Brief Definitive Report

Interferon γ Selectively Inhibits Very Primitive $CD34^{2+}CD38^-$ and Not More Mature $CD34^+CD38^+$ Human Hematopoietic Progenitor Cells

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Summary

To assess the effects of interferon γ (IFN-γ) on very primitive hematopoietic progenitor cells, $CD34^{2+}CD38^-$ human bone marrow cells were isolated and cultured in a two-stage culture system, consisting of a primary liquid culture phase followed by a secondary semisolid colony assay. $CD34^{2+}CD38^-$ cells needed at least the presence of interleukin 3 (IL-3) and kit ligand (KL) together with either IL-1, IL-6, or granulocyte–colony-stimulating factor (G-CSF) in the primary liquid phase in order to proliferate and differentiate into secondary colony-forming cells (CFC). Addition of IFN-γ to the primary liquid cultures inhibited cell proliferation and generation of secondary CFC in a dose-dependent way. This was a direct effect since it was also seen in primary single cell cultures of $CD34^{2+}CD38^-$ cells. The proliferation of more mature $CD34^+CD38^+$ cells, however, was not inhibited by IFN-γ, demonstrating for the first time that IFN-γ is a specific and direct hematopoietic stem cell inhibitor. IFN-γ, moreover, preserves the viability of $CD34^{2+}CD38^-$ cells in the absence of other cytokines. IFN-γ could, therefore, play a role in the protection of the stem cell compartment from exhaustion in situations of hematopoietic stress and may be useful as stem cell protecting agent against chemotherapy for cancer.

Differentiation and proliferation of primitive hematopoietic progenitor cells is tightly regulated by colony-stimulating factors (CSF) and by cytokines that act in synergy with CSF to stimulate the development of progenitor cells into mature cells (1). Recently several reports have demonstrated direct stimulatory effects of IFN-γ on hematopoietic progenitor cells in synergy with other hemoregulatory cytokines such as IL-3 (2–4), GM-CSF (2–4), *c-kit* ligand (KL) (5) and the combination of IL-3 and erythropoietin (epo) (6). Moreover, IFN-γ stimulates the expansion of progenitor cells induced by IL-3, IL-6, IL-1, epo, and KL (7) and the growth of acute myeloblastic leukemia cells supported by IL-3 (6). The growth of G-CSF–responsive granulocytic progenitors however is directly inhibited by IFN-γ (4). The direct effects of IFN-γ on very primitive progenitor and stem cells, however, have not been reported thus far. Very primitive human hematopoietic progenitor and stem cells are characterized by a high expression of CD34 and the absence of CD38 expression ($CD34^{2+}CD38^-$ cells). Upon differentiation and lineage commitment, the expression of CD38 increases while the expression of CD34 decreases ($CD34^+CD38^+$ cells) (8, 9). To study the effects of IFN-γ on the early stages of the development of these very primitive human progenitor cells, we used a precolony-forming cell (pre-CFC) assay (10, 11) where the effects of the presence of IFN-γ in primary cultures of $CD34^{2+}CD38^-$ cells on the output of secondary CFCs was studied. Here we report that IFN-γ is a potent and selective direct inhibitor of $CD34^{2+}CD38^-$ and not of $CD34^+CD38^+$ cells. These findings indicate that IFN-γ may play a role in protecting the stem cell compartment from exhaustion in situations of hematopoietic stress and that IFN-γ could be useful for the specific protection of hematopoietic stem cells against chemotherapy for cancer.

Materials and Methods

Bone Marrow Cells. Bone marrow samples were aspirated by sternal puncture from hematologically normal patients undergoing cardiac surgery, after informed consent according to the regulations of the Ethics Committee of the University of Antwerp, in tubes containing 2 ml IMDM (GIBCO, Paisley, UK) and 5 U/ml preservative free heparin (Novo Industries, Bagsvaerd, Denmark). Cells were separated on a lymphocyte separation medium (LSM; Boehringer Mannheim GmbH, Penzberg, Germany) density gradient and washed twice. Remaining RBC were lysed using an NH_4Cl containing lysing solution.

Cytokines and Monoclonal Antibodies. Supernatant of the 43A1 hybridoma (IgG_3, kindly donated by Dr. H. J. Bühring, University of Tübingen, Tübingen, Germany) (12) was used as a source

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of anti-CD34. FITC-conjugated rabbit anti–mouse immunoglobulin $F(ab')_2$ fragments (RAM) were purchased from Dako (Glostrup, Denmark). PE-conjugated anti-CD38 (IgG_1) antibodies as well as isotype-specific control antibodies were purchased from Becton Dickinson (Erembodegem, Belgium). Monoclonal neutralizing anti-IFN-γ antibody (clone GZ-4, $IgG_1\kappa$) and isotype-matched irrelevant control antibody (antidigoxigenin, $IgG_1\kappa$) were kindly donated by Dr. M. Kubbies (Boehringer Mannheim GmbH). Recombinant human IFN-γ (specific activity 2×10^7 U/mg), IL-6 (10^8 U/mg), and IL-1 (5×10^7 U/mg) were obtained from Boehringer Mannheim GmbH. Recombinant human G-CSF, GM-CSF, and IL-3 were a kind gift of Dr. S. C. Clark (Genetics Institute, Cambridge, MA), while recombinant human KL was kindly donated by Dr. M. Kubbies. Erythropoietin (epo) was purchased from Cilag (10^5 U/mg; Brussels, Belgium).

Cell Sorting. Bone marrow cells were incubated at 10^7 cells/ml with 43A1 supernatant in a 1:10 dilution for 20 min at 4°C, washed twice in IMDM containing 2% FCS, incubated with fluoresceinated RAM (1/50 dilution) for 20 min at 4°C. After washing the cells twice in IMDM + 2% FCS, they were sorted on a FACStar Plus® cell sorter (Becton Dickinson) equipped with an air-cooled argon ion laser (ILT model 5500A; Ion Laser Technology, Salt Lake City, UT). Cells with a low to medium forward scatter and a low side scatter, a highly positive green (CD34) fluorescence, and an orange (CD38) fluorescence signal lower than the mean fluorescence of cells labeled with control antibody + 2 standard deviations were retained as $CD34^{2+}CD38^-$ cells; cells with an orange fluorescence above this threshold were retained as $CD34^+CD38^+$ cells. Purities were always >95%.

Pre-CFU Assay. Primary liquid cultures were performed in duplicate in 96-well flat-bottomed plates at 100 cells/well in IMDM, 10% FCS, and combinations of the following recombinant human cytokines: 100 ng/ml IL-1, 200 U/ml IL-6, 100 ng/ml G-CSF, 30 U/ml IL-3, 100 ng/ml KL, and varying concentrations of IFN-γ. After 14 d of primary culture, the number of cells in each well was counted using an inverted microscope at 250× magnification, after which the cells were harvested, washed three times in IMDM + 10% FCS, and plated in secondary methylcellulose cultures (0.9%) supplemented with 20% FCS, 1% BSA, 10^{-5} M 2-mercaptoethanol, 30 U/ml IL-3, 100 ng/ml G-CSF, 100 ng/ml GM-CSF, and 2 U/ml epo, which were found to be optimal concentrations for colony formation in preliminary experiments. These cultures were microscopically scored for colony formation after 14 and after 21 d of culture at 37°C in 7.5% O_2 and 5% CO_2 in a fully humidified incubator.

To ascertain that the effects of IFN-γ in these experiments were direct, the same experiments were performed at a single cell level. $CD34^{2+}CD38^-$ cells were sorted at 1 cell/well in 96-well V-bottomed plates (two plates per cytokine combination). In test sorts using fluorescent microbeads, on average <2% of the wells contained no beads and no wells were detected which contained more than one bead. Each well contained 100 μl of culture medium consisting of IMDM, 10% FCS, IL-1, IL-6, IL-3, and KL (concentrations as in the primary cultures described above) and either no IFN-γ or IFN-γ at 10^3 U/ml. After 14 d of culture (37°C, 7.5% O_2, 5% CO_2 in a fully humidified incubator), the number of wells where growth had occurred (primary colonies) was scored using an inverted microscope, the primary colonies were harvested, washed four times, and individually plated in secondary methylcellulose cultures as described above. Secondary colony formation was assessed after 14 and 21 d of secondary culture. Parallel experiments were performed using $CD34^+CD38^+$ cells (which constitute the remainder of the $CD34^+$ cells).

In a number of experiments, the cells were first cultured in the presence or absence of IFN-γ without any other cytokines for 4 d in 96-well flat-bottomed plates, after which the number of remaining cells was counted by phase contrast microscopy (250× magnification). Shrunken, dull-appearing cells with a ruffled cell membrane were considered to be dead and were not counted. The cells were then harvested, washed, and cultured in liquid culture medium for 14 d in the presence of KL (100 ng/ml), IL-1 (100 ng/ml), IL-6 (200 U/ml), and IL-3 (30 U/ml). After 14 d of culture the cells were harvested, washed, and plated out in secondary methylcellulose cultures as described above.

Statistics. In all experiments, the Student's t test for paired samples was used.

Results

Cytokine Requirements of $CD34^{2+}CD38^-$ Cells. The $CD34^{2+}CD38^-$ fraction comprises ~0.01–0.05% of human bone marrow cells and 1–5% of the $CD34^+$ cells (not shown). These cells hardly form any colonies when plated directly in semisolid methylcellulose cultures in the presence of combinations of CSF such as IL-3, GM-CSF, G-CSF, and epo (cloning efficiency <2%, results not shown). However, when cultured in primary liquid cultures for 14 d in the presence of combinations of multiple early acting factors (for a review, see reference 1), the $CD34^{2+}CD38^-$ cells give rise to committed progenitor cells which do form colonies in secondary methylcellulose cultures (CFC). To generate secondary CFC, $CD34^{2+}CD38^-$ require at least the presence of IL-3, KL, and either IL-1, IL-6, or G-CSF in the primary liquid cultures (Fig. 1). No secondary CFC were generated in the absence of either IL-3 (not shown) or KL (Fig. 1), and only few secondary CFC were produced in the presence of IL-3 and KL without any other synergistic factors in the primary liquid cultures (Fig. 1). The secondary colonies generated from $CD34^{2+}CD38^-$ cells were mostly myeloid with <2% of erythroid or mixed erythroid/myeloid colonies. Most of the myeloid colonies consisted of large macrophages. Addition of epo to the primary cultures had no effect on the number nor on the morphology of the secondary colonies (not shown). When the secondary cultures were scored at day 21, secondary high proliferative potential CFC (HPP-CFC, defined as macroscopic colonies of >2 mm diameter with a dense center, consisting mostly of large macrophage-like cells) were noted (see Fig. 3). These HPP-CFC are believed to arise from more primitive progenitor cells than other colony types (13, 14).

IFN-γ Inhibits the Proliferation and Differentiation of $CD34^{2+}CD38^-$ Cells. When IFN-γ was added to the primary liquid cultures of $CD34^{2+}CD38^-$ cells (100 cells/well) stimulated by cytokine combinations that induce proliferation of $CD34^{2+}CD38^-$ cells (see Fig. 1), cell proliferation, generation of secondary CFC, and of secondary HPP-CFC were profoundly inhibited in a dose-dependent way, with near complete inhibition occurring at 5,000 U/ml (Figs. 2 and 3). The inhibitory effect of IFN-γ was less pronounced, but still statistically significant, in cultures stimulated by IL-3+KL (Fig. 2). The inhibitory effect of IFN-γ was blocked by adding neutralizing antibodies to human IFN-γ to the cultures (Fig. 3), but not by adding isotype-matched irrelevant control antibodies.

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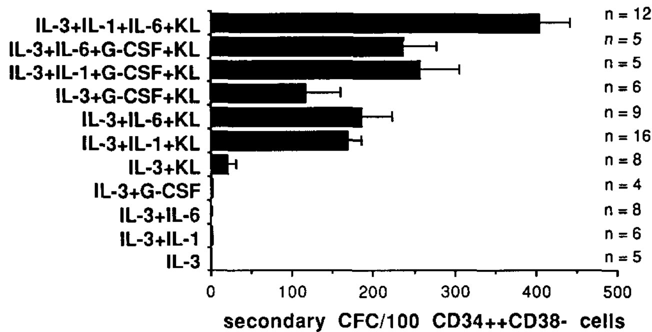

Figure 1. Effect of primary culture conditions of purified CD34²⁺CD38⁻ bone marrow cells (cytokine combinations mentioned on the y-axis) on the generation of secondary CFC. Results expressed as the number of secondary CFC/100 CD34²⁺CD38⁻ cells (mean ± SEM). The number of independent experiments is indicated on the right hand side of the figure (*n*).

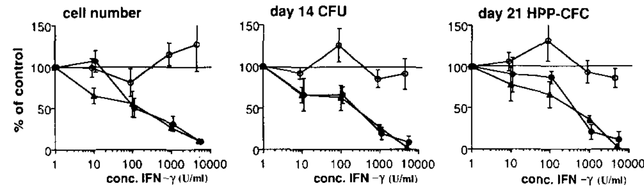

Figure 3. Dose response curve of the effect of IFN-γ (▲), IFN-γ + neutralizing anti-IFN-γ antibody (○), and IFN-γ + irrelevant control antibody (●) on the primary liquid culture total cell number (*left*), number of day 14 secondary CFC (*middle*), and day 21 secondary HPP-CFC (*right*) after 14 d of primary liquid culture of CD34²⁺CD38⁻ cells with IL-1, IL-6, IL-3, and KL. Results (mean ± SEM) are expressed as percentage of inhibition, compared with primary cultures without IFN-γ. The number of cells after 14 d of primary culture without IFN-γ was 1,318 ± 354, the number of day 14 secondary CFC 473 ± 64, and the number of day 21 secondary HPP-CFC was 61 ± 10 per input of 100 CD34²⁺CD38⁻ cells (pooled results of five independent experiments performed in duplicate).

To see whether this inhibition was a direct effect of IFN-γ, primary liquid cultures were performed at a single cell level, by sorting CD34²⁺CD38⁻ cells at one cell/well in 96-well plates in the presence of IL-3, IL-1, IL-6, and KL with or without IFN-γ. The wells in which growth had occurred as assessed by microscopic evaluation (primary colonies) were picked up, washed, and plated individually in methylcellulose cultures supplemented with IL-3, GM-CSF, G-CSF, and epo. In these single cell culture experiments, the presence of IFN-γ in the primary cultures inhibited the total number of secondary CFC to the same extent as in experiments where CD34²⁺CD38⁻ cells were cultured at 100 cells/well (−67.5 ± 13.7% vs. −69.1 ± 4.3%, respectively, at a concentration of 1,000 U/ml, $n = 4$), demonstrating that the inhibitory effect of IFN-γ on the proliferation of CD34²⁺CD38⁻ cells is a direct one. IFN-γ primarily inhibited the number of CD34²⁺CD38⁻ cells forming primary colonies (Table 1, *top*), whereas the number of secondary CFCs per individual primary colony was inhibited to a lesser extent (Table 1, *bottom*).

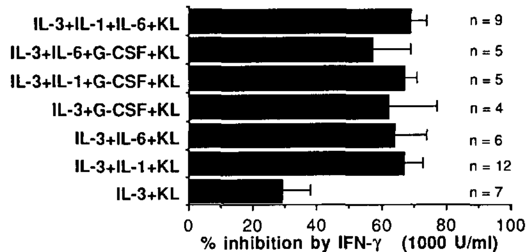

Figure 2. Relative inhibition induced by IFN-γ (1,000 U/ml) in the 14-d primary liquid cultures of CD34²⁺CD38⁻ cells, supported by the cytokine combinations mentioned on the y-axis, on the generation of secondary colonies (results expressed as mean ± SEM). The number of independent experiments is indicated on the right hand side of the figure (*n*). The IFN-γ–induced inhibition was statistically significant in all cases ($p < 0.05$, Student's *t* test for paired samples).

IFN-γ Does Not Inhibit More Mature CD34⁺CD38⁺ Cells. The same single cell culture experiments were performed using CD34⁺CD38⁺ bone marrow cells, which constitute the remainder of CD34⁺ cells (Table 1). Strikingly, only 6.7 ± 2.5% of the primary colonies generated from this population in the presence of IL-3, IL-1, IL-6, and KL contained secondary CFCs (compared with 95.3 ± 2.4% of the primary colonies derived from CD34²⁺CD38⁻ cells, $p = 0.0001$). This indicates that this culture system allows a very sharp functional distinction of very primitive CD34²⁺CD38⁻ cells from more mature CD34⁺CD38⁺ cells. In contrast to the effects of IFN-γ on CD34²⁺CD38⁻ cells, addition of IFN-γ to primary cultures of CD34⁺CD38⁺ cells had no effect on the number of primary colonies nor on the number of secondary CFC (Table 1). Moreover, IFN-γ did not inhibit colony formation in single cell methylcellulose cultures of CD34⁺CD38⁺ cells supported by epo, IL-1, IL-6, KL, and IL-3 (mean cloning efficiency 30.8 ± 2.6% without and 27.5 ± 3.2% with IFN-γ at 10^3 U/ml respectively, $n = 6$, $p = 0.35$). Taken together, these data indicate that IFN-γ acts as a highly selective and direct inhibitor of the proliferation and differentiation of very primitive CD34²⁺CD38⁻ cells and not of more mature CD34⁺CD38⁺ cells.

IFN-γ Stimulates the Survival of CD34²⁺CD38⁻ Cells. To see whether preincubation of CD34²⁺CD38⁻ cells in IFN-γ also affects their subsequent capacity to generate secondary CFC, CD34²⁺CD38⁻ cells where first cultured for 4 d either in culture medium (IMDM, 10% FCS) or in culture medium containing IFN-γ, after which the cells were washed and a pre-CFC assay was performed as described above. When, after 4 d of preculture in either medium without cytokines or medium containing IFN-γ (10^3 U/ml) the cells were washed and cultured for another 14 d in the presence of IL-3, KL, IL-6, and IL-1, significantly more secondary CFC were recovered from the cells that had been precultured for 4 d in the presence of IFN-γ than in the absence of IFN-γ (mean difference +117 ± 26%, $p = 0.02$, $n = 4$). This effect is due to a survival-enhancing effect of IFN-γ, since significantly

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Table 1. *Effects of IFN-γ on CD34²⁺CD38⁻ and CD34⁺CD38⁺ Cells in Single Cell Culture Experiments*

	No IFN-γ	IFN-γ 10^3 U/ml	Mean Δ*	n‡	p§
Percentage of primary colonies in single cell liquid cultures supported by IL-1, IL-6, IL-3, and KL					
$CD34^{2+}CD38^-$	25.4 ± 2.9	13.3 ± 2.7	−48.4 ± 6.7%	6	0.0008
$CD34^+CD38^+$	22.6 ± 3.1	22.0 ± 3.3	+1.8 ± 8.0%	6	0.83
Number of secondary CFC per individual primary colony					
$CD34^{2+}CD38^-$	73.4 ± 13.1	53.0 ± 18.1	−20.0 ± 5.3	4	0.033
$CD34^+CD38^+$	0.7 ± 0.4	0.2 ± 0.2	−0.5 ± 0.4	6	0.31

* Mean difference (Δ) expressed as relative difference in terms of percentage compared with cultures without IFN-γ.
‡ Number of independent experiments.
§ Statistics used Student's t test for paired samples.

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more morphologically intact cells, as determined by counting the cells in the culture wells by phase contrast microscopy, were recovered after 4 d of culture with IFN-γ than after 4 d of culture in medium without cytokines (68.5 ± 2.9% and 31 ± 4.8%, respectively, of the input cell number, $p = 0.0008$, $n = 5$, 10^3 U/ml IFN-γ). Both the effect on cell number and on the capacity to generate secondary CFC reached a plateau at an IFN-γ concentration of 50 U/ml (not shown), i.e., at a 2-log lower concentration than the concentration at which complete inhibition of proliferation and differentiation of pre-CFC was noted (see Fig. 2). These data show that IFN-γ promotes the survival of $CD34^{2+}CD38^-$ cells and that preincubation in IFN-γ does not inhibit their subsequent proliferative capacity.

Discussion

The assay used in this report identifies very primitive precursors of CFC, since $CD34^{2+}CD38^-$ cells that do not form colonies in semisolid media are stimulated to differentiate in suspension culture into CFC. $CD34^{2+}CD38^-$ cells, which, according to our data also contain precursors of HPP-CFC which are considered to be very early progenitors (13, 14), are known to be among the most primitive hematopoietic precursors (8, 9). The fact that in the secondary cultures, mostly colonies containing macrophages were recovered might indicate that a less primitive cell is detected here. However, many authors have shown that HPP-CFC also consist of mostly large macrophage-like cells (13, 14). Moreover and in accordance with our data, Lu et al. (15) showed that colonies with a high replating capacity generated from primitive $CD34^{3+}$ cord blood cells consisted mostly of large macrophage-like cells. The development of colonies originating from very early (stem) cells in these in vitro assays thus seems to be biased towards the macrophage lineage. Since this is a two-stage culture system, this assay allows the characterization of the direct effects of IFN-γ on the early phases of the development of primitive progenitor cells (from pre-CFC to CFC), without interference of any effects of IFN-γ on the terminal stages of differentiation (from CFC to mature cell), on which IFN-γ has been shown to have stimulatory effects (2–6).

This report for the first time identifies IFN-γ as a direct bidirectional regulator of hematopoiesis whose inhibitory effects display a very strong specificity for very primitive progenitor and stem cells, as evidenced by the direct inhibition by IFN-γ of the early stages of proliferation and differentiation of very primitive $CD34^{2+}CD38^-$ cells, but not of more mature $CD34^+CD38^+$ cells. The smaller decrease in secondary colony formation induced by IFN-γ in primary cultures of $CD34^{2+}CD38^-$ cells supported by IL-3+KL (Fig. 2) might be interpreted in the same context, since the $CD34^{2+}CD38^-$ cells stimulated by this cytokine combination are probably less primitive than cells which require a combination of three or four cytokines in order to proliferate. These effects are moreover undoubtedly direct since they were also seen in single cell culture experiments.

A number of recent reports, including one from our laboratory (4), demonstrated direct stimulatory effects of IFN-γ on human hematopoietic progenitor cells in synergy with IL-3 and GM-CSF (2–4). Some reports even suggested that IFN-γ has a selective stimulatory effect on more primitive progenitors in the murine system (5). However, in most of these reports the effects of IFN-γ on progenitor cells responsive to single CSF was assessed, indicating that more mature progenitor cells were studied. We show in this report that IFN-γ does not inhibit, nor stimulate the proliferation and differentiation of more mature $CD34^+CD38^+$ population stimulated by IL-3, IL-1, IL-6, and KL and epo. We could thus not confirm the data of Brugger et al. (7) who showed that IFN-γ stimulates the expansion of CFC induced by the same four cytokine combination using peripheral blood $CD34^+$ cells. Differences in target cell populations and purity, or in cell isolation procedure and culture could account for this discrepancy. Earlier reports showed suppressive effects

of IFN-γ on committed progenitor cells (16–18). However, in these reports, various crude conditioned media were used to stimulate progenitor cell proliferation, the experiments were performed with relatively unpurified bone marrow populations and single cell assays were never performed, so that most probably indirect effects were measured.

Other cytokines that have been identified as negative regulators of hematopoiesis, i.e., TGF-β (19–21) and some members of the chemokine-family of cytokines, among which macrophage inflammatory protein 1α (MIP-1α) (22–24), tend to display a selectivity for the inhibition of primitive progenitor cells, but, in contrast to IFN-γ (2–4), they also inhibit committed early human erythroid and myeloid progenitor cells responsive to single CSF or to combinations of two growth factors (20–24). Among the more mature progenitor cells, only the G-CSF–induced proliferation of relatively mature progenitors committed to the neutrophilic lineage is directly inhibited by IFN-γ (4).

Quite surprisingly, besides inhibiting the growth factor–induced proliferation of $CD34^{2+}CD38^{-}$ cells, IFN-γ also maintains their viability in the absence of other cytokines. To the best of our knowledge, such a phenomenon has not been described for TGF-β and MIP-1α. We have already shown that IFN-γ promotes the survival of more mature human committed erythroid and myeloid progenitor cells (25). IFN-γ probably inhibits apoptosis of progenitor cells. However, due to the very limited number of $CD34^{2+}CD38^{-}$ cells that could be isolated from a bone marrow sample, we could not confirm this mechanism by either demonstrating a DNA-ladder or by flow cytometry.

Since IFN-γ is an inflammatory cytokine that at the same time inhibits proliferation and cell death of very primitive progenitor cells and stimulates proliferation of more mature progenitors, it might, in situations of increased demand for blood cells such as infection and inflammation, stimulate the expansion of committed progenitor cells and their proliferation and differentiation into mature cells (2–7), while at the same time sparing the cells of the very primitive stem cell compartment from recruitment and thus protecting this compartment from exhaustion. IFN-γ could therefore be useful in the setting of chemotherapy for cancer as a stem cell protecting agent against cell cycle–specific drugs. Our data furthermore support the hypothesis that IFN-γ is a candidate inhibitor of hematopoiesis in the setting of at least some cases of severe aplastic anemia (26). In view of the recent evidence that IFN-γ stimulates progenitor cell proliferation (2–7), this controversial hypothesis (26, 27) had become less attractive. The data presented here however indicate that IFN-γ can indeed inhibit hematopoiesis through a selective and direct effect on very primitive stem cells.

In conclusion, IFN-γ inhibits the proliferation of very primitive $CD34^{2+}CD38^{-}$ cells and not of more mature $CD34^{+}CD38^{+}$ cells and is thus a selective hematopoietic stem cell inhibitor. IFN-γ, moreover, promotes the survival of $CD34^{2+}CD38^{-}$ cells.

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