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Whereas multiple growth-promoting cytokines have been demonstrated to be involved in regulation of the hemopoietic stem cell (HSC) pool, the potential role of negative regulators is less clear. However, IFN-γ, if overexpressed, can mediate bone marrow suppression and has been directly implicated in a number of bone marrow failure syndromes, including graft-vs-host disease. Whether IFN-γ might directly affect the function of repopulating HSCs has, however, not been investigated. In the present study, we used in vitro conditions promoting self-renewing divisions of human HSCs to investigate the effect of IFN-γ on HSC maintenance and function. Whereas purified cord blood CD34+CD38− cells underwent cell divisions in the presence of IFN-γ, cycling HSCs exposed to IFN-γ in vitro were severely compromised in their ability to reconstitute long-term cultures in vitro and multilineage engraft NOD-SCID mice in vivo (>90% reduced activity in both HSC assays). In vitro studies suggested that IFN-γ accelerated differentiation of targeted human stem and progenitor cells. These results demonstrate that IFN-γ can negatively affect human HSC self-renewal. 


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3 Abbreviations used in this paper: HSC, hemopoietic stem cell; 7-AAD, 7-aminoactinomycin D; BM, bone marrow; CB, umbilical cord blood; CFC, colony-forming cell; EPO, erythropoietin; FL, flt3 ligand; LTC-IC, long-term culture-initiating cell; rh, recombinant human; SCF, stem cell factor; SF, serum free; SFT3, SCF + FL + thrombopoietin + IL-3; TPO, thrombopoietin.
from samples using Ficoll-Hypaque density gradient centrifugation (lymphoprep, 1.077 ± 0.001 g/ml; Nycomed Pharma). Positive selection of CD34<sup>+</sup> cells was performed by a MACS-CD34 isolation kit (Miltenyi Biotec), according to the manufacturer’s instructions. CB CD34<sup>+</sup> cells were run through a second column to obtain higher purity (91–94%) of CD34<sup>+</sup> cells. CD34-enriched cells were incubated with anti-CD38 PE and anti-CD34 FITC mAbs or with isotype-matched irrelevant control Abs (all from BD Biosciences), and subsequently sorted on a FACSVantage cell sorter (BD Biosciences). A conservative approach was taken to exclusively sort the 3% lowest CD34-expressing CD34<sup>+</sup> cells (CD34<sup>−</sup>CD38<sup>+</sup>), in an effort to obtain a highly purified population of primitive progenitor/stem cells. The purity of CD34<sup>−</sup>CD38<sup>+</sup> cells in the experiments was 96–98%.

**In vitro expansion cultures**

CD34<sup>+</sup> and CD34<sup>−</sup>CD38<sup>+</sup> CB cells were cultured at 1000 cells/ml for 7 or 12 days in serum-free (SF) medium (IMDM; BioWhittaker) supplemented with 10 ng/ml BSA, 10 µg/ml human insulin, 200 µg/ml human transferrin (BIT, StemCell Technologies), and a mixture of cytokines (100 ng/ml rhSCF, 100 ng/ml rhFL, 100 ng/ml rhTPO, and 20 ng/ml rhIL-3, defined as SFT3) in the absence or presence of 1000 U/ml rhIFN-γ. Following culture, cells were enumerated and evaluated functionally in vitro long-term cultures or by transplantation into NOD-SCID mice. In some experiments, cultured cells were further examined with regard to apoptosis, cell cycle status, and differentiation.

**Single-cell clonogenic assays**

As described previously (23), CD34<sup>+</sup>CD38<sup>+</sup> CB cells were seeded in Ter- aski plates (Nunc) at a density of 1 cell/well in 20 µl of SF medium (X-vivo 15; BioWhittaker) and 1% BSA (StemCell Technologies), supplemented with a mixture of cytokines (50 ng/ml rhSCF, 50 ng/ml rhFL, 50 ng/ml rhTPO, and 20 ng/ml rhIL-3) and different concentrations of rhIFN-γ. Wells (120/group) were scored for cell growth following 11–12 days of incubation at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air.

**Long-term culture-initiating cell (LTC-IC) assay**

Establishment and maintenance of long-term cultures were performed according to previously described procedure (23). Briefly, stroma cell feed ers were established by seeding a mixture (1:1) of two irradiated (8,000 cGy) murine fibroblast cell lines (M2-10B4 and sl/l, kindly provided by D. Hogge, Vancouver, Canada), engineered to produce high levels of human G-CSF, IL-3, and SCF (42), into 96-well collagen-coated microtiter plates (Nunc) containing LTC medium (Myelocolt; StemCell Technologies) supplemented with freshly dissolved 10<sup>-6</sup> M hydrocortisone 21-hemisuccinate (Sigma-Aldrich). These cell lines have been demonstrated to detect candidate HSCs with enhanced efficiency when compared with stromal derived from primary BM cells (42). Each well contained 10,000 cells from the mixture of two cell lines. The freshly isolated or expansion equivalents of 50 CD34<sup>+</sup>CD38<sup>+</sup> CB cells cultured in SFT3 in the absence or presence of rhIFN-γ for 7 days were seeded per stroma well (four replicates per group) and incubated for 6 wk at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air. All samples, 7-aminoactinomycin D (7-AAD; Sigma-Aldrich) was included and lineage analysis with anti-human CD34 FITC (progenitors), anti-human CD19 PE (B cells; BD Biosciences), anti-human CD15 PE, and anti-mouse CD45.1-PE Ab (BD Pharmingen) was performed. BM cells were counted and blocked with anti-mouse CD45.1-PE Ab (BD Pharmingen) as well as anti-mouse CD45.1-PE Ab (BD Pharmingen). BM cells from untransplanted mice (negative controls) and mixtures of 0.1% human cells in NOD-SCID BM (positive controls) were included. If engraftment was detected as human CD45/CD71 positive detection level 0.05%, lineage analysis with anti-human CD34 FITC (progenitors), anti-human CD19 PE (B cells; BD Biosciences), anti-human CD15 PE, and anti-human CD66b FITC (myeloid; both BD Pharmingen) combined with anti-human CD45 allopheyocyanin (BD Biosciences) was performed. For all samples, 7-aminoactinomycin D (7-AAD; Sigma-Aldrich) was included to gate out dead cells. A minimum of 5 × 10<sup>6</sup> BM cells was examined for each sample. Only mice with both positive myeloid and lymphoid engraftment (defined as >10 positive events each per 5 × 10<sup>6</sup> viable BM cells with maximum 1 event in corresponding controls) were evaluated as positive. If no engraftment was detected by flow cytometry or if the myeloid engraftment was questionable, BM cells were plated in methylcellulose supplemented with human-specific cytokines (25 ng/ml rhSCF, 25 ng/ml rhFL, 50 ng/ml rhGM-CSF) and 5 U/ml rhEPO at a density of 10<sup>4</sup> cells/35-mm plate, four replicates per group. CFU granulocyte-macrophage and burst-forming unit erythroid were scored after 10–12 days. No colonies were observed in the absence of cytokines or from BM of untransplanted mouse cultured with the same cytokines (L. Yang and S. E. W. Jacobsen, unpublished observations).

**Flow cytometric evaluation of differentiation, apoptosis, and cell cycle status of cultured CB progenitors**

To evaluate differentiation, CD34<sup>+</sup> CB cells were cultured for 9 or 12 days in SF medium (X-vivo 15; BioWhittaker) with 1% BSA (StemCell Technologies) or IMDM (BioWhittaker) with 20% FCS (BioWhittaker), supplemented with SFT3 and SFT3 + IL-6 + GM-CSF + G-CSF, respectively, in the absence or presence of 1000 U/ml rhIFN-γ. Following 2 wk of culture, adherent and nonadherent cells from each well were transferred to methylessulfoxide cultures containing rhSCF, rhFL, rhIL-3, rhGM-CSF, rh-CSF (all at 10 ng/ml), and rhEPO (5 U/ml). To ensure formation of a reliable number of colonies from the long-term culture, the content of each well was transferred to methylessulfoxide cultures at both a low (20%) and a high concentration (80% of cells). LTC colony-forming cells (LTC-CFCs) were scored after an additional 10–12 days of culture. Stromal cells without hemopoietic cells and cytokines were used as a negative control for the CFC assay.

**NOD-SCID-repopulating assay**

NOD-SCID mice (originally from The Jackson Laboratory, Bar Harbor, ME) were bred and housed under sterile conditions and maintained on autoclaved food and acidified water. All animal procedures were performed with consent from the local ethics committee at Lund University. At 8–12 wk of age, mice were irradiated with 350 cGy from a 137Cs source. The transplantation of 5 × 10<sup>5</sup> to 1 × 10<sup>6</sup> CD34<sup>+</sup> or 5000 CD34<sup>−</sup>CD38<sup>+</sup> CB cells together with 1 × 10<sup>6</sup> irradiated (1500 cGy) accessory cells (CD34-depleted CB cells) in 0.5 ml of medium was performed by tail vein injection within 4 h of irradiation. Mice were sacrificed after 6 wk by asphyxiation with CO<sub>2</sub>, and femora and tibiae were collected; and engraftment was evaluated by flow cytometry (FACS Calibur; BD Biosciences) using CellQuest analysis software (BD Biosciences), as described previously (23, 40, 41). Briefly, BM cells were counted and blocked with anti-mouse CD16/CD32 (BD Pharmingen,) and ChromPure mouse IgG, whole mole-
FIGURE 3. IFN-γ negatively modulates self-renewal of CD34⁺CD38⁻ candidate stem cells with LTC-CFC activity. Fifty CD34⁺CD38⁻ cells were cultured for 7 days in SF medium with SFT3 in the absence or presence of 1000 U/ml IFN-γ and subsequently evaluated for 6-wk LTC-CFC activity (see Materials and Methods). Also shown is the number of LTC-CFC generated from 50 freshly isolated CD34⁺ cells (data from cultures generated by 50 CD34⁺CD38⁻ cells after 6-wk LTC) are presented as means (SEM) of two individual experiments, each with four replicates cultures. +, p < 0.05, comparing −IFN-γ and +IFN-γ.

culture, cells were stained with anti-human CD34 FITC or allophycocyanin and a lineage mixture containing PE- or FITC-conjugated Abs (against CD11b, CD14, CD15, CD33, CD41, CD66b, and glycophorin A) and 7-AAD (to exclude nonviable cells). Control samples of cultured cells were stained with irrelevant isotype-matched control Abs. Samples were analyzed on a FACSCalibur.

Apoptosis of CD34⁺ CB cells was assessed by measuring redistribution of phosphatidylserine using annexin V-PE (BD Pharmingen) and uptake of annexin V-PE (5 µg/ml) in 100 µl of annexin V-binding buffer (BD Pharmingen) for 15 min, resuspended in annexin V-binding buffer, and analyzed on a FACSCalibur.

Cell cycle status of freshly isolated or cultured (5 days) CD34⁺ CB cells was determined, as described (45), with minor modifications. Cells were stained with anti-human CD34 allophycocyanin and a lineage mixture, followed by fixation and permeabilization with Cytofix/Cytoperm kit (BD Pharmingen) for 30 min. After washing, cells were stained with FITC-conjugated anti-Ki67 (Beckman-Coulter) or an isotype-matched irrelevant control Ab for 30 min. After 3 h of incubation in PBS containing 5% FCS supplemented with 7-AAD (5 µg/ml) at 4°C under dark conditions, samples were analyzed on FACSCalibur.

Results

IFN-γ inhibits clonal expansion of human candidate HSCs

We have recently demonstrated that in vitro culture under SF conditions in the presence of the early acting cytokines SFT3 efficiently promotes recruitment of murine and candidate human HSCs into proliferation with sustained stem cell function (32, 33). In this study, we used this system to investigate the effect of IFN-γ on self-renewal of human HSCs. CD34⁺CD38⁻ CB cells proliferated extensively in the presence of SFT3, and this expansion was inhibited by 35% in response to IFN-γ (Fig. 1).

To investigate whether IFN-γ might potentially block the first cell divisions of candidate HSCs, single cells were cultured in SFT3 in the presence or absence of different concentrations of IFN-γ (10–1000 U/ml; Fig. 2). Importantly, at all used concentrations, IFN-γ did not affect the number of proliferating clones. However, the size of the clones was reduced in a dose-dependent manner. These findings suggested that IFN-γ does not affect cytokine-induced recruitment of CD34⁺CD38⁻ candidate HSCs into proliferation, but rather inhibits their clonal expansion.

IFN-γ negatively affects maintenance of CD34⁺CD38⁻ CB LTC-IC under self-renewing conditions

To investigate the effect of IFN-γ on maintenance of candidate human HSCs, we cultured CD34⁺CD38⁻ CB cells in SFT3 in the presence or absence of IFN-γ, and subsequently evaluated the LTC-IC colony-forming cells (LTC-CFCs) in such cultures. In agreement with previous studies (23, 32), SFT3 promoted maintenance of high levels of LTC-CFC activity after 7 days of culture, comparable to uncultured input cells with an average of 343 LTC-CFCs derived from the initiating 50 CD34⁺CD38⁻ CB cells (Fig. 3). In the presence of IFN-γ, LTC-CFCs were reduced by as much as 89% (Fig. 3).

IFN-γ negatively modulates maintenance of multipotential NOD-SCID-repopulating HSCs under self-renewing conditions

The NOD-SCID xenograft assay is thought to detect more primitive human hematopoietic cells than the LTC-IC assay (40, 41, 46). In addition, the NOD-SCID assay allows evaluation of in vivo multilineage-repopulating ability of candidate human HSCs. In a total of three experiments using CD34⁺ cells and one using highly purified CD34⁺CD38⁻ cells, SFT3 cultures maintained high levels of multilineage NOD-SCID reconstitution activity that was almost completely abolished in the presence of IFN-γ (Table I and Fig. 4). This was reflected in the mean human reconstitution being reduced from 11 to 0.3%. Furthermore, whereas 100% of mice transplanted with SFT3-cultured cells were multilineage reconstituted, only 18% of mice transplanted with cells exposed to IFN-γ were positive for human myeloid reconstitution (Table I).

Table I. IFN-γ potently inhibits self-renewal of in vivo multilineage NOD-SCID-reconstituting cells

<table>
<thead>
<tr>
<th>Cells Transplanted</th>
<th>EE³</th>
<th>−IFN-γ</th>
<th>+IFN-γ⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1 CD34⁺</td>
<td>100,000</td>
<td>23 (18-12-5-58-20)</td>
<td>5/5</td>
</tr>
<tr>
<td>Expt. 2 CD34⁺</td>
<td>50,000</td>
<td>14 (3-1-1-1-1-1-1-1-1)</td>
<td>7/7</td>
</tr>
<tr>
<td>Expt. 3 CD34⁺</td>
<td>50,000</td>
<td>14 (3-1-1-1-1-1-1-1-1)</td>
<td>7/7</td>
</tr>
<tr>
<td>Expt. 4 CD34⁺CD38⁻</td>
<td>5,000</td>
<td>12 (14-2-26-11-1)</td>
<td>5/5</td>
</tr>
<tr>
<td>Mean</td>
<td>11</td>
<td>Total 21/21</td>
<td>Mean 0.3</td>
</tr>
</tbody>
</table>

¹ IFN-γ used at 1,000 U/ml.
² CD34⁺ or CD34⁺CD38⁻ CB cells were, as indicated, cultured for 7 days in SF medium with SFT3 before transplantation of the expansion equivalent (EE) of the indicated number of starting cells.
³ Percentage of human engraftment (representing total human reconstitution independent of whether both myeloid and B cell reconstitution were observed) is presented as mean values (individual mice in parentheses).
⁴ Frequency of mice with multilineage (B cell and myeloid) human reconstitution.

*p < 0.05, comparing −IFN-γ and +IFN-γ.
IFN-γ promotes differentiation of human hematopoietic progenitor/stem cells under self-renewing conditions

To investigate potential mechanisms by which IFN-γ might negatively affect maintenance of HSCs, we examined whether IFN-γ might promote differentiation at the expense of self-renewal. Following 12 days of culture under self-renewing conditions (SFT3), 18% of cells continued to express the stem/progenitor cell Ag CD34, which was down-regulated upon myeloid differentiation. In contrast, only 2% of cells cultured under the same conditions, but in the presence of IFN-γ, remained CD34+ (Table II), compatible with IFN-γ promoting differentiation rather than self-renewal of CD34+ stem/progenitor cells. To further investigate this, CD34+ cells were also cultured under conditions more efficiently promoting lineage differentiation (SFT3 + IL-6 + G-CSF + GM-CSF). Under these conditions, the maintenance of cells with a stem/progenitor CD34+ as well as lineage-negative phenotype was reduced significantly in the presence of IFN-γ (Table II). Specifically, whereas 40% of CD34+ cells cultured in the absence of IFN-γ remained negative, as much as 92% of cells cultured in the presence of IFN-γ became positive for myeloid lineage Ags. In further support of IFN-γ promoting myeloid differentiation and in line with the reduced CD34 expression, the number of CFU-C was reduced almost 3-fold in IFN-γ-containing cultures (Table II).

IFN-γ did not seem to significantly affect apoptosis of cultured progenitors because a combined annexin V and 7-AAD staining demonstrated comparable numbers of apoptotic cells in control and IFN-γ-supplemented cultures (Fig. 5). Cellular maintenance also appeared unaffected by IFN-γ, as CD34+ CD38- cells cultured in the absence and presence of IFN-γ showed indistinguishable forward scatter profiles (L. Yang and S. E. W. Jacobsen, unpublished observations). Furthermore, IFN-γ did not affect the fraction of cells in G1/S of CD34+ progenitors cultured in SFT3 (Fig. 6). Thus, IFN-γ might, at least in part, negatively affect the maintenance of primitive hematopoietic progenitor/stem cells by promoting their differentiation rather than self-renewal.

Discussion

Clinical and experimental studies have strongly implicated IFN-γ as a key mediator of BM failure in a number of diseases associated with inflammation, such as graft-vs-host disease, myelodysplastic syndromes, and aplastic anemia (10, 11, 21, 47), and recent studies suggest that the ability of drugs, such as cyclosporine, to inhibit IFN-γ production might explain their beneficial effects on hematopoiesis in patients with immune-mediated BM suppression (47). Furthermore, IFN-γ produced by human stromal microenvironment has been demonstrated to negatively affect hematopoiesis (16, 47). In agreement with this, IFN-γ inhibits the in vitro growth of hematopoietic progenitors, including primitive human CD34+ CD38- BM cells (15). However, whether or not such growth inhibition is associated

Table II. IFN-γ promotes differentiation of in vitro expanded CD34+ CB cells

<table>
<thead>
<tr>
<th>Cytokinesa</th>
<th>Ag</th>
<th>IFN-γ</th>
<th>+IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFT3</td>
<td>CD34</td>
<td>18%</td>
<td>2%</td>
</tr>
<tr>
<td>SFT3 + IL-6 + GM-CSF</td>
<td>CD34</td>
<td>3%</td>
<td>1%</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Lin</td>
<td>60%</td>
<td>92%</td>
</tr>
<tr>
<td>CFU-C</td>
<td></td>
<td>48 (2)</td>
<td>18 (1.5)*</td>
</tr>
</tbody>
</table>

a CD34+ cells were cultured either in SF medium supplemented with SFT3 for 12 days or in FCS-containing medium supplemented with SFT3 + IL-6 + GM-CSF + G-CSF for 9 days.

b Cultured cells were stained with anti-CD34 and anti-lineage Abs against CD11b, CD14, CD15, CD33, CD41, CD66b, and glycophorin A and analyzed by FACS for potential down-regulation of CD34 expression and up-regulation of lineage Ags as an indication of differentiation. Data are means from four experiments.

c CFU-C derived from 2000 CD34+ cells cultured in FCS containing medium supplemented with SFT3 + IL-6 + GM-CSF + G-CSF for 9 days. CFU-C were cultured in SCF + FL + G-CSF + GM-CSF + IL-3 + EPO and scored after 12 days. Data are from three individual experiments. Mean (SEM), p < 0.05, comparing −IFN-γ and +IFN-γ.
with sustained, enhanced, or reduced HSC function had not been investigated before these studies.

Using recently developed conditions promoting in vitro self-renewal of candidate human HSCs (32), we found that IFN-γ negatively affects in vitro maintenance of cycling human CB HSCs capable of multilineage reconstitution in vivo. Importantly, IFN-γ did not affect cytokine-induced recruitment of CD34<sup>+</sup>CD38<sup>−</sup> HSC into proliferation, but rather reduced the number of subsequent cell divisions, as reflected in reduced clonal expansion. However, the relatively limited reduction in cellular proliferation was accompanied by a striking loss in HSC function as well as enhanced differentiation, suggesting that IFN-γ might negatively affect HSC maintenance, by promoting HSC commitment and differentiation rather than self-renewal.

Whereas in recent studies demonstrated a comparable negative effect of TNF on CB and adult BM candidate HSCs (23), we in this study only investigated the effect on CB stem and progenitor cells. Thus, although we would postulate that IFN-γ would have similar suppressive effects on BM HSCs, as in this study demonstrated for CB stem and progenitor cells, this remains to be established.

Importantly, the conclusion that IFN-γ potently suppresses HSC self-maintenance in vitro was supported by evaluation of two key properties of human HSCs, the ability to maintain long-term cultures in vitro (42) and their in vivo multilineage-repopulating activity (41, 46). Although our data support that the ability of IFN-γ to reduce the reconstituting potential of self-renewing HSCs is due to enhanced differentiation, it remains possible that IFN-γ might also affect HSC potential through effects on HSC adhesion/engraftment, although IFN-γ was only present in the culture supporting self-renewing divisions and not in the LTC-IC assays.

In these studies, we found no indication that the suppressive role of IFN-γ involves effects on cell cycle or apoptosis. However, these conclusions are significantly limited by the fact that functionally defined HSCs represent a minority of the investigated CD34<sup>+</sup> as well as CD34<sup>+</sup>CD38<sup>−</sup> populations. Thus, we cannot exclude HSC-specific effects of IFN-γ on cell cycle and apoptosis, as such effects might have been obscured by the presence of large numbers of more committed progenitors.

Although the present results demonstrated that IFN-γ at high concentrations negatively affects HSC maintenance, it remains uncertain to what degree these concentrations of IFN-γ are relevant for acute and chronic inflammatory BM-suppressive syndromes, in particular because the local concentrations of IFN-γ in the BM have not been established.

In conclusion, the present studies provide the first evidence that the suppressive effects of IFN-γ in BM failure syndromes might involve direct targeting and suppression of repopulating HSCs.

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