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IFN-γ abrogates IL-7-dependent proliferation in pre-B cells, coinciding with onset of apoptosis

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SUMMARY

These studies investigated the mechanism by which interferon-γ (IFN-γ) inhibits the interleukin-7 (IL-7)-dependent proliferation of BALB/c bone marrow B-cell precursors *in vitro*. Low concentrations (1 U/ml) of recombinant murine IFN-γ (rmIFN-γ) caused a ~80% suppression of IL-7 colony-forming units (CFU) formation in semi-solid media, in part through a direct affect on isolated B220$^+$ pre-B cells. IFN-γ did not induce apoptosis in small resting pre-B cells in BALB/c bone marrow. There was no difference in the proportion of apoptotic B220$^+$ pre-B cells in IFN-γ-treated cultures compared to cultures treated with IL-7 alone. However, IL-7-responsive pre-B cells generated from bone marrow had a 30–50% loss in cells in S + G_2/M phases of the cell cycle and an increase of up to twice as many in apoptotic cells within 48 hr of exposure to IFN-γ. Notably, expression of the tyrosine phosphatase B220 was increased in the IFN-γ-treated pre-B cells. Interestingly, although there was no substantial change in IL-7 receptor mRNA expression upon IFN-γ treatment, a small decrease in binding of biotinylated IL-7 to IFN-γ-treated pre-B cells was observed. These results suggest that IFN-γ inhibits IL-7 responsiveness in pre-B cells, resulting in a subtle down-regulation of IL-7 binding, inhibition of proliferation and, ultimately, apoptosis.

INTRODUCTION

B-cell development is inhibited in a number of immunodeficiency states, including graft-versus-host disease,[1,2] autoimmune disease[3] and malnutrition.[4,5] While it is clear that interleukin-7 (IL-7) is important in driving the normal proliferation of pre-B cells,[6] it remains to be seen how B-cell development may be down-regulated during various immunodeficiency states.

Recently, there has been evidence that interferon-γ (IFN-γ) is responsible, in part, for the delayed reconstitution of B-cell development after bone marrow transplantation in mice undergoing a minor antigen graft-versus-host reaction.[1] Human B-cell lymphocytic leukaemia (BCLL) cells also secrete IFN-γ and such patients are lymphopenic early in the course of their disease.[7] Further, it has been reported that IFN-γ inhibits IL-7 colony-forming units (CFU),[1,8] growth of pre-B cells in long-term bone marrow cell cultures,[8] and causes arrest of proliferation and resultant apoptosis in IL-7-dependent murine pre-B-cell lines and clones.[9]

We have analysed normal murine bone marrow pre-B cells and determined that IFN-γ (1) inhibits the IL-7 responsiveness of pre-B cells directly; (2) is not a potent inducer of apoptosis when compared to glucocorticoids; (3) has little or no effect on IL-7 receptor mRNA expression; but (4) partially down-regulates IL-7 binding to pre-B cells and up-regulates B220 expression in pre-B cells; and (5) inhibits further proliferation of IL-7-dependent pre-B cells leading to their apoptotic death.

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MATERIALS AND METHODS

Mice and IL-7 colony assay

BALB/c mice 2–4 months of age were obtained from the University of Miami breeding facility and used in all experiments. Bone marrow was flushed from femurs and tibias with Hanks' balanced salt solution (HBSS)/1% fetal bovine serum (FBS) and red blood cells (RBC) removed using a hypotonic ammonium chloride lysis buffer. Cells were suspended at 2×10^5 cells/ml in 0·9% methylcellulose in α-minimal essential media (MEM) with 30% FBS and 10^{-4} M 2-mercaptoethanol (2-ME) and cultured at 1 ml/well in triplicate cultures in 12-well plates. Recombinant murine IL-7 (rmIL-7; BioSource International, Camarillo, CA) was added to cultures at 50 U/ml and rmIFN-γ (BioSource International) at 0·25–50 U/ml. Colonies of >25 cells were enumerated after culture for 7–10 days at 37° with 5% CO_2 using an inverted microscope. For some experiments, B220$^+$ cells were obtained from bone marrow by positive selection on anti-B220 (14.8 monoclonal antibody)-coated Petri dishes. Cells obtained with this method were routinely >85% B220$^+$ and >90% viable.

Liquid cell culture initiation

Bone marrow cells devoid of RBC were adjusted to 10^6 viable cells/ml in RPMI with 5% FBS and 2×10^{-5} M 2-ME and placed

in 24-well plates for culture. IL-7 and IFN-γ were added at 25 U/ml to cultures as indicated. Dexamethasone (Sigma Chemical Co., St Louis, MO) was dissolved in ethanol, diluted into RPMI and added to indicated cultures at a final concentration of 1 μM. Cultures were incubated at 37° under an atmosphere of 5% CO_2 in air. IL-7-dependent pre-B cells (6C3/BP-1$^+$ CD43$^+$ B220$^+$) were obtained by culture for 7 days with 25 U/ml IL-7, washed by centrifugation, and recultured at 10^6 cells/ml with IL-7 ± IFN-γ.

Cell-surface staining for flow cytometry

At various times after liquid culture initiation, cells were harvested from 24-well plates and viable and non-viable (intact membranes, but blue) cells enumerated using 0·2% trypan blue. Aliquots ($2–10 \times 10^5$) of cells were placed into tubes and incubated with biotinylated anti-6C3 (PharMingen, San Diego, CA) or anti-CD43 (S7; PharMingen) for 20 min at 4°. After washing by centrifugation with HBSS/0·1% bovine serum albumin (BSA)/0·02% NaN_3, cells were incubated with FITC-conjugated anti-B220 (RA3.6B2; Caltag Laboratories, South San Francisco, CA) for 20 min at 4°. Biotinylated antibodies were fluorescently labelled by incubation with streptavidin-conjugated phycoerythrin (PE; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 20 min at 4°. IL-7 receptor (IL-7R) staining was performed using biotinylated IL-7 followed by incubation with avidin–PE using a kit from R&D Systems (Minneapolis, MN) exactly as the manufacturer's instructions. IL-7-dependent pre-B cells were washed extensively and incubated at 37° for 2 hr before staining for IL-7R in order to reduce any residual unlabelled IL-7 bound to the receptors. After washing, cells were resuspended at 10^6/ml for analysis using a FACScan (Becton Dickinson, Mountain View, CA). In some experiments, viable and non-viable cells were enumerated by incubation with 1 μg/ml 7-amino actinomycin D (7-AAD; Molecular Probes, Eugene, OR) for 2 min before FACS analysis.

Cell-cycle analysis and quantification of apoptotic cells using flow cytometry

Cells to be used for cell cycle analysis were stained with biotinylated anti-6C3 or unlabelled anti-B220 (14.8 culture supernatant; ATCC, Rockville, MD). Anti-6C3 and 14.8 antibodies were fluorescently labelled by incubation with streptavidin–FITC (PharMingen) and anti-rat IgG–FITC (Boehringer Mannheim, Indianapolis, IN), respectively. After washing, cells were suspended in HBSS with 50% FBS, fixed by drop-wise addition of ice-cold 75% ethanol to a final concentration of 50%, and held on ice for at least 1 hr. After extensive washing, the cells were suspended in 50 μg/ml propidium iodide (Sigma) with 50 μg/ml RNAse A (Boehringer Mannheim) in HBSS for 1 hr at room temperature. Cells were analysed by flow cytometry using a FACScan. Apoptotic cells were quantified as previously described.[10,11]

Analysis of IL-7 receptor mRNA expression using reverse transcription and amplification via the polymerase chain reaction

IL-7-dependent pre-B cells or 70Z/3 cells (ATCC) were treated with IFN-γ as described above, harvested, and lysed by suspension in 4 M guanidinium isothiocyanate. RNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated at −20° first with propanol followed by 70% ethanol. Reverse transcription of the isolated RNA was performed using oligo $d(T)_{16}$ as primer (GeneAmp RT-PCR kit; Perkin-Elmer Cetus, Norwalk, CT). cDNA sequences were amplified using murine IL-7R MAPPing amplimers (5′ primer: 5′CCCCATAACGATTACTTCAAGGCTCTTGG3′; 3′ primer: 5′AGAGTTTGGCAGCAAGTCTTGATACACAGG3′), obtained from Clontech Laboratories (Palo Alto, CA). Murine β-actin MAPPing amplimers (Clontech) were used as an internal control. Polymerase chain reaction (PCR) was performed for 30 cycles as follows: 1 min at 94°, 2 min at 60°, and 3 min at 72°. PCR products were visualized on a 1·5% agarose gel stained with 5 μg/ml ethidium bromide and photographed under UV light.

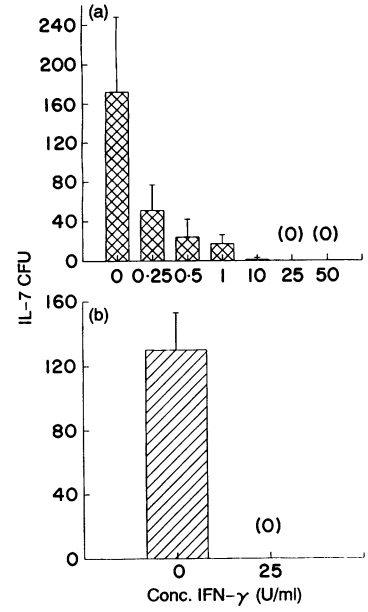

Figure 1. IFN-γ inhibits IL-7 colony formation in both whole bone marrow and in isolated B220$^+$ cells. Either (a) whole bone marrow or (b) panned B220$^+$ bone marrow cells (2×10^5 or 10^5 cells/well, respectively) were cultured in 0·9% methylcellulose semi-solid media for 7 days with 50 U/ml rmIL-7 and the indicated concentration of rmIFN-γ. Colonies of > 25 cells were enumerated and data expressed as the mean ± SD of triplicate cultures. Data are representative of three separate experiments.

RESULTS

IFN-γ inhibits the formation of IL-7 CFU *in vitro*

Consistent with previous reports,[1,8] formation of IL-7-dependent pre-B-cell colonies from BALB/c bone marrow cells (IL-7 CFU) was inhibited by IFN-γ in a dose-dependent manner. There were virtually no IL-7 colonies found in cultures with IFN-γ concentrations as low as 10 U/ml (Fig. 1a). At 0·25 U/ml IFN-γ, IL-7 CFU were less than 50% of control cultures (Fig. 1a). To determine if IFN-γ was altering accessory cell function or directly affected B-lineage cells, BALB/c bone marrow B220$^+$ cells were highly enriched by positive selective on anti-B220 (14.8) antibody-coated panning plates, harvested (> 85% B220$^+$ cells), and placed into IL-7 CFU cultures. Twenty-five U/ml IFN-γ completely inhibited IL-7 CFU from B220$^+$ cells, indicating a direct effect on B-lineage cells (Fig. 1b).

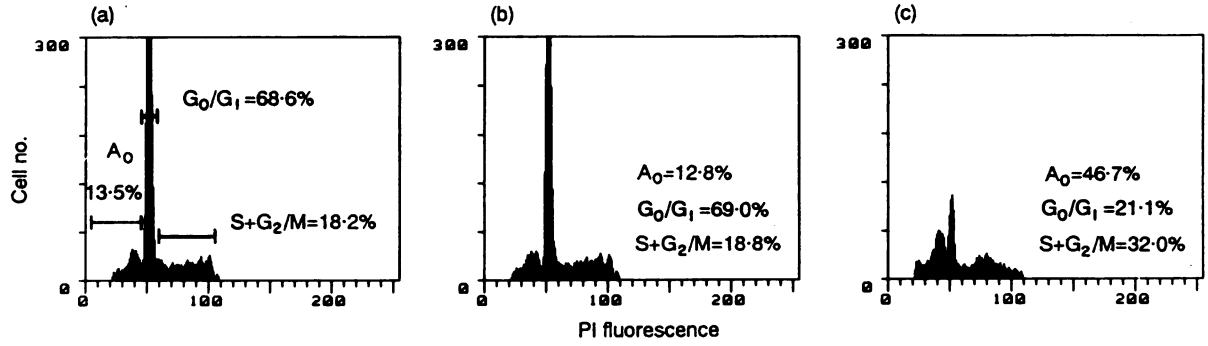

Figure 2. Cell cycle histograms of B220$^+$ bone marrow cells upon culture with IL-7 and IFN-γ or dexamethasone. Whole bone marrow cells were cultured for 24 hr with 25 U/ml rmIL-7 and either 25 U/ml rmIFN-γ or 1 μM dexamethasone, harvested, stained with anti-B220–FITC and propidium iodide, and analysed using flow cytometry. Apoptotic cells are found in the A_0 region of the DNA histograms as indicated in (a). The percentages of B220$^+$ cells in G_0/G_1 and S+G_2/M phases of the cell cycle are indicated. Cultures were treated with (a) IL-7, (b) IL-7+IFN-γ, (c) IL-7+dexamethasone. Data are representative of four separate experiments.

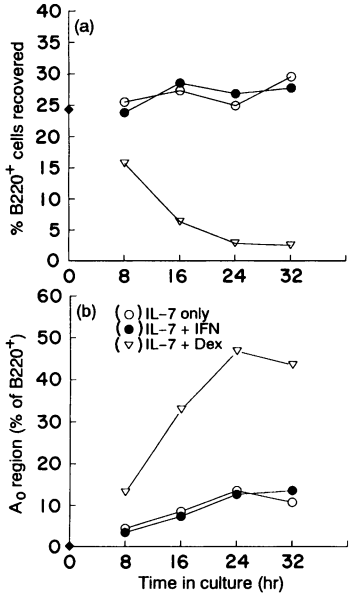

Figure 3. The proportion of B220$^+$ bone marrow cells and apoptotic B-lineage cells in bone marrow cultures exposed to IFN-γ and dexamethasone. Bone marrow cells were cultured for 32 hr with 25 U/ml rmIL-7 and either 25 U/ml rmIFN-γ or 1 μM dexamethasone. Cells were harvested at 8-hr intervals and stained with anti-B220–FITC and propidium iodide and analysed using flow cytometry. (a) Represents the percentage B220$^+$ cells recovered from the cultures and (b) represents the percentage of recovered B220$^+$ cells which resided in the A_0 region (apoptotic) of PI DNA histograms. Data are representative of three separate experiments.

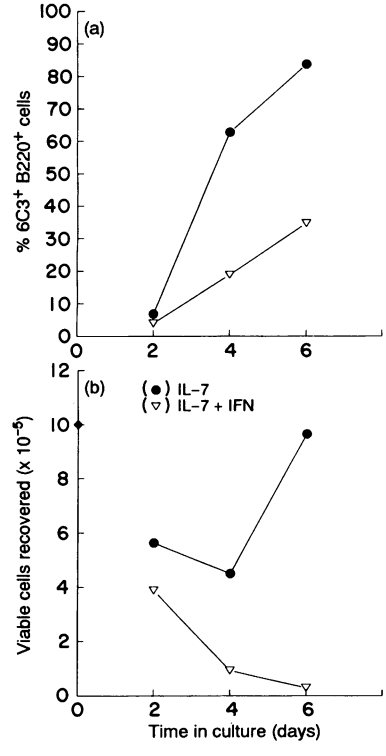

Figure 4. Development of IL-7-dependent pre-B cells from whole bone marrow cells in the presence of IFN-γ. Bone marrow cells were cultured with 25 U/ml rmIL-7±25 U/ml IFN-γ for 6 days. Cells were harvested at 2-day intervals, stained with biotinylated anti-6C3/avidin-PE and anti-B220–FITC, and analysed using flow cytometry. The percentage of recovered viable cells which were B220$^+$ and brightly stained for 6C3 is shown in (a). The number of viable cells recovered from the cultures over 6 days is indicated in (b). Data are representative of three separate experiments.

IFN-γ does not cause apoptosis of B-lineage cells in primary bone marrow cultures

To investigate if IFN-γ inhibited IL-7 CFU by simply inducing cell death among pre-B cells, BALB/c bone marrow cells were placed into liquid culture together with IL-7 and either IFN-γ or dexamethasone (Dex). It has been reported previously by Garvy *et al.* that glucocorticoids such as dexamethasone rapidly induce apoptosis in B220$^+$ bone marrow cells.[11] Propidium iodide (PI) fluorescence was utilized in a flow cytometric assay to assess apoptosis. It has been shown previously that apoptotic cells form a discreet hypodiploid peak (the A_0 region) in DNA histograms.[10,11] Incubation of bone marrow cells with IFN-γ for 24 hr resulted in very few B220$^+$ B-lineage cells appearing in the A_0 region of DNA histograms compared to cells treated with Dex (Fig. 2). While approximately 13% of B220$^+$ cells were in the A_0 region in both IL-7+IFN-γ-treated and IL-7-only treated cultures, ~40% of B220$^+$ cells were apoptotic in the Dex-treated cultures (Fig. 2). Treatment with media alone or

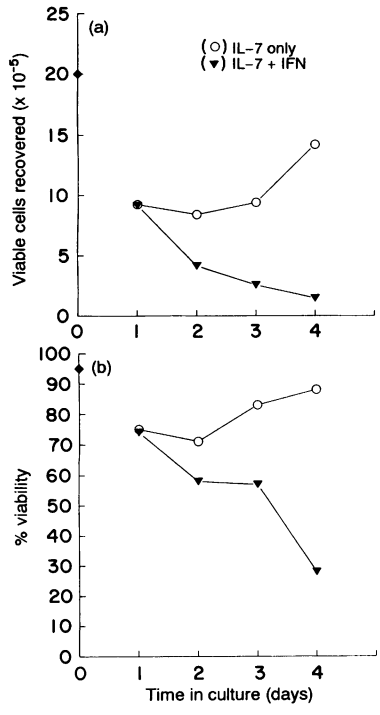

Figure 5. Recovery of viable IL-7-dependent pre-B cells from cultures exposed to IFN-γ. IL-7-dependent pre-B cells were obtained by culturing whole bone marrow cells with 25 U/ml rmIL-7 for 7 days. IL-7-dependent pre-B cells were recultured at 10^6 cells/ml with 25 U/ml IL-7 ± 25 U/ml rmIFN-γ and harvested at 24-hr intervals for analysis. Viable cells recovered were determined using trypan blue exclusion. (a) Shows the number of viable cells recovered and (b) shows the percentage viability over a 96-hr period. Data are representative of four separate experiments.

media plus IFN-γ also resulted in comparable low levels of apoptosis (< 20%) after 24 hr in culture (data not shown). Although the proportion of apoptotic (A_0 region) B220$^+$ cells increased slightly in the control and IFN-γ-treated wells over 32 hr, significant apoptosis was only observed in the Dex-treated wells at ⩾ 8 hours (Fig. 3). The number of viable and non-viable cells recovered from the cultures was not different between the IL-7-treated control and the IFN-γ-treated cultures; however, there was a nearly 50% loss of viable cells in the Dex-treated cultures by 16 hr (data not shown).

The proportion (and absolute number) of B220$^+$ cells recovered at 32 hr was also no different between IFN-γ + IL-7-treated cultures compared to the IL-7-treated control cultures (Fig. 3). However, within 8 hr the proportion of B220$^+$ cells in the Dex-treated cultures had decreased by 40% compared to control cultures, and by 24 hrs almost 90% of the B220$^+$ cells had disappeared (Fig. 3).

It has been reported previously that bone marrow cells which respond to IL-7 *in vitro* express both B220 and the 6C3/BP-1 antigen on their surfaces.[12] Increased numbers and proportions of B220$^+$ 6C3$^+$ pre-B cells were observed in cultures of bone marrow cells plus IL-7 after 4 days. The proportion of B220$^+$ cells with bright 6C3 staining was not different between the IL-7-treated and IL-7 + IFN-γ-treated cultures over the first 32 hr (data not shown). However, extended kinetics indicated that while the IL-7-treated cultures had increased numbers of B220$^+$ 6C3^{hi+} cells as a result of proliferation of IL-7 responsive pre-B cells by day 2, B220$^+$ 6C3^{hi+} developed to a lesser extent in IL-7 + IFN-γ-treated wells (Fig. 4).

IFN-γ causes a decrease in proliferating IL-7-dependent pre-B cells

It was reported previously that IFN-γ arrests proliferation of stromal cell/IL-7-dependent pre-B-cell lines and clones.[9] Here, we have generated IL-7-responsive pre-B cells in primary bone marrow cultures which have the surface phenotype 6C3$^+$ CD43$^+$ B220$^+$. These IL-7-responsive pre-B cells possess ~30–40% cells in the S + G_2/M phases of the cell cycle (Fig. 6), have demonstrable IL-7 receptor mRNA in PCR assays (Fig. 7), and bind biotinylated IL-7 (Fig. 8). Upon harvest and reculture with IL-7, a decrease in viable pre-B cells (determined by trypan blue exclusion) was followed by increased cell numbers within 4 days (Fig. 5). However, in the IFN-γ + IL-7-treated cultures, there was a decline in viable cells over this period (Fig. 5).

The proportion of IL-7-responsive pre-B cells which were 6C3$^+$ B220$^+$ after IFN-γ + IL-7 treatment was not different from that of control IL-7-treated cells (Fig. 6). However, IFN-γ-treated cells had a twice as great an increase in the proportion of cells which were found in the apoptotic A_0 region of DNA histograms and a 30% decline in cells in S + G_2/M at day 2 after reculture (Fig. 6). These data were consistent over five separate experiments and indicated that IFN-γ caused an arrest in proliferation that led to apoptotic cell death.

IL-7 receptor mRNA expression is retained in IL-7-dependent pre-B cells and 70Z/3 cells upon culture with IFN-γ

It has been reported previously that there are two IFN regulatory elements 5′ of the murine IL-7R promotor.[13] To determine if IFN-γ altered IL-7R mRNA expression, PCR analysis was performed on both IL-7-dependent pre-B cells and 70Z/3 pre-B cells after treatment with IL-7 + IFN-γ. Generation of cDNA from mRNA with reverse transcriptase using oligo $d(T)_{16}$ as primer and PCR analysis were performed using 10^5, 10^4, and 10^3 cell equivalents. These indicated a decrease in intensity of the 600 bp IL-7R PCR product band with cell number (Fig. 7). However, culture of IL-7-dependent pre-B cells or 70Z/3 cells with IFN-γ for either 2 or 3 days, respectively, resulted in no detectable change in IL-7R mRNA, as detected by PCR, compared to cells treated with IL-7 alone (Fig. 7).

IFN-γ results in partial loss of IL-7 binding concomitant with up-regulation of surface B220 expression in pre-B cells

Biotinylated IL-7 was used to examine cellular binding of IL-7 to the IL-7R by flow cytometric analysis. Incubation of either IL-7-responsive bone marrow pre-B cells or 70Z/3 pre-B tumour cells with IFN-γ for 48 hr resulted in a reproducible reduction in bound IL-7 fluorescence intensity (Fig. 8). Coincident with down-regulation of IL-7 binding capacity, there was a significant upward shift in B220 expression in both IL-7-dependent pre-B cells and in 70Z/3 pre-B cells (Fig. 8). The shift in B220 expression was reproducibly observed within 24 hr of culture with IFN-γ. Decreased binding of IL-7 to 70Z/3 cells treated with IFN-γ was observed along with increased expression of

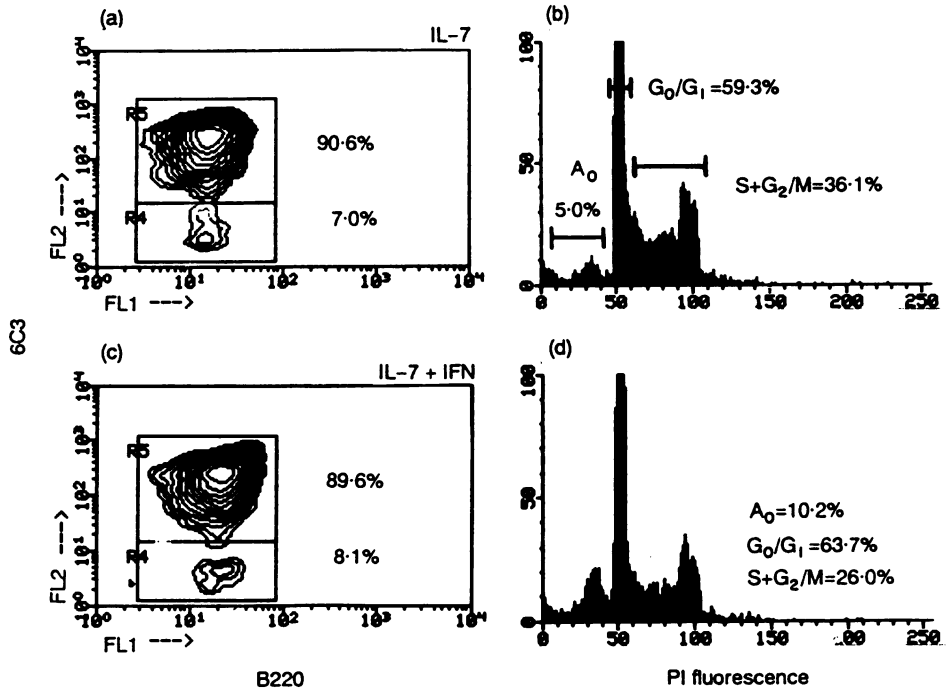

Figure 6. IFN-γ inhibits proliferation but does not change the phenotype of IL-7-dependent pre-B cells. IL-7-dependent pre-B cells obtained by culturing bone marrow with IL-7 for 7 days were recultured for 48 hr with 25 U/ml rmIL-7 (a and b) or IL-7 + 25 U/ml rmIFN-γ (c and d), harvested, and stained with biotinylated anti-6C3/avidin–PE and anti-B220–FITC (a and c) or with PI (b and d) and analysed using flow cytometry. The percentages of $6C3^+$ $B220^+$ cells [upper box (R5) in a and c] and $6C3^-$ $B220^+$ cells [lower box (R4) in a and c] are indicated. The percentage of cells in A_0, G_0/G_1, and $S+G_2/M$ regions of DNA histograms (b and d) are indicated. Data are representative of four separate experiments.

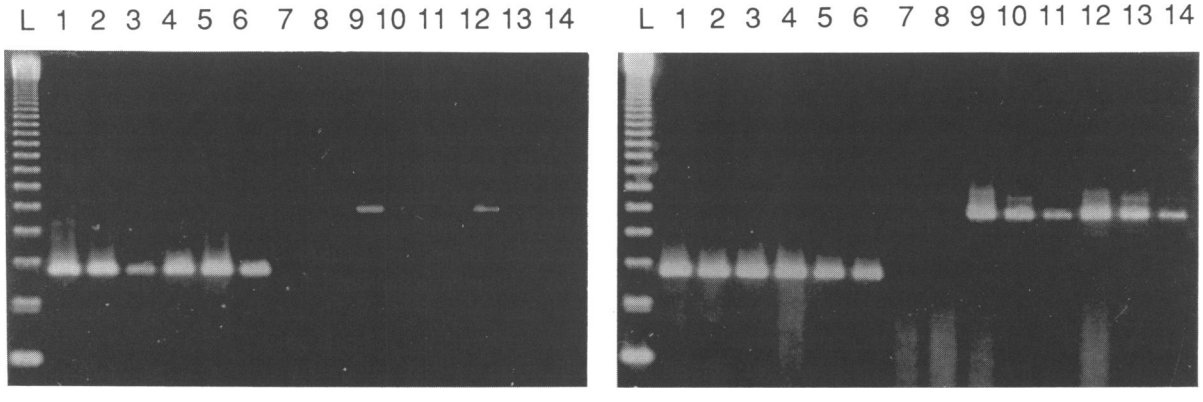

Figure 7. IL-7 receptor mRNA expression in pre-B cells is unaffected by IFN-γ. IL-7-dependent pre-B cells (a) and 70Z/3 pre-B tumour cells (b) were incubated either with media (lanes 1–3, 7, 9–11) or with 25 U/ml rmIFN-γ (lanes 4–6, 8, 12–14) for 2 and 3 days, respectively. (All IL-7-dependent pre-B-cell cultures contained 25 U/ml IL-7.) RNA from 10^5 (lanes 1, 4, 7, 8, 9, 12), 10^4 (lanes 2, 5, 10, 13) or 10^3 (lanes 3, 6, 11, 14) cells was used to generate cDNA using reverse transcriptase (RT) followed by amplification by PCR using IL-7R or β-actin amplimers. PCR products were visualized from ethidium bromide stained agarose gels. L, 123 bp ladder; lanes 1–6, β actin; lanes 7 and 8, IL-7R minus RT; lanes 9–14, IL-7R plus RT. Data are representative of three separate experiments.

surface B220; however, proliferation was not substantially impaired in this cell line in bulk cultures and cell viability was not affected by IFN-γ treatment (data not shown).

DISCUSSION

Cytokines serve as important positive and negative regulators of B lymphopoiesis. IL-7 acts to drive proliferation of late pro-B/early pre-B cells in both murine and human systems.[6,14] In mice, IL-7 has been shown to cause increased expression of the surface aminopeptidase A molecule, 6C3/BP-1, on pre-B cells.[6,12,15] 6C3/BP-1 function is important in allowing pre-B-cell responses to IL-7.[12,16] A number of inhibitors of pre-B-cell proliferation have been identified including transforming growth factor-β (TGF-β)[6] and IL-1.[17] Previously, IFN-γ has been shown to promote κ light chain transcription in pre-B-cell lines (including 70Z/3)

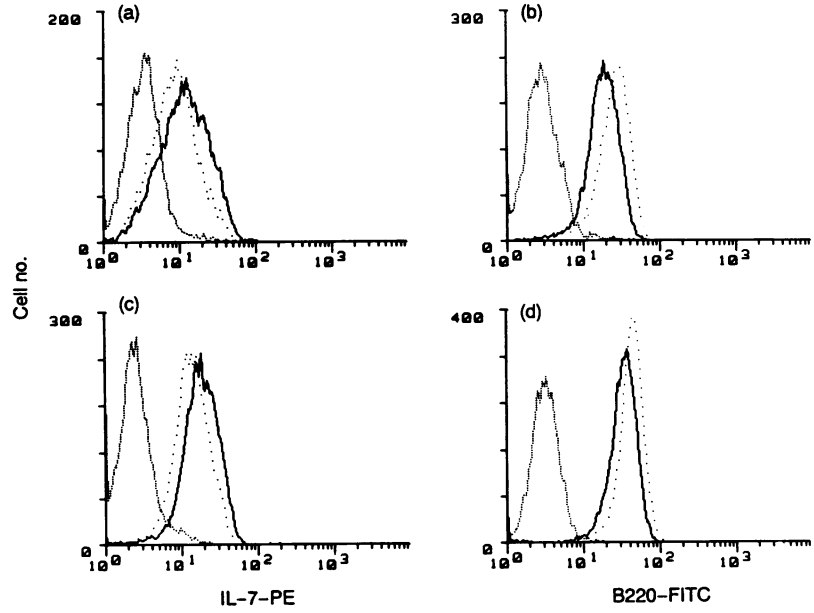

Figure 8. IFN-γ causes decreased IL-7 binding and increased surface B220 expression in pre-B cells. IL-7-dependent pre-B cells (a and b) or 70Z/3 pre-B cells (c and d) were cultured for 48 hr with 25 U/ml murine rIL-7 or media, respectively, ±25 U/ml IFN-γ. Cells were harvested, washed extensively, cultured for 2 hr to remove IL-7, and stained with biotinylated IL-7/avidin–PE and anti-B220–FITC. Histograms show IL-7–PE (a and c) and B220–FITC (b and d) fluorescence. Histograms with solid lines are from either media or IL-7-treated cells with equivalent results. Histograms on the far left are negative controls (avidin–PE or irrelevant FITC staining) and dotted lines are from IL-7 (or media) + IFN-γ-treated cells with equivalent results. Representative of six separate experiments.

and therefore may influence pre-B-cell development.[18] Recently, we and others have reported that IFN-γ suppressed IL-7-mediated pre-B-cell colony formation by normal bone marrow cells.[1,8] We have now expanded these studies to suggest mechanisms by which IFN-γ detrimentally affects B-cell development.

IFN-γ completely inhibited IL-7 CFU formation at concentrations of less than 1 U/ml. The low concentrations of IFN-γ required to inhibit pre-B-cell proliferation implies that a small number of IFN-γ-secreting cells may exert significant local effects on IL-7-responsive B-lineage cells. In contrast, concentrations as high as 1000 U/ml IFN-γ have been shown to be necessary for inhibition of bone marrow myeloid cell proliferation and colony formation in response to granulocyte colony-stimulating factor (G-CSF) or IL-3.[19] We have shown that low frequencies (<2%) of IFN-γ-producing bone marrow T cells activated during murine graft-versus-host responses are sufficient to inhibit IL-7-mediated pre-B-cell colony formation *in vitro*.[1] Notably, normal murine bone marrow lymphocytes exhibit IFN-γ mRNA detectable by *in situ* hybridization.[20] Therefore, IFN-γ production in the bone marrow may regulate pre-B-cell development in both healthy and disease states.

IFN-γ apparently can act directly on pre-B cells since IL-7 CFU formation was completely inhibited among IFN-γ treated, isolated B220$^+$ B-lineage cells. Although this does not preclude a possible effect on other cell types, it implies that IFN-γ may be a direct negative regulator of IL-7-mediated functions during pre-B-cell growth and development.

Cell death by apoptosis is considered a natural consequence for pre-B cells which have dysfunctional gene rearrangements or fail to respond to appropriate growth and differentiation signals.[21] In addition, as in thymocytes, glucocorticoids such as dexamethasone can induce apoptosis in pre-B cells.[11] However, the data presented here indicate that IFN-γ is not a potent inducer of apoptosis in normal resting murine pre-B cells. IFN-γ did inhibit IL-7 responses among pre-B cells as evidenced by the failure to increase 6C3/BP-1 expression and decreased proliferation in primary bone marrow cultures. However, IFN-γ failed to accelerate apoptotic cell death among predominantly small resting pre-B cells, a result in contrast to the effects of dexamethasone which substantially increased pre-B-cell apoptosis.

IFN-γ inhibited proliferation among IL-7-responding pre-B cells, eventually resulting in their apoptotic death. Expression of 6C3 and CD43 antigens did not change with IFN-γ treatment of IL-7-responsive pre-B cells, suggesting that these cells were not induced to further differentiate. Consistent with these findings, Grawunder *et al.* found that stromal cell/IL-7-dependent pre-B cells, upon exposure to IFN-γ, were inhibited from further proliferation, underwent apoptotic death, and failed to differentiate to Ig$^+$ B cells upon IL-7 withdrawal.[9] Although IFN-γ has been shown in other cell systems to inhibit proliferation,[19] the biochemical mechanism has not been elucidated.

Apoptosis in immortalized B-lineage cells has been reported as a consequence of growth factor deprivation.[22,23] Withdrawal of IL-7 upon reculture of IL-7-dependent pre-B cells caused arrest in proliferation and apoptosis similar to that seen in IL-7 + IFN-γ treated IL-7-responsive pre-B cells (data not shown). It has been reported previously that there are two IFN response elements (IRE) 5′ of the murine IL-7R promoter.[13] Therefore, it was reasonable to suspect that IFN-γ may down-regulate IL-7R transcription in pre-B cells, thereby effectively depriving the cells of IL-7-mediated proliferation signals. However, there were no detectable changes observed in IL-7R mRNA expression, using PCR analysis, in both IL-7-dependent pre-B cells and in 70Z/3 cells upon culture with IFN-γ. Interestingly, our studies did demonstrate a small, but reproducible, decrease

in IL-7 binding in both IL-7-dependent pre-B cells and in 70Z/3 pre-B cells upon exposure to IFN-γ. The decrease in IL-7 binding among IL-7-dependent pre-B cells was limited to large cells (data not shown), suggesting that the decreased IL-7 binding may be partially responsible for decreased proliferation and the predominance of small, resting cells in the IFN-γ-treated cultures. The binding of IL-7 to pre-B cells exhibited heterogeneity in affinity, which may be indicative of negative cooperativity due to possible IL-7R dimerization and/or association with other proteins.[24,25] Furthermore, as noted above, the 6C3/BP-1 aminopeptidase A may cleave IL-7, resulting in altered binding.[12,16] Since IL-7R mRNA continues to be produced in pre-B cells exposed to IFN-γ, the negative effects on IL-7 binding may occur through the above processes. However, further investigation is required to determine if the small, reproducible downward shift in IL-7 binding seen in IFN-γ-treated cells has any biological significance.

Unexpectedly, surface B220 expression was reproducibly up-regulated in IFN-γ-treated IL-7 pre-B cells and in 70Z/3 cells. Similar results were obtained when either the 14.8 or RA3.6B2 monoclonal antibodies, which are specific for different epitopes on the same molecule,[26] were used for surface staining, suggesting that the effect was not simply due to differential exon usage in expression of this surface protein. B220 is a member of the leukocyte common antigen (LCA or CD45) family of surface glycoproteins. These molecules differ in their extracellular domains, but are all identical in their cytoplasmic domain.[27,28] The intracellular domain in both B- and T-lineage cells has been shown to have tyrosine phosphatase activity.[29,30] Interestingly, stimulation of human thymocytes and pre-B cell lines through the IL-7R results in tyrosine phosphorylation of a number of proteins and functions as an early event in IL-7-mediated signalling.[14,31] Up-regulation of B220 tyrosine phosphatase expression in IFN-γ-treated pre-B cells may act to negate IL-7-mediated tyrosine phosphorylation and inhibit IL-7R-dependent signalling pathways. These issues are currently under investigation.

It is clear from the studies presented here that IFN-γ can have detrimental effects on normal B-cell development. This may have important implications in situations characterized by excessive IFN-γ production as a result of inflamation or malignancy. Patients with graft-versus-host reactions as a result of allogeneic bone marrow transplantation or patients with BCLL may have increased levels of circulating IFN-γ.[7] Based upon our studies, neutralization of excessive IFN-γ production in such situations would be expected to improve B-lineage cell production within the bone marrow.

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