DNA synthesis, assessed by [3H]-thymidine and [3H]-deoxyadenosine incorporation into DNA. These results indicate that Leu-13 is part of a novel IFN-inducible signaling pathway which may modify the growth and adhesive properties of leukemic B cells under physiologic or therapeutic conditions.

INTERFERONS (IFN) are a group of naturally produced cytokines that exert diverse immunomodulatory, antiproliferative, and antitumor effects in vitro and in vivo. IFN-α therapy elicits a variable antileukemic response in related B-cell lymphoproliferative disorders including hairy cell leukemia (HCL) and chronic lymphocytic leukemia (CLL). In this regard, it is now well established that IFN-α is highly effective in the treatment of HCL with response rates of 80% to 90% reported. In contrast, IFN-α is generally ineffective in advanced B-cell CLL (B-CLL), with response rates of 18% observed. However, recent studies have indicated that IFN may produce clinical benefit in some CLL patients with early stage disease.

It has been proposed that the consistent clinical response to IFN-α in HCL results from direct effects on leukemic B-cell growth, differentiation, and recirculation in addition to activation of host immune effector cells. In a previous report we have shown that IFN-α inhibits DNA synthesis in HCL lymphocytes stimulated with low molecular weight B-cell growth factor (BCGF) in vitro. Genet et al recently confirmed and extended these observations, demonstrating that short-term exposure of hairy cells to IFN-α in vivo inhibits subsequent BCGF-dependent proliferation. These studies have suggested that IFN directly regulates the clonal expansion of malignant B cells in vivo by inhibiting responsiveness to growth factors. IFN-α has also been reported to induce differentiation of normal B lymphocytes, B-HCL, and B-CLL cells in vitro, as assessed by transformation to a plasmacytoid morphology, increased accumulation of intracellular immunoglobulin (Ig), and augmented IgM secretion. Furthermore, IFN-α/β has been shown to act directly on lymphocytes to increase their uptake and retention in lymph nodes in an animal model. Thus, the rapid reduction in circulating leukemic B cells observed in IFN-α-responsive HCL and CLL patients may reflect a direct effect of IFN-α on the recirculation of neoplastic B cells, possibly involving the modulation of homing receptors or adhesion molecules.

It is now clear that although IFN-α binding to high affinity cell surface receptors is required, it is not necessarily sufficient to elicit a biologic response. These observations have served to emphasize the role of postreceptor binding events in mediating the effects of IFN-α. In this regard, IFN-α induces the expression of a number of gene products in IFN-sensitive B-lymphoblastoid cell lines and leukemic B cells including major histocompatibility complex (MHC) class I and II molecules, 2'-5'-oligoadenylate synthetase, and several functionally undefined proteins. Although information is now available regarding the transcriptional regulation of IFN-inducible genes, the role of IFN-inducible proteins in eliciting the immunomodulatory and antileukemic effects of IFN-α remains a critical question.

Recently Jaffe et al reported that the expression of a 16-Kd cell surface protein, designated Leu-13, is inducible by IFN-α and IFN-γ on cultured umbilical cord endothelial cells. Previously we showed that Leu-13 is expressed on T and B lymphocytes as well as on vascular endothelial cells examined in frozen tissue sections. Monoclonal antibody (MoAb) to the Leu-13 antigen was found to trigger T-cell aggregation and inhibit T-cell proliferation that is induced by MoAbs to CD3. These observations prompted us to investigate the effects of IFN-α on leukemic B-cell expression of Leu-13 and to determine whether monoclonal anti-Leu-13 modulated responsiveness of malignant B cells to BCGF. In this report we show that IFN-α augments the cell surface density of Leu-13 on normal B lymphocytes and on leukemic B cells isolated from HCL, CLL, and prolymphocytic leukemia (PLL) patients. Our studies further show that...

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2583
signaling of leukemic B cells with monoclonal anti-Leu-13 upregulates the function of a novel adhesion pathway and potentiates the inhibitory effects of IFN-α on BCGF-driven proliferation. These data suggest that the IFN-inducible cell surface protein Leu-13 plays an important role in mediating the immunomodulatory and antileukemic effects of IFN-α.

MATERIALS AND METHODS

Antibodies. MoAb anti-Leu-13 is an IgG,k protein that has been previously characterized. MoAbs specific for CD5 (anti-Leu-1, an IgG,k) and CD8 (anti-Leu-2a, an IgG,k) have been previously described. MoAbs specific for anti-Leu-14 and anti-Leu-M5, specific for the CD22 and CD11c antigens, respectively, are commercially available (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Anti-Leu-16, an IgG,k specific for the CD20 antigen, was obtained commercially (Becton Dickinson). Anti-B4, an IgG,k specific for the CD19 antigen, was kindly provided by Dr L. Nadler (Dana-Farber Cancer Institute, Boston, MA). L368, an IgG,k directed against B-2 microglobulin, was a gift from Dr R. Levy (Stanford University, School of Medicine, Stanford, CA). Murine 4C11 hybridoma protein, an IgG,x directed against the phosphorylcholine binding site on the T15 idiotype (kind gift of Dr R. Ward, Roswell Park Memorial Institute, Buffalo, NY), was used as a control antibody in this study. Highly characterized MoAbs specific for cell surface adhesion molecules included: MoAbs specific for the a chain of leukocyte-function-associated (LFA) antigen-1, TS1/22 (kindly provided by Dr T.A. Springer, Harvard Medical School, Boston, MA) and 2F12 (kindly provided by Dr J. Ritz, Danafarber Cancer Institute, Boston, MA); and a MoAb specific for intercellular adhesion molecule-1 (ICAM-1), RR1/10 (kind gift from Dr R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Rabbit polyclonal antibodies to human IFN-α were obtained from Interferon Sciences Inc, New Brunswick, NJ.

Cell separations. Enriched populations of leukemic B cells were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation as described previously. Briefly, peripheral blood mononuclear cells (PBMC) were incubated 45 minutes at 37°C with 5 mg/mL carbonyl iron and iron-ingesting cells were depleted with a magnet (Dynal Inc, Great Neck, NY). Following the subsequent removal of plastic adherent cells, the nonadherent population was rosetted one to two times with 2-aminoethylisothiouronium bromide-treated SRBC (AET-SRBC). The nonadherent population was then incubated for 1 hour at 37°C with 106 U/m2 body surface area of recombinant IFN-α, and MoAb to the Leu 13 antigen, cells were pulsed as indicated with either 0.6 μmol/L [3H]-thymidine (Tdr) (6.7 Ci/mmol; New England Nuclear, Boston, MA), 10 μmol/L [3H]-Tdr, or 0.6 μmol/L [3H]-deoxyadenosine (29 Ci/mmol; Amersham Corp, Arlington Heights, IL) for 4 hours, and DNA was harvested using a Beckman cell harvester and counted in a Beckman scintillation counter. For [3H]-deoxyadenosine incorporation, a 1-hour incubation at 37°C in 0.1 mol/L NaOH was performed before precipitation with 10% trichloroacetic acid to ensure hydrolysis of labeled RNA, according to the method of Gewart et al. All cultures were performed in triplicate and the standard deviation between replicates was less than 5%. Cells were cultured for 3, 5, 7, and 9 days and maximal incorporation was

Flow cytometric analysis of B-cell phenotypes. The relative expression of the Leu-13 antigen on normal and malignant B cells was determined by indirect immunofluorescence analysis using a FACS 440 (Becton Dickinson, Sunnyvale, CA). Following Fe receptor (FcR) blockade with goat serum for 10 minutes on ice, 5 × 10^6 B cells were incubated with saturating amounts of monoclonal anti-Leu-13 (100 μg/mL) for 1 hour, washed once, and incubated for 30 minutes with goat F(ab')2 anti-mouse IgG-fluorescein isothiocyanate (FITC) (Organon Teknika-Cappel, Malvern, PA) at a dilution of 1:30. As a control, murine 4C11 hybridoma protein, an IgG,x specific for an irrelevant antigen, was substituted for anti-Leu-13 in the first step. We previously showed that these labeling conditions are required to optimally stain the Leu-13 antigen with 81% fluorescence intensity is proportional to the number of antibody binding sites per cell. A total of 4,000 events were analyzed and specific fluorescence intensity, reported as mean channel fluorescence based on a linear scale of 0 to 255 channels, was calculated by subtracting control fluorescence values.

IFN-α receptor analysis. Analysis of IFN-α binding to malignant B cells was performed in competitive binding assays as previously described. Briefly, recombinant human IFN-α (rhIFN-α) (kindly provided by Dr P. Trotta, Schering Corp, Bloomfield, NJ) was iodinated to a final specific activity of 63 mCi/mg. IFN-α receptor expression was determined by incubating 5 × 10^6 cells with 0.5 ng of [125I]-IFN-α and increasing amounts of unlabeled ligand for 2 hours at 4°C. Under these conditions maximal binding of [125I]-IFN-α occurred within 45 minutes and IFN-α was not internalized or degraded. At the end of the incubation, cell-bound and free [125I]-IFN-α were separated by layering samples over 0.2 mL of a 2:1 mixture of di-n-butylphthalatediodinyl phthalate in microfuge tubes at 4°C. Following centrifugation for 1 min at 10,000g, supernatants were aspirated and microfuge tubes containing cell pellets were counted in a Beckman gamma counter with 81% efficiency. Scatchard analysis of the binding data was performed using the LIGAND computer program to facilitate the identification of high affinity binding sites (dissociation constant [Kd] of approximately 1 to 6 × 10^-10 mol/L).

Analysis of DNA synthesis in leukemic B cells. The incorporation of [3H]-nucleosides into DNA in malignant B cells was studied by culturing 2 × 10^6 cells/well in 96-well plates in a total volume of 0.2 mL RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY), 0.01% Staphylococcus aureus Cowan I (SAC I; Calbiochem, La Jolla, CA), 10% low molecular weight BCGF (vol/vol) (Cellular Products, Buffalo, NY), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine as previously described. Consistent with previous reports, this BCGF preparation supported both short-term and long-term proliferation of normal and leukemic B cells but did not stimulate the proliferation of T cells. Leukemic B cells failed to incorporate significant amounts of [3H]-thymidine in the absence of BCGF and SAC I (<500 cpm/2 × 10^6 cells). Following incubation of these cultures with rhIFN-α and MoAb to the Leu 13 antigen, cells were pulsed as indicated with either 0.6 μmol/L [3H]-thymidine (Tdr) (6.7 Ci/mmol; New England Nuclear, Boston, MA), 10 μmol/L [3H]-Tdr, or 0.6 μmol/L [3H]-deoxyadenosine (29 Ci/mmol; Amersham Corp, Arlington Heights, IL) for 4 hours, and DNA was harvested using a Beckman cell harvester and counted in a Beckman scintillation counter. For [3H]-deoxyadenosine incorporation, a 1-hour incubation at 37°C in 0.1 mol/L NaOH was performed before precipitation with 10% trichloroacetic acid to ensure hydrolysis of labeled RNA, according to the method of Gewart et al. All cultures were performed in triplicate and the standard deviation between replicates was less than 5%. Cells were cultured for 3, 5, 7, and 9 days and maximal incorporation was
routinely detected on day 7. The results reported in this study are based on data obtained following 7 days of culture.

Quantitation of aggregation. The number of normal and leukemic B cells in aggregates was quantitated essentially as previously described by Rothlein et al. To block potential FcR sites, 2 × 10^5 B cells were incubated 30 minutes with 50 pg/mL of anti-Leu-2a, an IgG1 specific for an irrelevant antigen. Cells were then transferred to 96-well plates and incubated in a total volume of 100 μL of 10% FCS/RPMI 1640 supplemented with 1,000 IU/mL IFN-α and 5 pg/mL anti-Leu-13. Ascites containing MoAbs that block aggregation mediated by the interaction of LFA-1 (TS1/22* and 2F1229) and ICAM-1 (RR1/10.4) were included in cultures at a final dilution of 1:200. In preliminary studies it was established that MoAbs TS1/22 and 2F12 inhibited phorbol myristate acetate (PMA)-induced aggregation of B cells from normal donors to the same extent and, therefore, both antibodies were used throughout this study. After incubation for 18 hours at 37°C, cells were gently resuspended and the number of nonaggregated cells was determined by counting 10 μL of the suspension in a hemocytometer. The total number of cells was determined after the addition of 10 mmol/L EDTA and incubation at 4°C for 30 minutes. Percent aggregation was determined by the following equation:

\[
\text{% Aggregation} = \left(1 - \frac{\text{number of nonaggregated cells}}{\text{total number of cells}}\right) \times 100
\]

RESULTS

IFN-α-induction of Leu-13 antigen in leukemic B cells in vitro and in vivo. The relative cell surface density of Leu-13 antigen on normal and leukemic B cells was determined by immunofluorescence flow cytometry (Table 1). The initial levels of Leu-13 antigen detected on leukemic B cells measured at zero time were relatively low compared with normal B lymphocytes, but highly variable among different patient samples. Direct comparison of samples incubated 48 hours in the presence or absence of 1,000 IU/mL rIFN-α showed that IFN-α markedly increased Leu-13 antigen density on leukemic B cells isolated from 5 of 5 HCL, 3 of 3 PLL, 11 of 13 early stage (stage 0 through II) CLL, and 4 of 4 advanced stage (stage III) CLL patients. IFN-α similarly upregulated Leu-13 expression on peripheral B lymphocytes isolated from normal donors. IFN-α failed to upregulate Leu-13 antigen density in 2 of 13 experiments involving B cells from early stage CLL patients (stage 0 CLL patient J.B. and stage I CLL patient R.R.), possibly reflecting a defective in vitro response to IFN-α in a subset of B-CLL patients. Analysis of leukemic B cells from five patients indicated that equivalent concentrations of rIFN-γ (Scher-

<table>
<thead>
<tr>
<th>Disease</th>
<th>Stage</th>
<th>Patient</th>
<th>0 h</th>
<th>48 h</th>
<th>48 h + IFN-α</th>
<th>R/C</th>
<th>K_r (x 10^-10 mol/L)</th>
</tr>
</thead>
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<td>CLL</td>
<td>0</td>
<td>J.B.</td>
<td>ND</td>
<td>85.0</td>
<td>70.1</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>J.G.</td>
<td>4.3</td>
<td>43.0</td>
<td>124.4 (70.0)</td>
<td>1,680</td>
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<td></td>
<td></td>
<td>J.I.</td>
<td>20.6</td>
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<td>121.4 (70.4)</td>
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<td>ND</td>
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<tr>
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<td></td>
<td>W.K.</td>
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<td>124.0</td>
<td>425</td>
<td>2.1</td>
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<td></td>
<td></td>
<td>C.T.</td>
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<td>25.8</td>
<td>83.7</td>
<td>380</td>
<td>1.8</td>
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<tr>
<td></td>
<td>I</td>
<td>E.B.</td>
<td>10.4</td>
<td>33.2</td>
<td>113.0 (68.0)</td>
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<td></td>
<td></td>
<td>E.C.</td>
<td>14.0</td>
<td>16.8</td>
<td>141.7</td>
<td>3,431</td>
<td>17.4</td>
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<td></td>
<td></td>
<td>B.F.</td>
<td>0.0</td>
<td>44.1</td>
<td>119.1</td>
<td>1,100</td>
<td>4.3</td>
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<td></td>
<td></td>
<td>R.R.</td>
<td>6.4</td>
<td>23.9</td>
<td>16.3</td>
<td>223</td>
<td>1.6</td>
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<td></td>
<td></td>
<td>B.W.</td>
<td>0.0</td>
<td>25.9</td>
<td>61.8</td>
<td>539</td>
<td>3.5</td>
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<td></td>
<td>II</td>
<td>A.B.</td>
<td>5.0</td>
<td>12.4</td>
<td>138.9</td>
<td>2,091</td>
<td>3.1</td>
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<td></td>
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<td>W.F.</td>
<td>15.3</td>
<td>54.8</td>
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<td>1,066</td>
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<td></td>
<td>M.P.</td>
<td>18.2</td>
<td>94.3</td>
<td>146.9 (98.0)</td>
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<td>1.9</td>
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<tr>
<td></td>
<td></td>
<td>R.G.</td>
<td>2.3</td>
<td>23.3</td>
<td>109.2</td>
<td>945</td>
<td>2.5</td>
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<td></td>
<td></td>
<td>H.L.</td>
<td>7.0</td>
<td>12.8</td>
<td>77.0</td>
<td>&lt;50</td>
<td>—</td>
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<td></td>
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<td>I.P.</td>
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<td>2.1</td>
<td>31.7</td>
<td>949</td>
<td>7.9</td>
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<tr>
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<td></td>
<td>F.W.</td>
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<td>96.1</td>
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<td>4.9</td>
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<td>13.0</td>
<td>83.1</td>
<td>538</td>
<td>1.0</td>
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<td>G.P.</td>
<td>0.0</td>
<td>9.9</td>
<td>40.5</td>
<td>1,018</td>
<td>6.5</td>
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<td></td>
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<td>7.2</td>
<td>63.3</td>
<td>5,898</td>
<td>12.3</td>
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<td>1,707</td>
<td>7.5</td>
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<tr>
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<td>T.L.</td>
<td>ND</td>
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<td>144.3</td>
<td>544</td>
<td>4.4</td>
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<td></td>
<td>E.R.</td>
<td>ND</td>
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<td>47.3</td>
<td>330</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B.W.</td>
<td>8.3</td>
<td>1.1</td>
<td>118.5</td>
<td>2,259</td>
<td>4.7</td>
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<td></td>
<td></td>
<td>S.Z.</td>
<td>ND</td>
<td>16.9</td>
<td>58.4</td>
<td>1,000</td>
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<td>Normal donor§</td>
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<td>26.0</td>
<td>32.0</td>
<td>118.0</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td>44.0</td>
<td>62.0</td>
<td>106.0</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses represent specific linear fluorescence of Leu-13 following incubation of cells for 48 hours with 1,000 IU/mL IFN-γ (Schering Corp.).

Abbreviation: ND, not determined.

*Leu-13 antigen density was assessed by flow cytometry at 0 hours and following culture for 48 hours ± 1,000 IU/mL IFN-α.
†The limit of detection of high affinity IFN-α receptors was greater than 50 receptors/cell (R/C).
‡An enriched population of peripheral blood B cells was isolated from normal donors (>80% CD19* and <7% CD3*).
Table 2. IFN-α Induction of Leu-13 Antigen In Vivo

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patient</th>
<th>Pre-IFN-α Treatment</th>
<th>Days Post-IFN-α Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL</td>
<td>N.C.</td>
<td>1.9</td>
<td>44.9 19.2 ND ND 21.0</td>
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<tr>
<td>HCL</td>
<td>B.W.</td>
<td>10.1</td>
<td>52.9 57.2 88.4 136.6 103.3</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*Peripheral WBC counts were greater than 44 x 10^3/mm³ and percent lymphocytes was greater than 95% at these time points.

...ing Corp) were also capable of increasing Leu-13 expression, although less efficiently than IFN-α. The induction of Leu-13 antigen by IFN-α shown by flow cytometric analysis in Table 1 was confirmed in selected patient samples by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, as previously described for endothelial cells.2 The constitutive expression of cell surface IFN-α receptors on leukemic B cells was determined in competitive IFN-α binding studies (Table 1). A significant number of specific high affinity receptors for IFN-α were detected in a majority of patient samples examined. However, in these studies the number of high affinity IFN-α receptors expressed on leukemic B cells did not correlate (P > .05) with the level of upregulation of the Leu-13 antigen. For example, similar increases in Leu-13 density were detected in B-HCL cells from patients T.L. and B.W. expressing 544 and 2,259 receptors/cell, respectively.

To determine if the Leu-13 antigen was modulated by IFN-α in vivo, the expression of Leu-13 was monitored in two patients undergoing IFN-α therapy. Leu-13 antigen density on leukemic B cells was evaluated by immunofluorescence analysis immediately before therapy and up to 28 days following the initiation of IFN-α treatment. The data shown in Table 2 indicate that the expression of the Leu-13 molecule was augmented in circulating leukemic B cells during the course of IFN-α therapy. Although the relationship between the level of induction of Leu-13 in vivo and clinical responsiveness to IFN-α was not determined in this limited study, these data show that modulation of Leu-13 by IFN-α is not restricted to in vitro culture conditions.

Dose-dependence, cycloheximide sensitivity, and kinetics of Leu-13 induction by IFN-α. B cells from two HCL patients and one CLL patient were incubated with increasing concentrations of rIFN-α for 48 hours before the assessment of the Leu-13 antigen density by immunofluorescence analysis. The results shown in Fig 1 demonstrate that the Leu-13 antigen was markedly increased by IFN-α in a dose-dependent manner in vitro. Significantly, induction of the Leu-13 antigen occurred at relatively low doses of IFN-α ranging from 0.1 to 100 IU/mL, demonstrating that this antigen can be regulated under both physiologic and pharmacologic conditions.

To determine if protein synthesis was required for the induction of Leu-13 by IFN-α, leukemic B cells from CLL patient N.W. were incubated with and without 50 μg/mL cycloheximide for 2 hours before induction with 1,000 IU/mL IFN-α (Fig 2). In the positive control, exposure of B-CLL cells to IFN-α alone for 18 hours significantly increased the Leu-13 cell surface antigen density (Fig 2b; specific linear fluorescence = 118.2) relative to untreated cells (Fig 2a; specific linear fluorescence = 5.6). Consistent with the report of Jaffe et al.2 on endothelial cells, cycloheximide pretreatment of leukemic B cells abrogated the induction of the Leu-13 antigen (Fig 2c; specific fluorescence intensity = 11.1), suggesting that upregulation of cell surface Leu-13 requires new protein synthesis rather than mobilization from intracellular precursor pools.

Isolated B cells from two HCL patients were incubated with 1,000 IU/mL IFN-α in vitro for the indicated period of...

![Fig 1. Dose-dependent induction of Leu-13 by IFN-α. Leukemic B cells were incubated with increasing concentrations of rIFN-α for 48 hours. The relative expression of Leu-13 antigen was determined by indirect immunofluorescence analysis.](image)

![Fig 2. Cycloheximide inhibits Leu-13 induction by IFN-α. B-CLL cells were cultured 18 hours either in medium alone (A) or in the presence of 1,000 IU/mL IFN-α (B) before indirect immunofluorescence analysis. Relative immunofluorescence intensity following staining with second antibody control (---) and anti-Leu-13 (—) are shown. In (C), leukemic B cells were preincubated 2 hours with 50 μg/mL cycloheximide before the addition of IFN-α. Cell viability following cycloheximide treatment was greater than 90%.](image)
time to determine the kinetics of Leu-13 antigen induction (Fig 3). IFN-α rapidly induced cell surface Leu-13 antigen on leukemic B cells with increased antigen density detectable as early as 8 hours on B-HCL cells from patient B.W. (Fig 3). Increased expression of the Leu-13 antigen was maintained for greater than 5 days in these experiments.

IFN-α augments the capacity of anti-Leu-13 to trigger homotypic aggregation of leukemic B cells. Leukemic B cells from CLL and HCL patients reportedly express low levels of LFA antigen-1 and ICAM-1, which mediate adhesions between lymphocytes triggered by activators of protein kinase C (PKC). This observation may account for the fact that CLL cells have a reduced capacity to aggregate in the presence of PKC-activators, including PMA. Because we have previously shown that anti-Leu-13 induces peripheral blood T cells to aggregate in vitro it was of interest to determine the capacity of anti-Leu-13 and IFN-α to modulate the adhesiveness of leukemic B cells. A representative experiment is shown in Fig 4. Following the blocking of potential FcR sites with anti-Leu-2a, an IgG, specific for an irrelevant antigen, leukemic B cells isolated from an HCL patient (S.Z.) were cultured at a final concentration of 10^6 cells/mL for 18 hours at 37°C in the presence of (a) medium, (b) 1,000 IU/mL IFN-α, (c) 5 μg/mL anti-Leu-13, or (d) 1,000 IU/mL IFN-α and 5 μg/mL anti-Leu-13. While medium alone (Fig 4a) or 1,000 IU/mL IFN-α (Fig 4b) did not directly induce aggregation, anti-Leu-13 triggered the formation of small aggregates (Fig 4c). Aggregation of leukemic B cells was markedly increased by coculture with IFN-α and anti-Leu-13 (Fig 4d). Aggregation triggered by IFN-α and anti-Leu-13 occurred rapidly (3 to 8 hours) in parallel with the induction of the Leu-13 antigen. In repeated experiments, similar results were obtained using lower doses of IFN-α (eg, 10 and 100 IU/mL) and B-CLL, HCL, and PLL cells, which generally expressed less than 25% the amount of LFA-1α subunit detected on normal peripheral blood B cells (data not shown). Leukemic B-cell aggregation did not occur in the presence of 10 mmol/L EDTA or following culture at 4°C. MoAbs directed against several other cell surface molecules expressed on B-CLL at similar densities as IFN-induced Leu-13, including CD5 (anti-Leu-1, mean channel fluorescence ± SD = 89 ± 13, n = 10), CD19 (anti-B4, mean channel fluorescence ± SD = 116 ± 19, n = 10), and β-2-microglobulin (L368, mean channel fluorescence ± SD = 110 ± 12, n = 4), did not induce B-CLL cellular adhesion (<10% cells in aggregates, data not shown). MoAbs anti-B, and L368 further serve as controls for possible involvement of FcR-mediated cellular cross-linking because they have the same isotype as anti-Leu-13.

To determine if the aggregation triggered by IFN-α and anti-Leu-13 resulted from increased intercellular LFA-1-ICAM-1 binding, the number of normal and leukemic B cells in aggregates was quantitated following incubation in the presence of MoAbs that specifically block adhesion via this pathway (Table 3). IFN-α and anti-Leu-13 induced cellular adhesion was stable, allowing quantitation of the degree of aggregation, and was inhibited by the inclusion of rabbit polyclonal antibody to human IFN-α. The results shown in Table 3 indicate that PMA induces significantly less aggregation of leukemic B cells (22% aggregation, SD = 11%, n = 6; legend) as compared with normal donor B lymphocytes (71% aggregation) and confirm the data reported by Inghirami et al. Aggregation of normal donor B cells induced by PMA was inhibited 65% and 58%, respectively, by MoAbs to LFA-1α (TS1/22 and 2F12) and ICAM-1 (RR1/1) (Table 3), as previously described. In contrast, IFN-α and anti-Leu-13 triggered similar aggregation of normal B lymphocytes (80% aggregation) and leukemic B cells (84% aggregation, SD = 6%, n = 6) while MoAbs TS1/22, 2F12, and RR1/1 did not significantly inhibit aggregate formation. Thus, these studies indicate that the aggregation signal provided by IFN-α and anti-Leu-13...
involves an adhesion pathway distinct from a LFA-1–ICAM-1–dependent interaction.

**Effect of IFN-α and anti-Leu-13 on BCGF-induced [³H]-nucleoside incorporation.** IFN-α has previously been shown to inhibit [³H]-thymidine incorporation in the Daudi B-lymphoblastoid cell line without altering overall DNA synthesis by decreasing thymidine transport and thymidine kinase activity.\(^\text{23}\) These studies have raised the concern that inhibition of DNA labeling with exogenous [³H]-thymidine in leukemic B cells may not reflect changes in DNA synthesis after IFN treatment. Therefore, leukemic B cells isolated from a HCL patient (B.W.) were cultured for 7 days with SAC I/BCGF and increasing concentrations of IFN-α in the presence or absence of 5 μg/mL anti-Leu-13 and then pulsed with either 0.6 μmol/L [³H]-thymidine, 10 μmol/L [³H]-thymidine, or 0.6 μmol/L [³H]-deoxyadenosine. The data shown in Fig 5 indicate that [³H]-thymidine incorporation assessed following incubation with 0.6 μmol/L [³H]-thymidine reflects changes in DNA synthesis in leukemic cells because (1) comparable inhibition of [³H]-thymidine incorporation was detected at 0.6 μmol/L thymidine and at higher thymidine concentrations of 10 μmol/L, at which uptake reportedly is not transport dependent,\(^\text{33}\) and (2) incorporation of another nucleoside precursor, [³H]-deoxy-

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patient</th>
<th>Stimulation</th>
<th>Control</th>
<th>Anti–LFA-1α</th>
<th>Anti–ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal donor</td>
<td>—</td>
<td>PMA</td>
<td>71</td>
<td>18</td>
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<tr>
<td>CLL (stage 0)</td>
<td>J.I.</td>
<td>IFN-α/anti-Leu-13</td>
<td>80</td>
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<td>81</td>
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<tr>
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<td>IFN-α/anti-Leu-13</td>
<td>75</td>
<td>75</td>
<td>82</td>
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<tr>
<td>CLL (stage 0)</td>
<td>B.F.</td>
<td>IFN-α/anti-Leu-13</td>
<td>93</td>
<td>90</td>
<td>89</td>
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<tr>
<td>CLL (stage 0)</td>
<td>M.P.</td>
<td>IFN-α/anti-Leu-13</td>
<td>86 (25)</td>
<td>79</td>
<td>78</td>
</tr>
<tr>
<td>CLL (stage 0)</td>
<td>M.G.</td>
<td>IFN-α/anti-Leu-13</td>
<td>92 (6)</td>
<td>95</td>
<td>86</td>
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<tr>
<td>HCL</td>
<td>H.K.</td>
<td>IFN-α/anti-Leu-13</td>
<td>79</td>
<td>87</td>
<td>86</td>
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</tbody>
</table>

Percent aggregation induced by 5 ng/mL PMA was as follows: J.I. (31), C.T. (10), B.F. (20), M.P. (21), M.G. (10), and H.K. (42). Values in parentheses represent the percent aggregation that occurred in the presence of 1,000 IU/mL IFN-α, 5 μg/mL anti-Leu-13 and 1,000 neutralizing units of rabbit polyclonal antihuman IFN-α.

\(*\text{The number of cells in aggregates} (\% \text{aggregation}) \text{ was quantitated 18 hours after the simultaneous addition of MoAbs to cell surface adhesion molecules and either PMA (5 ng/mL) or IFN-α/anti-Leu-13. Less than 5\% of the cells aggregated in the absence of either PMA or IFN-α/anti-Leu-13. Data represent the average of duplicate wells; standard deviation between replicates was }<5\%.\)

\(\dagger\text{An enriched population of peripheral blood B cells was isolated from a normal donor (>80\% CD19\′ and <7\% CD3\′).}\)
SIGNALING OF LEUKEMIC B CELLS BY ANTI-LEU-13

Fig 5. \[^{[3}H\]-nucleoside incorporation in leukemic B cells. B-HCL cells (B.W.) were cultured in 10% BCGF/0.01% SAC I with increasing concentrations of IFN-α in the absence (closed symbols) or presence (open symbols) of 5 pg/mL anti-Leu-13. On day 7 cells were pulsed 4 hours with 0.6 pmol/L \[^{[3}H\]-thymidine (0.0), 10 pmol/L \[^{[3}H\]-thymidine (▲), or 0.6 pmol/L \[^{[3}H\]-deoxyadenosine (■). Based on these studies, the effect of IFN-α and anti-Leu-13 on SAC I/BCGF-induced thymidine incorporation in leukemic cells was further evaluated. Representative data from experiments involving 20 CLL, 3 PLL, and 5 HCL patients are shown in Fig 6. Consistent with our previous report,\(^{1,2}\) IFN-α inhibited DNA synthesis in B-HCL cells to a variable extent. Culture of leukemic B cells with anti-Leu-13 in the absence of IFN-α consistently decreased \[^{[3}H\]-thymidine incorporation, which may partially reflect the moderate increase in Leu-13 antigen observed during culture in the absence of IFN-α (Table 1). However, the most significant inhibition of DNA synthesis in B-CLL, HCL, and PLL cells was detected following culture with the combination of IFN-α and anti-Leu-13. In two control experiments shown in Fig 6, equivalent concentrations of anti-Leu-1, an IgG, did not significantly inhibit thymidine incorporation in CD5-positive B-CLL cells (specific fluorescence intensity of anti-Leu-1 on E.C. and R.G. B-CLL cells was 85.0 and 98.0, respectively), supporting the conclusion that antibody-dependent cytotoxicity mechanisms are not involved in this system. Of particular interest, anti-Leu-13 inhibited DNA synthesis in leukemic B cells from both early and late stage CLL patients (stage 0 through II v stage III) as well as in B cells that were not highly responsive to the antiproliferative effects of IFN-α in vitro (eg, CLL-W.F., PLL-G.P., HCL-S.Z.). However, it is noteworthy that IFN-α was capable of increasing the Leu-13 antigen density on these cells (Table 1). These results indicate that signaling of leukemic B cells with a MoAb to the IFN-inducible Leu-13 antigen in vitro adenosine, was similarly inhibited by IFN-α and anti-Leu-13. Similar results were obtained using leukemic B cells from other HCL and CLL patients (data not shown).

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Fig 6. IFN-α and anti-Leu-13 inhibit \[^{[3}H\]-thymidine incorporation in leukemic B cells. Leukemic B cells were cultured in 10% BCGF/0.01% SAC I in combination with increasing concentrations of IFN-α alone (■—■) or in the presence of 5 μg/mL anti-Leu-13 (■—■). In two experiments (CLL-E.C., CLL-R.G.) 5 μg/mL of anti-Leu-1 was included in cultures as a control (□—□). On day 7 cells were pulsed 4 hours with 0.6 μmol/L \[^{[3}H\]-thymidine.
can trigger an antiproliferative signal in cells that are either sensitive or resistant to the direct growth inhibitory effects of IFN-α.

DISCUSSION

Despite intensive investigation, the mechanisms of action of IFN-α in eliciting an antitumor response in B-cell leukemia are currently not well understood. In the present study we have shown that IFN-α acts both in vitro and in vivo to upregulate the cell surface density of Leu-13 on leukemic B cells in a majority of patients with CLL, PLL, and HCL. Induction of Leu-13 antigen by IFN-α in vitro occurred rapidly in a dose-dependent manner and required new protein synthesis. It was of interest that IFN-γ, which binds to cell surface receptors distinct from the IFN-α receptor, was less effective at increasing the final Leu-13 density on leukemic B cells. Similar levels of induction of Leu-13 by IFN-α were observed in these malignant B cells despite reported differential response rates to IFN-α therapy in these patient groups. Furthermore, Scatchard analysis of IFN-α binding to leukemic B cells did not show a significant correlation (P > .05) between IFN-α cell surface receptor expression and either patient diagnosis or the extent of upregulation of Leu-13 antigen. These results are consistent with recent reports showing that the level of IFN receptor expression on HCL and CLL cells did not correlate either with the clinical response to IFN-α or the degree of induction by IFN of the cytoplasmic protein 2′,5′-oligoadenylate synthetase in vitro. Taken together, these studies indicate that the interaction of IFN-α with a limited number of high affinity cell surface receptors is sufficient to signal the induction of proteins including Leu-13.

Our studies show that signaling of the IFN-inducible cell surface protein Leu-13 with a specific MoAb triggers homotypic aggregation of normal and leukemic B cells. Several lines of evidence support the conclusion that aggregation mediated by anti-Leu-13, an IgG1,κ, results from an active process rather than trivial cross-linking of cell surface antigens on adjacent cells: (1) aggregation occurs at 37°C but not at 4°C; (2) aggregate formation was not inhibited by preblocking FcR sites with anti-Leu-2a, a murine IgG1,κ; and (3) aggregation was not mediated by MoAbs to major leukemic B-cell surface antigens expressed at similar levels as IFN-α-induced Leu-13 (eg, CD5, CD19, and β2-microglobulin). Taken together these studies indicate that homotypic aggregation triggered by anti-Leu-13 is not the result of either passive agglutination or FcR dependent pathways.

It was of interest in the present study to determine if the homotypic adhesion triggered by IFN-α and anti-Leu-13 involves the interaction of LFA-1 and ICAM-1 because these well-characterized molecules are known to mediate aggregation of B lymphocytes. Numerous studies have clearly indicated that these adhesion molecules are important in mediating cell-cell interactions involved in the immune response and lymphocyte trafficking. Additionally, a recent report has suggested that the expression of LFA-1 on malignant cells is also required to initiate an antitumor response to lymphoid malignancies. Results obtained in the present study confirm previous reports showing that leukemic B cells from CLL and HCL patients express low levels of cell surface LFA-1 and aggregate poorly when stimulated with phorbol esters. In contrast, homotypic aggregation of leukemic B cells was triggered consistently by the combination of IFN-α and anti-Leu-13 although the cell surface density of LFA-1 and ICAM-1 was not altered (data not shown). Moreover, aggregation of normal and leukemic B cells triggered by IFN-α and anti-Leu-13 was not inhibited by MoAbs to either LFA-1 or ICAM-1. Because MoAb to the α subunit of LFA-1 (TSI/22) blocks aggregation mediated by the interaction of LFA-1 with both ICAM-1 and ICAM-2, our data indicate that IFN-α and anti-Leu-13 upregulated a previously undescribed leukocyte adhesion pathway in B lymphocytes. Based on these results, it is tempting to speculate that the decrease in lymphocyte recirculation observed in HCL and CLL patients during IFN therapy and in animals treated with IFN involves signaling of this adhesion pathway at the lymphocyte level.

Our studies further demonstrate that signaling of leukemic B cells with monoclonal anti-Leu-13 potentiates the inhibitory effects of IFN-α on BCGF-stimulated DNA synthesis. This finding is of interest because BCGF has been proposed to have a central role in the clonal expansion of malignant B cells in HCL. In this regard, Ford et al have shown that hairy cells synthesize and secrete an autostimulatory factor that is functionally and biochemically similar to the low molecular weight form of BCGF produced by T cells. We and other investigators have shown that malignant B cells from HCL patients are highly sensitive in vitro to the proliferative signal provided by exogenous low molecular weight BCGF, and that exposure of leukemic cells to IFN-α either in vitro or in vivo inhibits this proliferative response. These observations were confirmed in the present study using methods which distinguish between DNA synthesis per se and incorporation of [3H]-thymidine into DNA. Furthermore, in this system the proliferative response of leukemic B cells to BCGF and SAC I in vitro was shown to be significantly suppressed by the combination of IFN-α and anti-Leu-13. Thus, the IFN-inducible molecule Leu-13 appears to be a potent inhibitor of responsiveness to BCGF in B-HCL cells.

In CLL, the role of BCGF in the expansion of neoplastic cells has not been fully elucidated. The proliferative response of B-CLL cells to BCGF is highly heterogeneous compared with B-HCL cells, and B-CLL cells reportedly express low levels of BCGF receptors. In the present study we showed that B-CLL cells synthesize DNA in response to low molecular weight BCGF, although this response is heterogeneous among different patient samples. IFN-α inhibited BCGF/SAC I-driven proliferation in B-CLL cells in some experiments; however, this was not a consistent finding. In contrast, anti-Leu-13 consistently suppressed BCGF-dependent DNA synthesis in B-CLL cells from early and advanced stage patients. Of particular interest, leukemic B cells that were not highly responsive to the direct antiproliferative effects of IFN-α in vitro, Leu-13 was upregulated by IFN-α.
and anti-Leu-13 inhibited BCGF-dependent DNA synthesis. These results indicate that resistance to the growth inhibitory effects of IFN-α in B-cell leukemia can be circumvented via signaling of the IFN-inducible protein Leu-13.

Although these studies do not directly address the potential activation of effector mechanisms including T-cell and natural killer (NK) cell mediated cytotoxicity, the antiproliferative actions of IFN-α and anti-Leu-13 most likely involve a direct effect on leukemic B cells because: (1) hairy cells are relatively resistant to NK cytosis even in the presence of IFN-α\(^{29}\); (2) murine IgG\(_2\) antibodies, in contrast to IgG\(_3\), do not mediate antibody-dependent cellular cytotoxic activity in human peripheral blood leukocytes (PBL)\(^{51}\); and (3) anti-Leu-1, an IgG\(_3\), failed to mediate an antiproliferative effect in B-CLL cultures. Therefore, the capacity of anti-Leu-13 to potentiate the antiproliferative effect of IFN-α on BCGF-stimulated growth of leukemic B cells would appear to occur independently of effector cytotoxicity mechanisms.

The importance of IFN-inducible proteins in mediating the antiproliferative effects of IFN-α has been implicated in several recent studies. Hillman et al\(^{52}\) have reported that resistance to the growth inhibitory effects of IFN in the Namalva B cell line is associated with the absence of expression of a 17-Kd IFN-inducible cell surface protein. In these studies, IFN resistance could be reversed by the addition of partially purified 17-Kd protein to Namalva cells. Kessler et al\(^{53}\) have recently examined the pathways involved in the transcriptional regulation of IFN-inducible proteins in IFN-sensitive and resistant B-cell lines. These investigators demonstrated a failure to upregulate specific IFN-inducible proteins in some IFN-resistant B-cell lines that was associated with a defect in the ability of IFN-α to activate DNA-binding factors necessary to trigger IFN-stimulated gene transcription. In the present study, defective upregulation of Leu-13 in response to IFN-α in vitro was observed in only two experiments involving leukemic B cells from early stage CLL patients. In a vast majority of patient samples studied, IFN-α effectively increased the cell surface density of Leu-13 on leukemic B cells. Thus, although the molecular mechanisms involved in the upregulation of Leu-13 are not presently known, our data indicate that resistance to the antiproliferative effects of IFN-α on leukemic B cells in vitro is not generally associated with a defect in the regulation of synthesis of the IFN-inducible protein Leu-13. IFN resistance in B-cell leukemia may therefore occur at another level, possibly through the inappropriate signaling of IFN-inducible cell surface proteins.

Taken together, these studies provide insight into the mechanism of action of IFN-α in leukemic B cells. The data presented support the conclusion that the IFN-inducible protein Leu-13 plays a role in regulating the growth and cellular adhesion properties of leukemic B cells. Moreover, evidence indicating that resistance to the antiproliferative effects of IFN-α can be circumvented via signaling of the IFN-inducible protein Leu-13 may have future therapeutic implications. These initial studies suggest that the combined use of IFN-α and MoAbs to IFN-inducible proteins may represent a new approach for achieving therapeutic effects in B-cell leukemias that are nonresponsive to IFN-α treatment.

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Monoclonal antibody to the interferon-inducible protein Leu-13 triggers aggregation and inhibits proliferation of leukemic B cells

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