

Effects of Interferon α on Autocrine Growth Factor Loops in B Lymphoproliferative Disorders

By Helen E. Heslop,*[‡] Alessandra C. M. Bianchi,*
Frank T. Cordingley,* Martin Turner,[§] W. Chandima P. De Mel,*
A. Victor Hoffbrand,* and Malcolm K. Brenner*[‡]

From the *Department of Haematology, Royal Free Hospital, London NW3 2QG, UK; the [‡]Department of Hematology/Oncology, St. Jude Childrens Research Hospital, Memphis, Tennessee 38101; and [§]Charing Cross Stanley Research Centre, London W6 8LW, UK

Summary

The B lymphoproliferative disorders B chronic lymphocytic leukemia (B-CLL) and hairy cell leukemia (HCL) produce a number of autocrine growth factors, including tumor necrosis factor (TNF), interleukin 6 (IL-6), and IL-1, all of which may induce positive feedback growth loops. If such malignancies depend on these autocrine growth loops for survival, their interruption may be therapeutically valuable. Interferon α (IFN- α) abrogates TNF- or IL-6-induced proliferation of HCL and B-CLL cells in vitro and has therapeutic activity in these diseases. We have investigated the possibility that IFN- α may act by interrupting autocrine growth factor loops. If purified B-CLL or HCL cells are cultured in the presence of TNF, there is induction of mRNA for TNF, IL-1 α , IL-1 β , and IL-6. However, culture in the presence of IFN- α in addition to TNF reduced the level of mRNA for all these cytokines, compared with cells cultured in TNF alone. While cytokine mRNA levels were diminished, levels of mRNA for the ribonuclease activator 2-5A synthetase were increased. Analysis of the kinetics of cytokine mRNA production showed that levels fall shortly after the rise of 2-5A synthetase mRNA. IFN- α may produce these effects by shortening the half-life of cytokine mRNA, since TNF mRNA half-life in B-CLL and HCL cells is substantially reduced when the cells are cultured with IFN- α . These data suggest that IFN- α may mediate its therapeutic effects in these malignancies by blocking autocrine growth factor loops.

Autocrine production of growth factors to which a malignant cell expresses receptors has long been a postulated mechanism for tumor growth (1) and has recently been shown to occur in several hematological malignancies. Acute myeloid leukemia blast cells produce granulocyte/macrophage CSF and express receptors for this cytokine, which promotes clonogenic growth (2). Chronic malignancies of B cells consistently show evidence of autocrine growth, since the neoplastic cells of multiple myeloma (3) produce IL-6, while B chronic lymphocytic leukemia (B-CLL)¹ and hairy cell leukemia (HCL) produce and respond to a variety of cytokines, including IL-1 (4, 5), IL-6 (6, 7), and TNF (8, 9). Levels of constitutive cytokine production are variable (6, 7, 10), but induction of high levels can be attained after exposure to cytokine proteins (8).

If these malignancies are dependent on autocrine growth factors for survival, interruption of the autocrine loop would be of therapeutic value. TNF promotes viability of cells ob-

tained from patients with HCL or B-CLL (8) and also induces these malignant B cells into cell cycle so that culture with TNF protein induces expression of mRNA for *c-myc*, *c-fos*, and *c-jun* (11). TNF also promotes increased thymidine incorporation (8, 12). We have previously shown that IFN- α almost completely abrogates TNF-induced enhancement of thymidine uptake in cells from patients with B-CLL and HCL, while IFN- γ has no significant effect (8). IFN- α has been shown to have therapeutic activity in HCL and B-CLL in vivo (13, 14), and we now show that IFN- α may exert this therapeutic effect by reducing expression of mRNA for autocrine growth-promoting cytokines in these malignant cells.

Materials and Methods

Patient Details. 12 patients with B lymphoproliferative disorders (eight with BCLL, three with BCLL/PL, and one with HCL) were studied on 18 occasions in various parts of this study. Clinical data and diagnosis (FAB classification) (15) are shown in Table 1.

Cytokines. rTNF (sp act, 6.63×10^6 U/mg) was a gift of

¹ Abbreviations used in this paper: B-CLL, B chronic lymphocytic leukemia; HCL, hairy cell leukemia.

Knoll/BASF, Ludwigshafen, FRG, and rIFN- α (sp act, 2.5×10^6 U/mg) was a gift of Kirby Warrick, Slough, UK.

Probes. The TNF probe (an 800-bp EcoRI fragment) was provided by Dr. M. Shepard, Genentech Inc., San Francisco, CA. The IL-1 β probe was a 530-bp BamHI-NdeI fragment, and the IL-1 α probe was a 460-bp EcoRI-BamHI fragment, both provided by Dr. P. Lomedico, Hoffman-La Roche, Inc., Nutley, NJ. The IL-6 probe was a 517-bp EcoRI-HindIII fragment provided by Dr. E. Kawashima, Glaxo IMB, Geneva, and the 7B6 probe was a 708-bp PstI-DraII fragment provided by Prof. U. Torelli, University of Modena, Modena, Italy. The tubulin probe was a 1.4-kb PstI fragment provided by Dr. K. Philpott, Clinical Research Centre, Harrow, UK. The B-actin probe, a 2-kb HindIII fragment, was provided by Dr. S. Katzav, St. Jude Childrens Research Hospital, Memphis, TN, and the 2-SA synthetose probe was provided by Dr. K. Goneshaguru, Royal Free Hospital, London, UK.

Lymphocyte Separation. Venous blood taken into preservative-free heparin was layered onto lymphoprep (Nyegaard, Oslo, Norway), and the mononuclear cells in the interface were depleted of monocytes by adherence to plastic for 90 min at 37°C. The population was then depleted of T cells by double E-rosetting. The composition of the resulting PBL population was assessed by immunophenotyping, and contained <0.5% CD3⁺ T cells or CD14⁺ monocytes and (in patients 2–12) >98% CD5⁺ B-CLL cells (Table 1). All cell preparation and culture was undertaken in RPMI 1640 (Gibco Laboratories, Uxbridge, UK) supplemented with 2 mM glutamine (Gibco Laboratories) and 100 IU/ml streptomycin/penicillin (Gibco Laboratories) in the presence of 10% FCS (Sera-Lab, Crawley, Sussex, UK). Cells were cultured in medium containing 250 U/ml TNF or 250 U/ml TNF and 500 U/ml IFN- α , and were harvested at various time points for RNA extraction. The doses of cytokines chosen were those at which optimum effect had been demonstrated in thymidine incorporation assays (8).

Phenotyping. Phenotyping was performed by indirect immunofluorescence using a microplate method (16). OKT3 (CD3; Ortho Diagnostic Systems, Inc., Westwood, MA), RFT1 (CD5, a gift

of Professor G. Janossy, Immunology Department, Royal Free Hospital), and Leu-M3 (CD14; Becton Dickinson & Co., Mountain View, CA) were used as first layer mAb, while fluorescein- or rhodamine-conjugated goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) was used for the second layer. 500 cells were analyzed using an immunofluorescent microscope. All purified B-CLL preparations contained >98% CD5⁺ B cells, while >98% of HCL patient T-depleted lymphocytes had morphological and tartrate-resistant acid phosphatase staining characteristics of HCL cells.

mRNA Extraction. Cytoplasmic RNA was extracted from PBLs using the detergent lysis method (17). Briefly, cells were washed in PBS and resuspended in hypotonic buffer (10 mM Tris HCl, pH 7.8, 150 mM NaCl) containing 10 mM vanadyl ribonuclease complex (Bethesda Research Laboratories, Gaithersburg, MD) and 1% NP-40 (Shell Chemicals). The nuclei were pelleted by centrifugation, and the cytoplasmic extract was treated with 1% SDS, followed by phenol chloroform extraction and salt ethanol precipitation. RNA was quantified and either size fractionated on a 1% agarose/formaldehyde gel and transferred overnight to nitrocellulose or applied directly to nitrocellulose via a slot blot apparatus (Schleicher and Schuell, Inc.).

Filters were baked for 2 h at 80°C and then prehybridized in 50% formamide, 5 \times SSC, 5 \times Denhardt's solution, and 50 μ g/ml denatured salmon sperm DNA at 42°C for 4–8 h. cDNA probes were labeled by random oligopriming using [³²P]dCTP (Amersham International, Amersham, UK), and hybridization was carried out for 16 h at 42°C. Filters were washed twice in 2 \times SSC/0.1% SDS at room temperature and twice in 0.2 \times SSC/0.1% SDS at 65°C, and exposed to Fuji RX film for 1–7 d at -70°C with intensifying screens. Autoradiograms were scanned using a chromoscan (LKB Instruments, Inc., Gaithersburg, MD). Integrals obtained from the linear phase of the film were normalized with respect to the control probe.

Actinomycin D mRNA Half-Life Studies. Cultures of cells at 2×10^6 /ml in RPMI 1640 and 10% FCS were set up in the presence

Table 1. Patient Details

No.	Age/sex	WBC	Treatment	Diagnosis	Ig	Immunophenotype (percent positive)		
						CD5	CD2	CD19
		$\times 10^{-9}$						
1	62/F	55	Nil	HCL	IgG κ	9	10	77
2	74/M	164	Nil	B-CLL/PL	IgM κ	95	15	76
3	82/M	73	Nil	B-CLL	IgM κ	98	10	80
4	68/M	92	Nil	B-CLL/PL	IgM λ	95	2	93
5	66/M	274	Nil	B-CLL	IgM κ	97	2	92
6	77/F	34	Nil	B-CLL/PL	IgM λ	97	1	82
7	67/F	105	Nil	B-CLL	IgM κ	97	4	91
8	64/F	114	Nil	B-CLL	IgM κ	98	4	88
9	82/M	233	Nil	B-CLL	IgM κ	97	2	95
10	67/M	70	Nil	B-CLL	IgM κ	97	3	84
11	64/M	88	Chlorambucil	B-CLL	IgM κ	93	4	81
12	42/M	120	Nil	B-CLL	IgM κ	97	2	79

Immunophenotype was determined pre-depletion of monocytes and T cells.

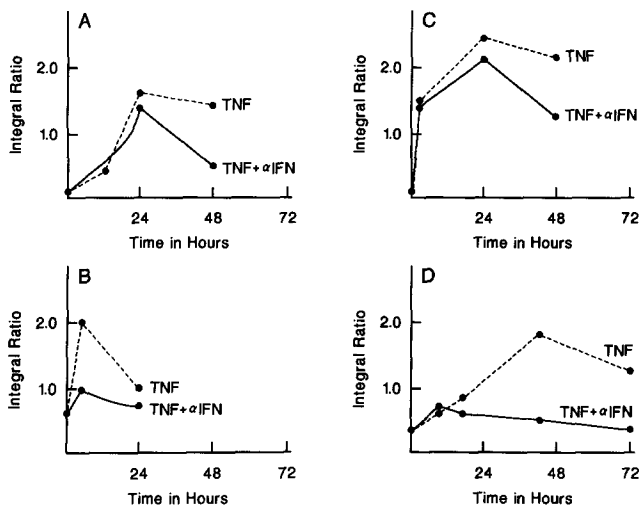


Figure 1. Detailed time courses of accumulation of mRNA for the autocrine growth factor TNF in one patient with HCL (A), two patients with B-CLL (B and C) and one patient with B-CLL/PL (D) whose cells were cultured in the presence of TNF or TNF and IFN- α , as described in Materials and Methods. Cytoplasmic RNA was extracted at various time intervals and applied to nitrocellulose filters in doubling dilutions. The filter was reprobed with the constitutively expressed protein 7B6 to confirm equal loading, and cytokine mRNA is expressed relative to 7B6 mRNA.

of 250 U/ml TNF or 250 U/ml TNF and 500 U/ml IFN- α (see Results). After 24 h of incubation, transcription was blocked by adding actinomycin D to culture medium at a final concentration of 5 μ g/ml. RNA was extracted from these treated cells 30–360 min later.

Results

Downregulation of Cytokine mRNA by IFN- α . Fresh cells obtained from patients with B lymphoproliferative disorders contain variable levels of mRNA for the growth factors TNF (10), IL-6 (6, 7), and IL-1 (5). Levels of cytokine mRNA can be increased by culture in the presence of the growth factor proteins themselves, which thereby accelerate the onset of autocrine growth when the cells are studied *ex vivo* (8). To determine the effects of IFN- α on this positive feedback

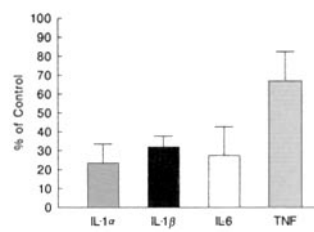


Figure 3. Effect of IFN- α on TNF, IL-1 α , IL-1 β , and IL-6 mRNA, accumulating at 24 h with and without IFN- α . Data shown are from all patients examined in this part of the study ($n = 5$). For each cytokine, bars represent the mean percent \pm SD of cytokine mRNA in the presence of TNF and IFN- α , compared with

that in the presence of TNF alone. Paired t test shows highly significant effect for IFN- α on all cytokine mRNA levels ($p < 0.01$).

growth loop, we stimulated CLL and HCL cells with TNF, and measured the effect of IFN- α on steady-state cytokine mRNA concentrations. Fig. 1 shows detailed time courses of TNF mRNA expression in one patient with HCL (A), two patients with B-CLL (B and C), and one patient with BCLL-PL (D). In the presence of exogenous TNF protein alone, TNF mRNA levels rose above constitutive levels and peaked by 24–40 h. However, culture of cells in the presence of both TNF and IFN- α substantially abrogated this rise in cytokine message. IFN- α has a similar effect on the accumulation of mRNA for three other cytokines, IL-6 (Fig. 2 a), and IL-1 α and IL-1 β (Fig. 2 b). Fig. 3 summarizes these data for all patients examined in this part of the study and illustrates cytokine mRNA at 24 h of culture in the presence of both TNF and IFN- α or TNF alone. The data show consistent reduction in cytokine mRNA levels in the presence of IFN- α .

Mechanism by which IFN- α Downregulates mRNA. Suppression of cytokine mRNA accumulation did not represent a global effect on cellular steady-state accumulation of mRNA: levels of mRNA for structural proteins such as tubulin were unaffected by IFN- α . Moreover, levels of mRNA encoding the enzyme 2-5 A synthetase substantially increased after culture with IFN- α (Fig. 4). 2-5A synthetase is a potent inducer of ribonucleases (18), which in turn increase degradation of certain mRNA species. We determined if increased degradation was indeed the mechanism by which IFN- α reduced steady-state cytokine mRNA. Fresh mRNA transcription was inhibited with actinomycin D, allowing measurement of the half-life of cytokine mRNA. Cytokine mRNA is short lived in these malignant B cells compared with the

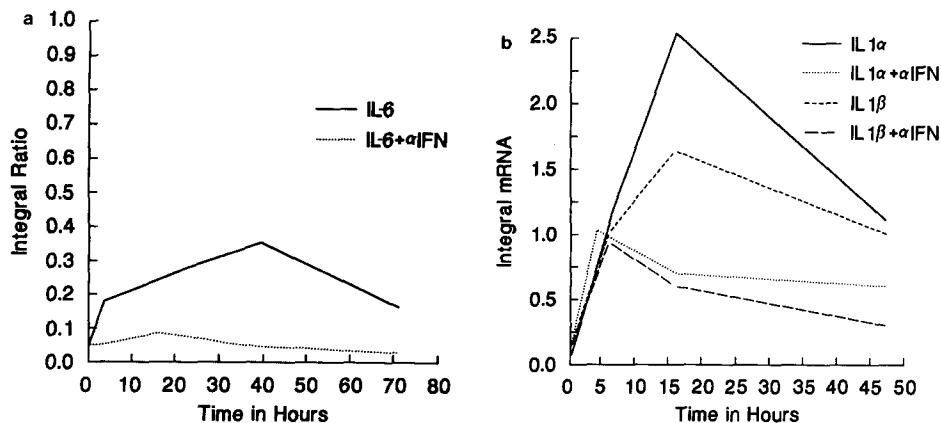


Figure 2. Detailed time course of accumulation of mRNA for the autocrine growth factors IL-6 (a), and rIL-1 α and IL-1 β (b) in representative B-CLL patients whose cells were cultured in the presence of TNF or TNF and IFN- α , as described Material and Methods. Cytoplasmic RNA was extracted at intervals and applied to nitrocellulose filters in doubling dilutions. The filter was reprobed with the constitutively expressed protein 7B6 to confirm equal loading, and cytokine mRNA is expressed relative to 7B6 mRNA.

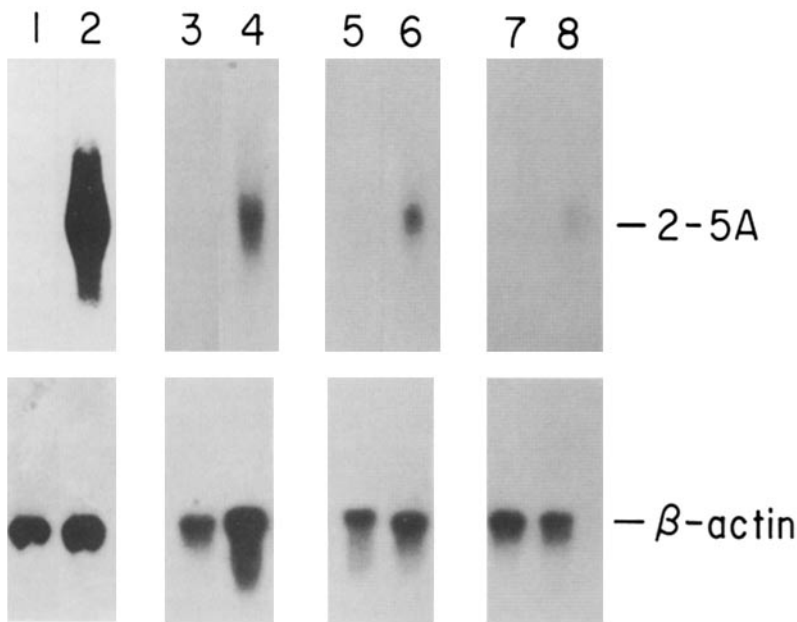


Figure 4. Induction of 2-5A synthetase mRNA by IFN- α . Purified B cells from HCL or B-CLL patients were cultured in medium alone or with IFN- α for 18 h. Northern blots from four representative patients are shown here. Pre-culture (lanes 1, 3, 5, and 7), and an absence of or low levels of mRNA for 2-5A synthetase were present; after culture with IFN- α (lanes 2, 4, 6, and 8), there was induction of mRNA for 2-5A synthetase.

mRNA for structural proteins, illustrated in Fig. 5 a for TNF and tubulin, respectively. However, Fig. 5 b shows that TNF mRNA half-life is further reduced by 33-50% in the presence of IFN- α .

Discussion

This study investigated one mechanism by which IFN- α may exert its therapeutic activity in B-CLL and HCL. The results of the current study show that IFN- α can interrupt

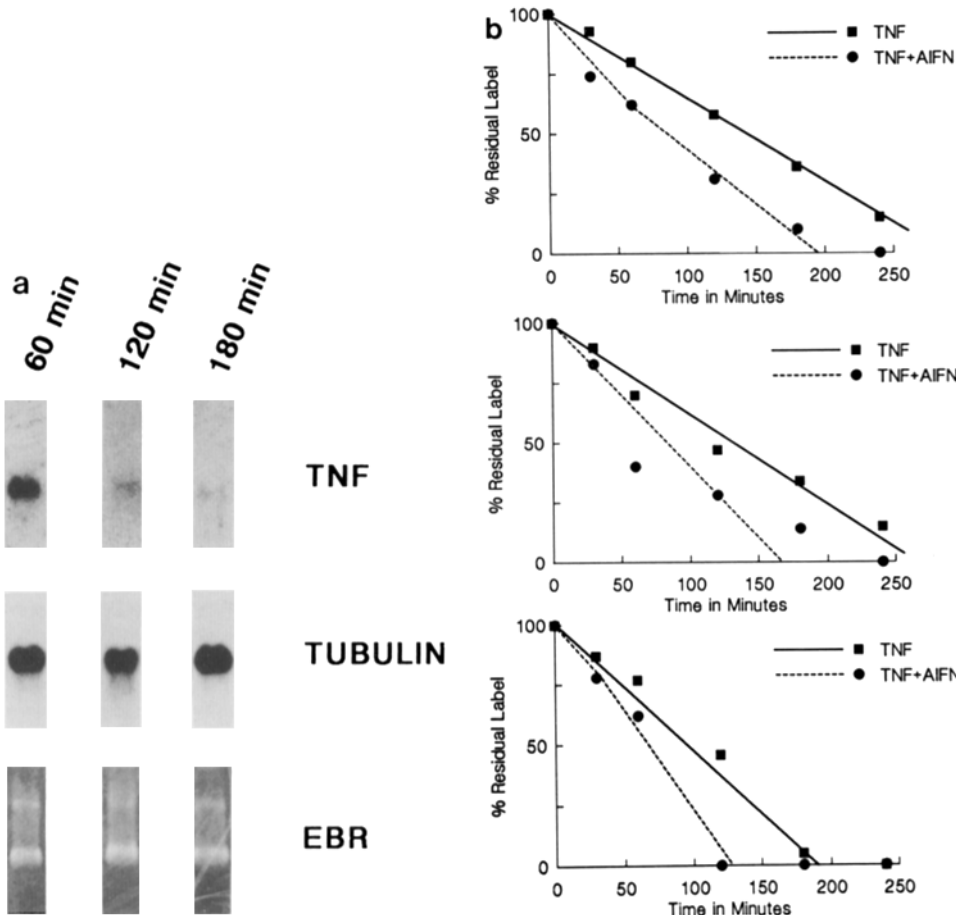


Figure 5. Effect of IFN- α on half-life of cytokine mRNA. B-CLL cells were cultured for 24 h in the presence of TNF or TNF and IFN- α . Actinomycin D was added to cultures, and mRNA was harvested at intervals for 4 h and run on a Northern blot. Ethidium bromide staining shows equal loading at all time points. Panel a shows that TNF mRNA degrades rapidly over this time course while Tubulin mRNA is unaffected, panel b shows that in three patients (1 HCL, 2 B-CLL), the half-life is reduced still further in the presence of IFN- α (in the examples shown by 33-50%).

the production of autocrine growth factors by HCL and B-CLL cells by reducing accumulation of mRNA for a number of cytokines, including IL-1 α and β , TNF- α , and IL-6. At the same time, IFN- α increases the expression of mRNA for the enzyme 2-5A synthetase, a ribonuclease activator. Analysis of the kinetics of cytokine mRNA production shows that levels fall shortly after the rise of 2-5A synthetase mRNA (Figs. 1 and 2). The fall in cytokine mRNA may therefore be a consequence of induction of ribonuclease activity by 2-5A synthetase, particularly as TNF mRNA half-life is reduced in the presence of IFN- α .

While accumulation of mRNA for a number of cytokines is reduced by IFN- α , mRNA levels for structural proteins such as tubulin are not detectably affected. Differences in the sensitivity of the mRNAs for cytokine and structural proteins to IFN- α -induced ribonucleases may reflect differences in their 3' untranslated regions. These 3' sequences regulate message instability (19, 20) and may confer susceptibility to IFN- α -induced ribonucleases. Preliminary observations show that these in vitro effects of IFN- α are reproduced in vivo. Treatment of patients with IFN- α rapidly reduces the content of TNF mRNA in B-CLL cells, while producing a corresponding increase in cellular 2-5A synthetase activity (De Mel et al., unpublished observations).

We suggest that IFN- α exerts its therapeutic effects by reducing endogenous growth factor production rather than

by enhancing antileukemic host cytotoxic effector mechanisms, as was first suggested (21). No correlation has been found between effector function and therapeutic response during IFN- α treatment of patients with HCL/B-CLL (22). Moreover, the leukemic target cells are almost entirely resistant to the cytotoxic effects of the activated killer cells generated during IFN- α treatment (22, 23). It is more difficult to assess the relative importance of each cytokine downregulated by IFN- α treatment. TNF (8, 12, 24), IL-1 (5), BCGF (25-27), and IL-6 (28) may all be capable of inducing growth and/or differentiation in B-CLL/HCL cells, so that the overall therapeutic effects of IFN- α may result from the downregulation of a multiplicity of endogenous growth factors.

If this concept of the mechanism of the effects of IFN- α in the B lymphoproliferative diseases is correct, then the variable sensitivity of individual neoplasms such as HCL, B-CLL, non-Hodgkins lymphoma, and myeloma to IFN- α treatment may reflect variation in dependence on autocrine growth loops. Moreover, within each disease, the sensitivity of an individual patient may depend on the degree to which the malignant lymphocytes depend for their growth on autocrine factors and how readily IFN- α induces 2-5 A synthetase activity. Our interpretation also leads to the prediction that antibodies to cytokine proteins or their receptors would have an additional contribution to make to the therapeutic action of IFN- α .

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Address correspondence to H. E. Heslop, Department of Hematology/Oncology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101.

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