Leukemia Inhibitory Factor Upregulates Cytokine Expression by a Murine Stromal Cell Line Enabling the Maintenance of Highly Enriched Competitive Repopulating Stem Cells


Attempts to maintain or expand primitive hematopoietic stem cells in vitro without the concomitant loss of their differentiative and proliferative potential in vivo have largely been unsuccessful. To investigate this problem, we compared the ability of three cloned bone marrow (BM) stromal cell lines to support the growth of primitive Thy-1.Sca-1.H-2K<sup>+</sup> cells isolated by fluorescence-activated cell sorting from the BM of Ly-5.2 mice treated 1 day previously with 5-fluorouracil. Sorted cells were highly enriched in cobblestone area-forming cells (CAFC), but their frequency was dependent on the stromal cell lines used in this assay (1 per 45 cells on SyS-1; 1 per 97 cells on PA6). In the presence of recombinant leukemia inhibitory factor (LIF), CAFC cloning efficiency was increased to 1 per 8 cells on SyS-1 and 1 per 11 cells on PA6, thus showing the high clonogenicity of this primitive stem cell population. More primitive stem cells with competitive repopulating potential were measured by injecting the sorted cells into lethally irradiated Ly-5.1 mice together with 10<sup>5</sup> radioprotective Ly-5.1 BM cells whose long-term repopulating ability has been "compromised" by two previous cycles of marrow transplantation and regeneration. Donor-derived lymphocytes and granulocytes were detected in 66% of animals injected with 50 sorted cells.

To determine the maintenance of competitive repopulating units (CRU) by stromal cells, sorted cells were transplanted at limiting dilution and after being cultured for 2 weeks on adherent layers of SyS-1, PA6, or SI7 cells. CRU represented 1 per 55 freshly sorted cells. CRU could be recovered from cocultures supported by all three stromal cell lines, but their numbers were ~sevenfold less than on day 0. In contrast, the addition of LIF to stromal cultures improved CRU survival by 2.5-fold on SI7 and PA6 cells (~two-fold to threefold decline), and enabled their maintenance on SyS-1. LIF appeared to act indirectly, because alone it did not support the proliferation of Thy-1.Sca-1.H-2K<sup>+</sup> cells in stroma-free cultures. Polymerase chain reaction (RT-PCR) analysis revealed that interleukin-1B (IL-1B), IL-2, IL-6, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, transforming growth factor-β, LIF, and Steel Factor (SLF) mRNAs were upregulated in SyS-1 within 1 to 6 hours of LIF-stimulation. To determine if increased expression of SLF by LIF-stimulated SyS-1 cells could account for their capacity to support stem cells, sorted cells were cocultured on simian CV-E cells that were transfected with an expression vector encoding membrane-bound SLF, or supplemented with soluble SLF. In both cases, SLF synergized with IL-6 produced endogenously by CV-E cells enabling CAFC growth equivalent to that on LIF-stimulated SyS-1. CAFC development on LIF-stimulated SyS-1 could also be completely abrogated by an anti-SLF antibody. These data provide evidence for a role of LIF in the support of long-term repopulating stem cells by indirectly promoting cytokine expression by BM stroma. Furthermore, we have used quantitative assays to show a maintenance of CRU numbers, with retention of in vivo function following ex vivo culture.

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MATURE BLOOD cells have a finite lifespan and must be replenished throughout adult life by the continued activity of hematopoietic stem cells (HSCs) that are operationally defined by their capacity to repopulate lymphoid and myeloid lineages upon transplantation into lethally irradiated or genetically deficient hosts. The majority of totipotent HSCs are normally quiescent, but are periodically recruited into active cell cycle in response to intrinsic factors that induce proliferation, or hematologic stress. When HSCs divide, they may generate large numbers of committed progenitors with a progressively restricted differentiation potential. Alternatively, HSCs may after only one or a few divisions return to the Go-phase of the cell cycle and retain the functional capacity to generate a clone of cells at a later time. The latter process is known as self-renewal.

The mechanisms that regulate HSC self-renewal versus their irreversible differentiation are poorly understood. Elucidation of these processes may facilitate the maintenance or expansion of totipotent stem cells in vitro, and would have immediate practical utility in a variety of clinical applications including bone marrow transplantation (BMT) and gene therapy. In vivo, these processes occur in close proximity to the BM microenvironment, which presumably provides the appropriate cell to cell interactions and/or cytokines. Long-term BM cultures (LTBMCs) closely mimic this environment, and have enabled HSC self-renewal, proliferation, and differentiation to occur in vitro.

However, the complexity of both the hematopoietic cells and stroma in LTBMCs presents difficulties for the identification of cytokines that may promote HSC self-renewal or proliferation in this setting. To date most studies of hematopoiesis in stroma-dependent cultures have relied on assays for spleen colony-forming units (CFU-S) or in vitro colony-forming cells to quantitate HSCs. Consistent with the functional differences between these relatively mature clonogenic cell types and more primitive HSCs with long-term repopulating potential, it was shown that the latter are poorly maintained in LTBMCs despite extensive production of CFU-S and more differentiated cell types. Therefore, analysis of the factors regulating the development of the most primitive HSCs requires quantitative assays that measure appropriate end-points. It was previously shown that some stem cells with competitive long-term repopulating ability (CRU) undergo self-renewal in LTBMCs. However,
because the adherent layer in such cultures is composed of a complex mixture of mesenchymal cell types, they are not readily amenable to the study of the mechanisms controlling CRU proliferation in vitro. Clonal stromal cell lines with the capacity to support early hematopoiesis have greatly simplified this approach. For example, day 12 CFU-S are amplified 16-fold when cultured for 6 days on the murine preadipose cell line, MC3T3-G2/PA6 (PA6).23 Murine BM cells cultured for 3 weeks on S17 stroma compete efficiently against fresh marrow for the repopulation of B, T, and myeloid lineages in lethally irradiated mice, although there is no evidence of an increase in repopulating stem cell numbers.24 Some murine stromal lines also support the maintenance and differentiation of primitive human hematopoietic cells. For example, MS-5 stromal cells promote the expansion of human CD34+CD38- BM cells with high proliferative potential, although they do not support their differentiation into more committed progenic progenitors or mature cells.25 Similar results were obtained with human CD34+HLA-DR+ cells cultured on murine M2-10B4 stroma.26 Maintenance of primitive long-term culture initiating cells (LTCICs) was further improved when M2-10B4 cells were engineered to express human granulocyte colony-stimulating factor (G-CSF) and interleukin-3 (IL-3).26 Murine SyS-1 stromal cells support the proliferation and lymphomyeloid differentiation of primitive human CD34-Thy-1-LIN- cells isolated from fetal BM and mobilized peripheral blood (PB).24 Taken together, these findings suggest that the culture of highly enriched HSC populations on cloned stromal cell lines may represent a more simple system for the study of early hematopoiesis in vitro.

The present study was designed to assess the capacity of different cloned BM stromal cell lines to support the most primitive murine stem cells with competitive long-term repopulating ability. Lymphoblasts expressing low levels of Thy-1.1,27-29 and high levels of Sca-128,29-33 and H-2K anti-
gens34 were enriched from the BM of mice treated 1 day previously with 5-FU by fluorescence-activated cell sorting (FACS). The frequency of CRU in the freshly sorted population, and among the cells generated after the sorted cells were cocultured for 2 weeks on stroma, was quantitated by limiting dilution analysis in vivo using a competitive repopulation assay. We show that CRU numbers decline sharply when cultured on monolayers of SyS-1, S17, or PA6 cells. However, the addition of leukemia inhibitory factor (LIF) to SyS-1, but not S17 or PA6 cocultures, facilitates a maintenance of input CRU numbers by induction of early-
acting cytokines including IL-6 and Steel Factor (SLF). This capacity of LIF-stimulated SyS-1 stroma to support early HSC development was recapitulated in CV-E cells that had been engineered to express membrane-bound SLF. These cells with low side light-scatter and intermediate/high forward light-scatter, and expressing low levels of Thy-1.1, and high levels of Sca-1 and H-2K uppermost 3% positive cells) antigens (Thy-1Sca-1H-2K) (see Fig 1) were sorted and counted with a hemacy-
tometer to verify the electronic count of the FACS. When these counts were within 10% of each other, the electronic sort count was used to calculate subsequent dilutions. Reanalysis of the sorted populations indicated a reproducible purity of >90% (Fig 1).

Cobblestone area-forming cell (CAFC) assays. CAFC development was measured on several cloned murine BM stromal cell lines. SyS-1, a subclone of AC6,35 and S1736 stromal cells were maintained in RPMI 1640 medium containing 5% FCS, 50 U/mL penicillin G, 50 μg/mL streptomycin, 2 mM L-glutamine and 0.05 mM/ L-2-mercaptoethanol (S1 medium). PA6 stromal cells37 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS, 50 U/mL penicillin G, 50 μg/mL streptomycin, 2 mM L-glutamine and 0.05 mM/ L-2-mercaptoethanol. An automated cell deposition unit (Becton Dickinson Immunocytochemistry Systems) was used to sort Thy-1Sca-1H-2K FUBM cells (3, 10, or 30 per well; 24 to 192 wells per concentration) into 96-well flat-bottom tissue culture plates seeded 1 day previously with 5,000 unirradiated stromal cells per well in 200 μL S1 medium. In some experiments, recombinant murine IL-3, IL-6, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), SLF (R&D Systems, Minneapolis, MN), human G-CSF (Amgen Inc, Thousand Oaks, CA) or human LIF were added at 10 ng/mL (SLF at 100 ng/mL). A polyclonal antimurine SLF blocking antibody or control goat immunoglobulin (R&D Systems)...
twice serially transplanted Ly-5.1 "compromised" BM cells. The presence of CRU in stroma-dependent cultures of sorted FUBM was determined by transplanting mice with 0, 1/8, 1/20, or 1/40 of a total culture, together with compromised Ly-5.1 BM cells as previously described. Reconstitution by donor stem cells was assessed for each recipient 10 weeks after transplantation by analysis of PB stained with anti-Ly-5.2 and lineage MoAbs as previously described. The number of CRU in the transplanted cell population was determined by plotting the proportion of donor negative (≥95% Ly-5.1+, or recipient mice not exhibiting trilineage engraftment) reconstituted recipients in each group against the number of sorted cells or the culture fraction injected. A line of best fit was generated using the maximum-likelihood method, and the frequency of CRU was then calculated by interpolation of the number of test cells or the culture fraction required to obtain a 37% negative response.

Cytokine expression analysis of stromal cells. Total RNA was prepared from unstimulated and LIF-stimulated (100 ng/mL LIF for 1 to 6 hours) SyS-1 cells using a guanidine isothiocyanate/phenol procedure (Tel-Test B Inc, Friendswood, TX). Polyadenylated RNA was prepared, and first strand cDNA synthesized from an oligo
d(T) primer (Superscript System, Gibco-BRL, Gaithersburg, MD). Cytokine-specific cDNAs were amplified from 100 ng of starting material by 35 cycles of the reverse transcriptase polymerase chain reaction (RT-PCR; 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes: Perkin Elmer [Foster City, CA] reagents and 9,600 Thermocycler) using pairs of oligonucleotide primers specific for each cDNA as follows:

IL-2: 5'-TGATGAGCACTGACAGCTGCAG and 5'-AGCTACACTCCAAATGTCAGGCAG; IL-4: 5'-CGAAGAACACACCCTTCTGCTGA and 5'-CCGTTCAAACTGAGGTCGACAG; IL-5: 5'-CGATGGAGCTCCCTTTAAGTAACTTG and 5'-GCCATTCCAGATCAGTGACGATT; IL-6: 5'-GAATGATTTCGAGGAGGCACTCATCC and 5'-CCCTTCTTCAGGCTGAATCG; IL-10: 5'-ATCCCTTTATCTGAGCACGTCCT and 5'-ATCCCTTTATCTGAGCACGTCCT; IL-12: 5'-GACCCGCGCATGAGCTGCTG and 5'-GACCTTGAGGAGGCACTCATCC; IFN-γ: 5'-AGGCAGGGCCCTGCCGAGGCTG and 5'-GACCTTGAGGAGGCACTCATCC; TNF-α: 5'-GACTCATTCATGGTCAGACAGG and 5'-GACTCATTCATGGTCAGACAGG; GM-CSF: 5'-AGAAGAACAAGACTCGAGATGAG and 5'-GAGAAGAACAAGACTCGAGATGAG; IL-4: 5'-GACTCATTCATGGTCAGACAGG and 5'-GACTCATTCATGGTCAGACAGG; IL-3: 5'-GACTCATTCATGGTCAGACAGG and 5'-GACTCATTCATGGTCAGACAGG; G-CSF: 5'-GACTCATTCATGGTCAGACAGG and 5'-GACTCATTCATGGTCAGACAGG; M-CSF: 5'-GACTCATTCATGGTCAGACAGG and 5'-GACTCATTCATGGTCAGACAGG; TGF-β: 5'-GACTCATTCATGGTCAGACAGG and 5'-GACTCATTCATGGTCAGACAGG; and IGF-1: 5'-GACTCATTCATGGTCAGACAGG and 5'-GACTCATTCATGGTCAGACAGG.

Quantitative analysis of IL-1β mRNA expression was performed by competitive reverse transcription-PCR (RT-PCR) as described previously. 

Expression of membrane-bound SLF in CV-E cells. A cDNA clone encoding murine membrane-bound SLF (mSLF; provided by J. Fu, SysteMinx Inc) was modified by PCR to enable cloning into the EcoRI and Xhol sites of pHOLIN-1. pHOLIN-1 is a mammalian expression vector comprising the SRα promoter, SV40 polyadenylation sequences, Epstein Barr virus (EBV) origin of replication ( OriP) and bacterial origin and ampicillin genes from pCPE4 (Invitrogen Corp, San Diego, CA). pHOLIN-1 and pHOLIN-1/mSLF were used at 1 to 100 µg/mL. Cultures were incubated at 37°C in 5% CO₂ in air, and fed weekly (except those containing antibodies which were fed daily) by replacement of one half of the medium. The growth of highly refractile round blast cells and optically-dense cobblestone areas was monitored by phase-contrast microscopy every 7 days. The frequency of CAFC in the starting population was determined by limiting dilution analysis of the final proportion of negative wells at each test cell concentration after 4 weeks.

Stroma-dependent liquid cultures. Four thousand Thy-1.1–Sca-1−1 H-2Kb FUBM cells were cultured on monolayers of 10⁵ unirradiated stromal cells in 1.0 mL S1 medium and fed as required. After 2 weeks, the nonadherent cells and trypsinized adherent layer were pooled, washed, and resuspended to 1.0 mL in DMEM containing 2% FCS. The cell concentration was defined as 1.0 culture/mL, and subsequent dilutions and limiting dilution analyses calculated on a per culture basis.

Quantitation of CRU. Competitive reconstitution assays were performed as previously described with some modifications. Ly-5.1 recipients were exposed to 10.5¹³ CsI administered in two equal doses, 3 hours apart, from a Nordion Gamma Cell 40 machine (J.L. Shepard & Associates, San Fernando, CA) at a dose rate of 0.81 Gy per minute. Irradiated animals were injected IV with 0 (PBS only), 25, 50, 100, or 250 sorted Thy-1.1–Sca-1−1 H-2Kb FUBM cells (Ly-5.2+) per mouse, together with 10⁵ I¹³ Sca-1−1 H-2Kb FUBM cells in each group against the number of sorted cells or the culture fraction injected. A line of best fit was generated using the maximum-likelihood method, and the frequency of CRU was then calculated by interpolation of the number of test cells or the culture fraction required to obtain a 37% negative response.
were introduced into monolayers of CV-E cells (a derivative of simian CV-1 cells that was derived by transfection with pEBNA [Invitrogen Corp]) by DEAE-Dextran mediated transfection.\(^\text{59}\) Transfected cells were procured after 24 hours using trypsin-EDTA, and plated at 10^4 per well in 96-well plates in 100 µL S1 medium. FACS purified murine stem cells were added 24 to 48 hours later.

### RESULTS

Cobblestone area-formation by sorted Thy-1^+Sca-1^+H-2K\(^d\) cells. The potential to generate "cobblestone" areas of hematopoietic cells on BM stromal layers or cloned adherent cell lines has been suggested as an in vitro assay for primitive hematopoietic precursors.\(^\text{43}\) We measured the frequency of CAFC in Thy-1^+Sca-1^+H-2K\(^d\) cells isolated from day 1 FUBM by culturing them on SyS-1 or PA6 stromal cells that have previously been shown to support the growth of primitive murine and/or human HSCs. The sorted population contains precursors able to generate large hematopoietic foci on both stromal cell types. A few colonies (15%) were visible by day 7 of culture, but most were not visible until between days 7 and 14 (62%), or days 14 and 28 (23%). No significant differences in the kinetics of CAFC development, or in the morphology of the cells generated, was observed on Sys-1 or PA6 stroma. Limiting dilution experiments were performed to determine the frequency of CAFC in the sorted population. On Sy-S1 stroma, 22 of 272 (8%), 33 of 176 (19%) and 31 of 68 (46%) wells seeded with 3, 10, or 30 Thy-1^+Sca-1^+H-2K\(^d\) FUBM cells exhibited hematopoietic foci after 4 weeks. The CAFC frequency derived from this data is 1 per 45 sorted cells (Table 1). On PA6 stroma, 5 of 183 (3%), 20 of 184 (11%) and 11 of 46 (24%) wells seeded with 3, 10, or 30 sorted cells were positive, corresponding to a CAFC frequency of 1 per 97 sorted cells.

Table 1. CAFC Development by Thy-1^+Sca-1^+H-2K\(^d\) FUBM Cells

<table>
<thead>
<tr>
<th>Stromal Cells</th>
<th>Cytokines Added</th>
<th>1/CAFC Frequency (95% Confidence Limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sys-S1</td>
<td>none</td>
<td>45 (37-58)</td>
</tr>
<tr>
<td>Sys-S1</td>
<td>IL-3</td>
<td>17 (15-21)</td>
</tr>
<tr>
<td>Sys-S1</td>
<td>SLF</td>
<td>14 (12-16)</td>
</tr>
<tr>
<td>Sys-S1</td>
<td>LIF</td>
<td>8 (7-9)</td>
</tr>
<tr>
<td>PA6</td>
<td>none</td>
<td>97 (73-144)</td>
</tr>
<tr>
<td>PA6</td>
<td>IL-3</td>
<td>34 (28-43)</td>
</tr>
<tr>
<td>PA6</td>
<td>SLF</td>
<td>13 (11-15)</td>
</tr>
<tr>
<td>PA6</td>
<td>LIF</td>
<td>11 (10-13)</td>
</tr>
</tbody>
</table>

Three, 10, or 30 Thy-1^+Sca-1^+H-2K\(^d\) FUBM cells per well were cultured on stromal cells in 96-well plates. IL-3 or LIF were used at 10 ng/mL; SLF was used at 100 ng/mL. Wells containing CAFC were counted after 4 weeks, and CAFC frequencies determined by limiting dilution statistics. Pooled data from 2 or 3 experiments per condition.

In an attempt to increase the cloning efficiency of CAFC in the sorted population, the experiments were repeated in identical conditions. Addition of SLF to CAFC assays resulted in equivalent growth (1 per 13 or 14 sorted cells) on both Sys-S1 and PA6 cells. However, the most striking effect on CAFC was observed when LIF was added to the cultures (Table 1). One in 8 and 1 in 11 Thy-1^+Sca-1^+H-2K\(^d\) FUBM cells proliferated on LIF-stimulated Sys-S1 and PA6 cells, respectively, thus showing the high clonogenicity of this enriched cell population. The sorted cells did not proliferate in stroma-free cultures containing IL-3, SLF, or LIF alone (data not shown), suggesting that these cytokines act either by synergizing with factors that are constitutively secreted by Sys-S1, or by upregulating their expression of growth factors that are not normally produced, or produced at very low levels by the stroma.

Loss of competitive long-term repopulating stem cells in stromal cultures of Thy-1^+Sca-1^+H-2K\(^d\) FUBM. To determine whether more primitive stem cells with in vivo repopulating potential were maintained in cocultures supported by the cloned stromal cell lines, quantitative repopulation assays were used to compare the number of competitive repopulating units inoculated into the cultures with that recovered 2 weeks later. The frequency of CRU in the freshly sorted Thy-1^+Sca-1^+H-2K\(^d\) FUBM population was determined by injecting groups of lethally irradiated Ly-5.1 mice with limiting numbers of sorted Ly-5.2^+ cells (0 to 250 per mouse) together with 10^4 "compromised" Ly-5.1 BM cells. The latter population contains normal numbers of progenitors with short-term proliferative potential and is radioprotective.\(^\text{36}\) However, CRU have been depleted by two previous cycles of serial BMT and regeneration to a frequency of approximately 1 per 250,000 cells.\(^\text{17,38,44}\) A total of 137 mice were transplanted in 5 experiments and 118 survived at least 10 weeks. Progeny of sorted Thy-1^+Sca-1^+H-2K\(^d\) stem cells (Ly-5.2^+) were detected in 85 of the survivors at this time. The degree of donor engraftment was directly proportional to the number of sorted FUBM cells injected (Table 2). Even in mice transplanted with as few as 25 Thy-1^+Sca-1^+H-2K\(^d\) FUBM cells, donor cells usually contributed to both B and T lymphocytes and granulocytes at a proportion similar to that in normal PB (Table 2). The frequency of CRU in the sorted population was determined by plotting the proportion of negative recipients in each group (<5% trilineage donor lineage).
cells) against the number of sorted cells injected, and analyzing the resulting graph by limiting dilution statistics (Fig 2). We used 10 weeks as an end-point for this analysis because it was previously shown that the results are not significantly different when recipients are assessed between 10 weeks and 1 year after transplant. The average frequency of CRU in the sorted population, calculated from the 5 experiments previously described, was 1.8% (ie, 1 per 55 cells; 95% confidence limits 1 per 43 to 1 per 74 cells). This value is comparable to that obtained using a similar protocol to enrich stem cells from the BM of C57BL/6 mice.

Four thousand Thy-1+ Sca-1+ H-2Khi FUBM cells (containing a mean of 73 CRU) were also cultured for 2 weeks on SyS-1, PA6, or S17 stromal cells without added cytokines, and then injected at limiting dilution into lethally irradiated Ly-5.1 mice together with "compromised" Ly-5.1 cocultures, respectively. Twenty-nine of 36, and 29 of 36 mice injected with 1/8, 1/20, or 1/40 of a SyS-1 coculture, respectively. Ten weeks after transplantation, BM cells as above. Thirty-three of the 36 mice injected with kines, and then injected at limiting dilution into lethally irradiated Ly-5.1 recipients together with 10⁶ "compromised" Ly-5.1 BM cells. Shown are the proportion of mice containing ≥5% donor-derived (Ly-5.2) lymphocytes and granulocytes in PB 10 weeks after transplantation (pooled data from 2 or 3 independent experiments). Control mice (4 to 5 per condition) injected with 0 cultures were all negative. The total number of CRU present in the cultures at the time of harvest was determined by statistical analysis of the proportion of positive mice in each dose group. Parentheses indicate 95% confidence limits.

Fig 2. Limiting dilution analysis of the frequency of CRU in Thy-1+Sca-1+H-2Khi FUBM. Zero (PBS only), 25, 50, 100, or 250 Ly-5.2+ sorted cells were injected into groups of 4 to 8 lethally irradiated Ly-5.1 mice together with 10⁶ "compromised" Ly-5.1 BM cells. Recipients with ≥5% donor cells contributing to lymphoid and myeloid lineages were scored as positive. A line of best fit was generated by analysis of the proportion of negative mice at each "test" cell dose using maximum likelihood statistics. Results are derived from pooled data from 5 independent experiments in which 118 mice (16 to 36 mice per dose of sorted cells) were analyzed 10 weeks after competitive repopulation.

Table 3. CRU Maintenance in Stromal-Dependent Cultures of Thy-1+Sca-1+H-2Khi FUBM Cells

<table>
<thead>
<tr>
<th>Culture Fraction per Mouse</th>
<th>Culture Fraction per Mouse</th>
<th>Proportion of Mice Repopulated by Sorted CRU</th>
<th>Proportion of Mice Repopulated by Sorted CRU</th>
<th>Proportion of Mice Repopulated by Sorted CRU</th>
<th>Proportion of Mice Repopulated by Sorted CRU</th>
</tr>
</thead>
<tbody>
<tr>
<td>SyS-1</td>
<td>SyS-1 + LIF</td>
<td>PA6</td>
<td>PA6</td>
<td>PA6 + LIF</td>
<td>PA6 + LIF</td>
</tr>
<tr>
<td>1/8</td>
<td>5/8</td>
<td>12/12</td>
<td>3/6</td>
<td>4/4</td>
<td>5/8</td>
</tr>
<tr>
<td>1/20</td>
<td>3/10</td>
<td>13/13</td>
<td>6/11</td>
<td>2/6</td>
<td>4/6</td>
</tr>
<tr>
<td>1/40</td>
<td>6/15</td>
<td>17/19</td>
<td>1/4</td>
<td>6/8</td>
<td>14/7</td>
</tr>
<tr>
<td>CRU per culture:</td>
<td></td>
<td>(18-51)</td>
<td>(4-16)</td>
<td>(45-145)</td>
<td>(4-16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(44-190)</td>
<td>(4-16)</td>
<td>(9-39)</td>
<td>(6-22)</td>
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<td></td>
<td></td>
<td>(13-58)</td>
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</table>

Four thousand Thy-1+Sca-1+H-2Khi FUBM cells (containing 73 CRU) were cultured on stromal cells with or without 10 ng/mL rhuLIF. After 2 weeks, cultures were injected at limiting dilution (0, 1/8, 1/20 or 1/40 culture/mouse) into lethally irradiated Ly-5.1 mice together with 10⁶ "compromised" Ly-5.1 BM cells. Shown are the proportion of mice containing ≥5% donor-derived (Ly-5.2) lymphocytes and granulocytes in PB 10 weeks after transplantation (pooled data from 2 or 3 independent experiments). Control mice (4 to 5 per condition) injected with 0 cultures were all negative. The total number of CRU present in the cultures at the time of harvest was determined by statistical analysis of the proportion of positive mice in each dose group. Parentheses indicate 95% confidence limits.

The absolute number of CRU recovered from these cocultures was calculated by statistical analysis of the proportion of repopulated mice in each group injected with the different fractions of cultured cells. SyS-1 cocultures seeded with 73 CRU on day 0 contained 10 CRU at the time of harvest, representing a net 86% decline in repopulating stem cell numbers after 2 weeks (Table 3). CRU numbers declined 81% and 86% on S17 and PA6, respectively, to 14 and 10 per culture. These results are in agreement with published reports which indicate a failure to maintain starting numbers of repopulating stem cells on prolonged culture in vitro.

Maintenance of competitive long-term repopulating stem cells in stromal cultures containing LIF. Since the addition of LIF to CAFC assays of sorted Thy-1+Sca-1+H-2Khi FUBM cells increased their cloning efficiency in vitro, competitive repopulation experiments were performed to determine whether LIF also promoted the maintenance of more primitive CRU. Sorted FUBM cells (containing 73 CRU) were cultured for 2 weeks on stromal cells in medium containing 10 ng/mL rhuLIF, and cultures then procured and injected at limiting dilution as previously described. Forty-five of 54 mice that were competitively transplanted with various fractions of a LIF-stimulated SyS-1 coculture survived 5 weeks. Similar to CAFC, very high levels of CRU activity were detected among the cells injected into these mice. Twelve of 12, 13 of 13, and 17 of 19 animals injected with 1/8, 1/20, or 1/40 of an LIF-stimulated SyS-1 coculture.

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MAINTENANCE OF STEM CELLS IN STROMAL CULTURES

Fig 3. RT-PCR analysis of cytokine gene expression in unstimulated and LIF-stimulated SyS-1 cells. Poly A'RNA was isolated from SyS-1 cells before (−) or 1 to 6 hours after (+) culture in medium containing 100 ng/mL rhuLIF. Cytokine specific cDNAs were amplified by PCR. In each pair of lanes, the upper band is the cytokine designated at the top of the gel. The authenticity of each amplified band was confirmed by asymmetric restriction enzyme digestion of the cDNA from low-melting point agarose gels. M, molecular weight markers (kb).

exhibited a mean of 63 ± 7%, 59 ± 6%, and 40 ± 7% donor PB cells 10 weeks after transplantation. Statistical analysis of this data indicated that 95 (95% confidence limits; 45 to 145) CRU were present in the cocultures at the time of harvest (Table 3). Therefore, the addition of LIF to SyS-1-cocultures facilitated the maintenance of competitive repopulating stem cells over 2 weeks.

All 36 mice transplanted with cells from LIF-stimulated S17 or PA6 cocultures survived 5 weeks. However, lower levels of CRU activity were detected in these cultures than in the LIF-stimulated Sys-1 cultures previously described. Four of 4 mice, 4 of 6 mice, and 6 of 8 mice injected with 1/8, 1/20, and 1/40 of an S17 coculture, respectively, contained ≈5% donor-derived PB cells 10 weeks after transplantation. Four of 4 mice, 2 of 6 mice, and 6 of 8 mice injected with 1/8, 1/20 and 1/40 of a PA6 coculture, respectively, were positive at this time. The degree of donor engraftment was similar to that in mice injected with the same fraction of Sys-1 cocultured cells (not shown). Statistical analysis indicated that 36 (95% confidence limits; 13 to 58) and 24 (95% confidence limits; 9 to 39) CRU were recovered from the LIF-stimulated S17 and PA6 cocultures, respectively, representing a 51% and 67% decline over 2 weeks (Table 3). Nevertheless, LIF improved the survival of CRU in S17 or PA6 cocultures by ∼twofold.

Effect of LIF on growth factor gene expression by SyS-1 stromal cells. The inability of LIF to stimulate the proliferation of Thy-1+Sca-1+H-2Kk FUBM cells in stroma-free cultures suggested that it acts indirectly on stem cells by stimulating cytokine expression by the stroma. To address this question, RT-PCR analysis was performed using polyA’RNA extracted from SyS-1 cells before and 1 to 6 hours after exposure to 100 ng/mL rhuLIF. SyS-1 cells constitutively express mRNAs encoding M-CSF, IL-7, SLF, and flk-2/flt3 ligand (Fig 3). In data not shown, mRNAs encoding PDGF, transforming growth-factor-β (TGF-β), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) were also detected in SyS-1 cells. Unstimulated SyS-1 cells do not express mRNAs encoding IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, G-CSF, GM-CSF, tumor necrosis factor-α, LIF, or interferon-γ (Fig 3 and data not shown). On LIF-stimulation, mRNAs for IL-2, IL-6, G-CSF, GM-CSF, and LIF itself were upregulated (Fig 3). Messenger RNAs encoding IL-7, M-CSF, and flk-2 ligand (Fig 3), as well as PDGF, TGF-β, bFGF, and IGF-1 (data not shown) were not altered by LIF-stimulation. Though not apparent in the RT-PCR analysis of Fig 3, Northern blot analysis revealed that SLF mRNA was increased ∼threefold in SyS-1 cells 2 hours after treatment with LIF (data not shown). This suggests that SLF may be a component of LIF-induced maintenance of HSCs in this system.

Antibodies to SLF block CAFC development by Thy-1+Sca-1+H-2Kk cells. To examine the role of SLF in the increased proliferation of stem cells on LIF-stimulated Sys-1 stroma, Thy-1+Sca-1+H-2Kk FUBM cells were cultured on LIF-stimulated SyS-1 cells in the presence of 1 to 100 µg/mL anti-SLF antibody. After 7 days, the wells were washed and the proliferation of any surviving CAFC elicited by the addition of a potent growth factor cocktail containing IL-1, IL-3, IL-6, G-CSF, M-CSF, GM-CSF, LIF, and SLF. Figure 4A shows that CAFC survival was inhibited in direct proportion to the amount of anti-SLF present in the cultures, with complete blocking at 66 to 100 µg/mL. Interestingly, the kinetics of inhibition were characteristic of a biphasic antibody-cytokine interaction, and may reflect a differential efficiency in neutralizing the soluble versus membrane-bound forms of SLF. In separate experiments, LIF-stimulated SyS-1 cocultures containing 100 µg/mL anti-SLF were blocked for 3 to 17 days before antibody removal and restimulation (Fig 4B). All wells that were stimulated with cytokines (without antibody) from day 0 developed large cobblestone areas over 4 weeks of culture. CAFC development was also unaffected when 100 µg/mL of control Ig was added to the cultures for up to 17 days. In contrast, 75% of the wells that contained anti-SLF antibody for only 3 days never developed hematopoietic foci, even after antibody removal and restimulation with cytokines. After 7 days in anti-SLF antibody, essentially no CAFC remained viable.

SLF expression by CV-E cells is sufficient to support CAFC development. Experiments were performed to reconstitute the stem cell supportive capacity of LIF-stimulated Sys-1 stroma in simian CV-E cells that are able to drive the replication of plasmids containing the EBV origin of replication (OriP). Parental CV-E cells form contact-inhibited monolayers, and produce low levels of simian IL-6 sufficient to stimulate the proliferation of KD83 cells, but are nevertheless ineffective at stimulating CAFC development by sorted FUBM cells (Fig 5). However, when Thy-1+Sca-
1H-2K® FUBM cells were cultured on CV-E stroma with exogenous SLF, 66 ± 8% of the wells developed hematopoietic foci after 4 weeks (Figs 5 and 6C). Consistent with the antibody blocking experiments above, SLF was absolutely necessary for CAFC proliferation. Seven other cytokines tested singly (IL-1, IL-3, IL-6, G-CSF, M-CSF, GM-CSF, and LIF) were unable to support cobblestone area-formation on CV-E cells. Only IL-3 was able to synergize with SLF to stimulate the development of CAFC in all wells.

The stem cell supportive capacity of LIF-stimulated SyS-

1 was reconstituted in CV-E cells by transfection with pHO-
LIN-1 expressing a cDNA encoding membrane-bound SLF
(mSLF). Thy-1Sca-1H-2K® FUBM cells did not proliferate
on parental, mock-transfected or pHOLIN-1 vector
transfected CV-E cells (Fig 6B). In contrast, CV-E cells
transfected with the pHOLIN-1/mSLF construct (Fig 6D)
supported the development of hematopoietic foci similar to
those developing on LIF-stimulated SyS-1 cells (Fig 6A).
As with the combination of exogenous SLF and IL-3, co-
transfection of CV-E cells with plasmids encoding mSLF
and IL-3 resulted in a dramatic increase in the size of the
colonies generated by Thy-1Sca-1H-2K® FUBM cells (Fig
6E and F). Individual foci, presumably derived from a single
stem cell, grew to well over 10^5 blast cells but then rapidly
derdifferentiated into granulocytes and macrophages under
these conditions.

DISCUSSION
Analysis of the mechanisms controlling hematopoietic stem cell self-renewal and proliferation in vitro requires a
simple system where the many factors of interest can be
evaluated independently. In addition, appropriate assays
must be used for the direct quantitation of the most primitive
stem cells that are present. LTBMCS provide an opportunity
to study these problems, but are complicated by the
highly heterogeneous composition of both the supportive
stromal layer and the BM suspension containing stem cells.
In this report, we used quantitative competitive repopulation
assays to compare the ability of three different cloned BM
stromal cell lines to maintain highly purified stem cells with
lymphomyeloid repopulating potential. HSCs were enriched

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using a modification of a previously published procedure using day 4 FUBM as the starting material. However, because stem cells in such suspensions are in cell cycle and may be regulated differently from those that are normally quiescent in vivo, the time of exposure to 5-FU was reduced to 24 hours. This period is sufficient to kill both day 8 and day 14 CFU-S, thus retaining the advantage of 5-FU treatment in easily eliminating mature progenitors in vivo, but is too short to activate the majority of stem cells with competitive repopulating potential. CRU were isolated from day 1 FUBM by sorting lymphoblastoid cells expressing low levels of Thy-1.1 and high levels of Sca-1 and H-2K<sup>α</sup> antigens. Transplantation experiments showed that as few as 25 sorted cells could regenerate and maintain lymphocytes and granulocytes in 44% of lethally irradiated mice, despite the requirement to compete with a compromised but protective graft of 10<sup>7</sup> congenic BM cells. Limiting dilution analysis established that CRU represented at least 1 per 55 sorted cells. This frequency is ~threefold higher than that in a phenotypically identical population isolated from day 4 FUBM of C57BL/6 mice, and is consistent with the increased recovery of stem cells in the Thy-1<sup>+</sup> fraction of Thy-1.1 strains of mice.

In an attempt to identify conditions that would maintain or expand highly purified stem cells in vitro, they were cultured on stromal cell lines that support early hematopoiesis. SyS-1 is a subclone of AC6 stromal cells that were originally isolated from a Whitlock-Witte BALB/c BM culture and supports the proliferation and limited differentiation of human CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>−</sup> stem cells. PA6 is a cloned preadipocyte cell line able to expand day 12 CFU-S, and maintain more primitive stem cells able to reconstitute erythropoiesis for 24 weeks in WBB6F<sub>1</sub>-W/W<sup>+</sup> mice. S17 stromal cells are equivalent to primary stroma from Dexter cultures in supporting pluripotent stem cells with in vivo repopulating potential. When Thy-1<sup>+</sup>Sca-1<sup>+</sup>H-2K<sup>α</sup> cells were cocultured on SyS-1, PA6, or S17 stroma without added cytokines, CRU numbers declined to ~14% of input after 2
weeks. These results are consistent with published reports showing a net decline in stem cell activity in LTBMCS.\textsuperscript{21, 22} For example, Fraser et al\textsuperscript{18} used limiting dilution analysis to measure the number of CRU present in 4-week-old LTBMCS initiated with retrovirally-marked BM cells. Concurrent with the expansion of some totipotent cells, revealed by analysis of proviral integration sites in multiple recipients transplanted with cultured cells, there was a slow but net 6.5-fold decrease in total CRU numbers. In analogous LTBMCS initiated with highly purified human CD34\textsuperscript{+}HLA-DR\textsuperscript{-}\textsuperscript{80} BM cells, \textsim{}25% of input LTCICs were detected after 5 weeks.\textsuperscript{56} Collectively, this experience bodes unfavorably for the use of conventional unsupplemented LTBMCS, or those supported by the three stromal cell lines tested in our study, for the prolonged maintenance or expansion of stem cells with repopulation potential.

A correlation between the frequency and phenotype of stem cells with short-term marrow repopulating ability and late-appearing CAFC has previously been noted, prompting the suggestion that cobblestone area formation may be used as an in vitro assay for repopulating stem cells.\textsuperscript{41-43} We measured the frequency of CAFC in the Thy-1\textsuperscript{+}Sca-1\textsuperscript{-}H-2K\textsuperscript{b} population and found that it was \textsim{}twofold higher when assayed on SyS-1 versus PA6 stromal cells. This observation underscores the heterogeneity among different stromal cell types, and highlights the need to standardize such assays for comparing results from different laboratories. Significantly, the frequency of CAFC detected on SyS-1 (1 per 45 cells) was comparable to that of CRU (1 per 55 cells), further supporting the hypothesis that these are closely related stem cell types, and that the SyS-1 assay may provide a quantitative measure of HSCs with in vivo repopulating ability. However, it must be noted that an exact comparison of the frequency of stem cells detected by in vitro and in vivo assays is confounded by the unknown seeding efficiency of the latter. CAFC cloning efficiency was increased by adding growth factors that mediate early hematopoiesis, but are either not produced, or produced at low levels by SyS-1 cells (i.e., IL-3, LIF and SLF). LIF also promoted the maintenance of input CRU over 2 weeks in SyS-1 cocultures, and improved CRU survival by twofold (net fivefold to sevenfold decline) in PA6 and S17 cocultures. Although S17 cells have previously been shown to maintain stem cells with competitive repopulating potential,\textsuperscript{24} these investigators did not use highly purified stem cells in their studies, and could not exclude the possibility that the factors(s) affecting stem cell maintenance were produced by accessory BM cells that were efficiently supported by S17. The properties of SyS-1 that account for its superiority over S17 and PA6 in supporting hematopoiesis are unknown, but are likely a reflection of the presence of different mesenchymal cell types in unseparated BM, and their distinct role in regulating stem cell development in vivo. LIF-stimulated SyS-1 cells produce several positive (eg, SLF, flk-2/flt3 ligand, GM-CSF) and negative (eg, TGF-\textbeta) regulators of early hematopoiesis. Unfortunately, no clear correlation between the presence of a particular stromal cell type to support hematopoiesis in vitro and its cytokine expression profile or phenotype has yet been noted.\textsuperscript{48}

Several studies have shown a regulatory role for LIF in early hematopoiesis. In vitro, LIF has no direct effect on CFCS,\textsuperscript{49-50} but does augment the proliferation of multipotential progenitors in response to IL-3.\textsuperscript{51, 52} Consistent with these findings, Thy-1\textsuperscript{+}Sca-1\textsuperscript{-}H-2K\textsuperscript{b} FUBM cells did not proliferate in stroma-free cultures or on CV-E cells supplemented with LIF alone. LIF knockout mice have reduced numbers of myeloid progenitors in the spleen, but contain stem cells capable of reconstituting hematopoiesis upon transplantation into lethally irradiated wild-type hosts.\textsuperscript{53} These data suggest that LIF may act by exerting its effects on the hematopoietic microenvironment. Recently, mice were constructed with a disruption in the LIF receptor gene.\textsuperscript{54} Despite severe placent al, skeletal, neural, and metabolic defects that resulted in perinatal death, the frequency of day 14 CFU-S, and burst forming unit-erythroid in the fetal liver were relatively normal, indicating the existence of LIF-independent compensatory mechanisms for the maintenance of HSCs in vivo.

The present study defines several components of the mechanism of LIF-regulated hematopoiesis on SyS-1 stromal cells in vitro. Although our results do not clarify the precise combination of growth factors required for stem cell maintenance in these cultures, three lines of evidence suggest that synergy between IL-6 and SLF likely accounts for our observations. Firstly, mRNAs encoding several cytokines (eg, IL-2, IL-6, G-CSF, GM-CSF, SLF) are upregulated in SyS-1 cells following LIF treatment. The induction of SLF mRNA is weaker by comparison, although reproducibly threefold higher than in unstimulated SyS-1 cells. The self-induction of LIF might also contribute to the sustained effect observed over two weeks of culture. The combination of IL-6 and SLF was previously shown to stimulate the proliferation of CFU-C and day 12 CFU-S in Thy-1\textsuperscript{+}Sca-1\textsuperscript{-} BM,\textsuperscript{55} and supports a moderate increase in the number of stem cells with 30-day radioprotective capacity\textsuperscript{56} and long-term competitive repopulating ability.\textsuperscript{57} Previous studies in our laboratory indicated that Thy-1\textsuperscript{+}LIN Sca-1\textsuperscript{-} stem cells proliferate in medium conditioned by LIF-stimulated SyS-1 cells, and that this is partially inhibited (\textsim{}50%) by blocking antibodies to IL-6 (S. Pronovost, A. Buckle, unpublished observations). Secondly, myelopoiesis was blocked in LIF-stimulated SyS-1 cultures containing anti-SLF antibody, and did not recover even after antibody removal and restimulation with cytokines. This indicates that SLF is necessary for both CAFC survival and maturation, though it likely acts synergistically with other cytokines (such as IL-6) that are more strongly upregulated by LIF. Similar results were obtained when unfractionated BM cells were cultured on PA6 or S17 stroma with 10 \mu g/mL ACK-2 (anti-c-kit ligand) antibody,\textsuperscript{24, 47} although ACK-2 did not impair HSCs able to reconstitute lethally irradiated mice.\textsuperscript{58} Sorted CRU are also unaffected in LIF-stimulated SyS-1 cocultures containing 10 \mu g/mL anti-SLF (S.J. Szilvassy, unpublished observations). However, because we showed this concentration of antibody failed to completely block CAFC development, the lack of effect on CRU remains inconclusive. The final evidence suggesting that LIF indirectly promotes hematopoiesis by upregulating IL-6 and SLF expression by stroma was our ability to reconstitute the CAFC supportive capacity of LIF-stimulated SyS-1 cells by transfecting CV-E cells with an expression plasmid encoding mSLF. CV-E cells constitutively express
simian IL-6, which likely synergizes with transfected or exogenously added SLF, and stimulates cobblestone area-formation by Thy-1"SLA-1"H-2K" FUBM cells.

These results raise the important future possibility that HSCs may be sustained in vitro on relatively simple stroma that have been molecularly engineered to express the appropriate spectrum of cytokines and/or extracellular matrix components that promote self-renewal in vivo. Curative regimens demanding marrow rescue could be dramatically enhanced if numerical expansion of repopulating stem cells in vitro were practicable, or if large numbers of lineage-committed progenitors could be generated without depleting stem cells required to sustain long-term hematopoiesis. Although the molecular mechanisms of stem cell self-renewal are not currently defined, it is clear that this approach represents a powerful tool in the identification of novel factors involved in HSC development, and could have far reaching implications for ex vivo stem cell expansion, gene therapy, and therapeutic transplantation.

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Leukemia inhibitory factor upregulates cytokine expression by a murine stromal cell line enabling the maintenance of highly enriched competitive repopulating stem cells

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