RAPID COMMUNICATION

Ex Vivo Expansion of Murine Marrow Cells With Interleukin-3 (IL-3), IL-6, IL-11, and Stem Cell Factor Leads to Impaired Engraftment in Irradiated Hosts

By Stefan O. Peters, Ellen L.W. Kittler, Hayley S. Remshaw, and Peter J. Quesenberry

In vitro incubation of bone marrow cells with cytokines has been used as an approach to expand stem cells and to facilitate retroviral integration. Expansion of hematopoietic progenitor cells has been monitored by different in vitro assays and in a few instances by in vivo marrow renewal in myeloablated hosts. This is the first report of studies, using two competitive transplant models, in which cytokine-treated cells, obtained from nonpretreated donors (eg, 2-fluorouracil), were competed with normal cells. A major assumption is that the expansion of progenitors assayed in vitro as high- and low-proliferative potential colony-forming cells (HPP- and LPP-CFCs) indicates an expansion of stem cells which will repopulate in vivo. This study shows that culture of marrow cells with four cytokines (stem cell factor, interleukin-3 [IL-3], IL-6, IL-11) induces significant expansion and proliferation of HPP-CFC and LPP-CFC. Cell-cycle analysis showed that these hematopoietic progenitors were induced to actively cell cycle by culture with these cytokines. In the first competitive transplant model, which uses Ly5.2/Ly5.1 congenic mice, cytokine-cultured Ly5.2 cells competed with noncultured Ly5.1 cells led to 5% ± 1% engraftment at 12 weeks and to 4% ± 2% engraftment at 22 weeks posttransplantation for the cytokine exposed cells. Noncultured Ly5.2 cells competed with cultured Ly5.1 cells led to 70% ± 1% engraftment at 12 weeks and to 93% ± 2% engraftment at 22 weeks posttransplantation. In the second model, which uses BALB/c marrow of opposite genders, cultured male cells lead to 13% ± 9% engraftment at 10 weeks and 2% ± 1% engraftment at 14 weeks posttransplantation; noncultured male cells lead to 70% ± 2% and 96% ± 2% engraftment at 10 and 14 weeks posttransplantation, respectively. Data presented here from two different competitive transplant studies show a defect of cytokine expanded marrow related to cell cycle activation which manifests as defective long-term repopulating capability in irradiated host mice. The engraftment defect is more profound at long time intervals, suggesting that the most striking effect may be on long-term repopulating cells.

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MATERIALS AND METHODS
Mice. C57BL/6 (Ly5.2) and B6. SJL (Ly5.1) mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). All animals were maintained in virus-free conditions and were fed food and acidified water ad libitum. Mice were purchased at either 16 to 18 g or 18 to 22 g and were housed for a period of 1 week before experimental use.

Culture of bone marrow (BM) with cytokines for 48 hours for expansion. BM cells were obtained by flushing tibia and femurs of 6- to 8-week-old Ly5.2 (C57BL/6) and Ly5.1 (B6. SJL) mice. Ten million BM cells were cultured in nonadherent tissue culture dishes at a density of 10^6 nucleated cells/mL in DMEM (low glucose), supplemented with 15% heat-inactivated fetal calf serum (FCS) with cytokines (recombinant murine SCF [rSCF]) at 50 ng/mL, rmIL-3 at 50 U/mL, rmIL-6 at 50 U/mL, and rmIL-11 at 50 ng/mL) at 37°C in a fully humidified atmosphere of 5% CO2 in air. After 48 hours all cultured cells were procured by vigorous pipetting with ice-cold phosphate-buffered saline (PBS) and cell scraping (as determined by microscopy).

In vitro agar colony-forming assays. Both freshly procured uncultured cells and cells obtained after 48 hours culture were counted performed using a relatively unique transplant model in which donor cell populations engraft into nonlymphomyeloablated hosts. Assessment of long-term repopulating ability is usually performed in myeloablated hosts and virtually all transplant and stem cell models have used irradiated myeloablated hosts. This is the first report of two competitive studies in which cytokine-treated cells, which were not obtained after chemotherapy (eg, day 2 post 5-FU), were competed with noncultured fresh marrow cells in lethally irradiated recipients. We have evaluated the effects of in vitro culture of marrow cells with interleukin-3 (IL-3), IL-6, IL-11 and stem cell factor (SCF) on progenitor cell expansion, cell-cycle status, and competitive repopulation in vivo.

SHORT-TERM in vitro incubation of murine or human marrow cells with a variety of single cytokines or growth factor combinations results in expansion of hematopoietic progenitor cells (HPC) as assayed in in vitro colony systems including multipotent colony-forming cells, long-term culture-initiating cells (LTC-IC), and low- and high-proliferative potential colony-forming cells (HPP- and LPP-CFCs). In a few instances in vivo long-term repopulation has been assessed, but not compared with normal unmanipulated marrow in a competitive transplant model. Similar in vitro cytokine incubations have been used to cycle hematopoietic cells to facilitate retroviral integration in various gene therapy approaches.

Using a marrow transplant model employing normal nonirradiated host mice we have previously shown that cytokine exposure in vitro or administration of fluorouracil (5-FU) in vivo results in markedly impaired long-term engraftment of these cells. In each of these experimental models (in vitro cytokines or in vivo 5-FU) the cell progenitor populations assessed by in vitro colony growth of HPP-CFC and LPP-CFC have been stimulated to proliferate. These studies were
and plated at a cell density of 25,000 per 35-mm plastic petri dish in a double-layer nutrient agar system. Underlays were α-Modified Eagle’s Medium (α MEM) with 15% FCS supplemented with vitamins, glutamine, and growth factors (colony-stimulating factor [CSF-1], granulocyte-macrophage-CSF [GM-CSF], G-CSF, IL-1α, IL-3, mSCF, and basic fibroblast growth factor [BGF]). Target cells were included in a 0.5% overlay with a final agar concentration of 0.33%. Dishes were gassed with a mixture of 5% O2, 10% CO2, and 85% N2 and incubated at 37°C for 14 days. In general, four dishes were set up from each animal. Plates were scored on a dissecting microscope for LPP-CFC (all cell clusters with more than 50 cells and not fulfilling HPP-CFC criteria) and HPP-CFC (highly dense colonies greater than 0.5 mm in diameter or moderately dense colonies greater than 1.0 mm in diameter).13

**Truncated thymidine suicide assay.** The in vitro HTdR suicide technique was performed as previously described.13,14 Briefly, 5 × 10^6 marrow cells per mL were incubated at 37°C for 20 minutes in Hanks’ balanced salt solution (HBSS) containing 200 μCi per mL 3H-TdR (New England Nuclear, Boston, MA; specific activity = 20.0 Ci/mmol/L) or a concentration of unlabeled “cold” thymidine equivalent to that in the 3H-TdR incubation. After this incubation excess cold thymidine was added and the cell suspension washed three times and then plated at an equivalent cell density of 25,000 per dish based on the original counts for progenitor cell growth in vitro in soft agar as described above. The fraction of cells in cycle was determined by percent reduction of HPP-CFC induced by 3HTdR exposure.

**Competitive BM transplantation of marrow from Ly5.2/Ly5.1 congenic C57Bl/6 and B6.SJL mice.** Cells were obtained and cultured as described above. Recipient mice were exposed to 12.0 Gy (7.0 Gy and 5.0 Gy given 3 hours apart) in a gamma cell 40 cesium source and 1 hour later injected intravenously (IV) with (1) a mixture of 50% cytokine-cultured Ly5.1 cells (equivalent to 20 × 10^6 starting cells harvested after 48 hours of incubation with cytokines) and 50% fresh noncultured Ly5.2 cells; (2) a mixture of 50% noncultured Ly5.1 cells and 50% cytokine-treated Ly5.2 cells (equivalent to 20 × 10^6 starting cells harvested after 48 hours of incubation with cytokines); and (3) a mixture of 50% cytokine-cultured cells (equivalent to 20 × 10^6 starting cells harvested after 48 hours of incubation with cytokines) and 50% fresh noncultured Ly5.2 cells; (2) a mixture of 50% noncultured Ly5.1 cells and 50% cytokine-treated Ly5.2 cells (equivalent to 20 × 10^6 starting cells harvested after 48 hours of incubation with cytokines) and 50% fresh noncultured Ly5.2 cells; (3) a mixture of 50% cytokine-cultured cells (equivalent to 20 × 10^6 starting cells harvested after 48 hours of incubation with cytokines) of both cell types. Volumes for injection ranged from 0.5 to 1.0 mL.

**RESULTS**

**Effect of culturing BM in growth medium and cytokines IL-3, IL-6, IL-11, and SCF for 48 hours on expansion of hematopoietic progenitor cells.** Expansion of HPC by culture of marrow in growth medium and cytokines (mSCF at 30 ng/mL, mIL-3 at 50 U/mL, mIL-6 at 50 U/mL, and huIL-11 at 50 ng/mL) for 48 hours was determined by comparing the contents of HPP-CFCs and LPP-CFCs of both freshly obtained uncultured marrow and cytokine-treated cells in a soft-agar colony assay. Cultured cells were procured by vigorous pipetting with ice-cold PBS and cell scraping until virtually no residual cells remained in the culture flasks as determined by microscopy. Cultured marrow cells from Ly5.2 (C57BL/6) and Ly5.1 (B6.SJL) mice were expanded by 49% and 206% for HPP-CFC and 14% and 166% for LPP-CFC in these murine strains, respectively (Fig 1). This expansion of cultured progenitors by cytokine-treatment was significant for HPP-CFC and LPP-CFC in Ly5.1 marrow (P < .01, Wilcoxon’s rank sum test), and was marginally significant (P = .1) in Ly5.2 marrow.

**Effects of the culture with cytokines on the cell-cycle status of HPC.** The cell-cycle status of primitive progenitor cells present in noncultured marrow and in marrow that was incubated for 48 hours in culture medium with the cytokines mSCF, IL-3, IL-6, and IL-11 was analyzed by comparing colony growth of marrow exposed to high activity tritiated thymidine (HTdR) to colony growth of marrow that was...
The amount of HPP-CFC and LPP-CFC killed in noncultivates that most of the cytokine-treated cells had been induced to enter active phases of the cell cycle. This was demonstrated by tritiated thymidine in these Ly5.2 mice (Fig 2A). This indicated that most HPP-CFCs and a mean of 86% of LPP-CFCs were killed by exposure to high-activity tritiated thymidine in this strain. A standardized-normal test comparing the mean differences of noncultured and cultured progenitors was highly significant (P = .01, Wilcoxon’s rank sum test) in the experiment in Ly5.2 recipients (Fig 3B) at 12 weeks posttransplantation. Engraftment levels of cultured Ly5.2 marrow cells competing with noncultured Ly5.1 cells were 5% ± 1% (n = 10) in Ly5.2 recipients (Fig 3A) and 7% ± 2% (n = 5) in Ly5.2 recipients (Fig 3B) at 12 weeks posttransplantation. Differences in engraftment between noncultured and cytokine-treated populations were significant (P < .01, Wilcoxon’s rank sum test) in the experiment in Ly5.2-recipients and was marginally significant (P = .1) in the experiment in Ly5.1-recipients. This engraftment defect of cytokine-treated marrow was even more pronounced when analyzed 22 weeks after transplantation (Fig 3C). Mean engraftment of cultured Ly5.2 donor marrow competing with fresh Ly5.1 cells was only 4% ± 2% (n = 10) at 22 weeks, six individual mice showing no engrafting cells. Fresh noncultured Ly5.2 marrow competed with cultured Ly5.1-cells gave a mean level of 93% ± 2% (n = 10) engraftment with three mice showing all Ly5.2 cells. Differences in engraftment between noncultured and cytokine-treated populations were significant at 22 weeks posttransplantation (P < .01).

A similar engraftment defect of expanded progenitor cells in active phases of the cell cycle could be shown in the other transplant model that uses male and female BALB/c donor populations into irradiated female BALB/c marrow recipients (Fig 4). We have previously shown a 2.3-fold increase of LPP-CFCs and maintenance of HPP-CFCs after culture with cytokines for 48 hours. Cell-cycle analysis had indicated that 90% of HPP-CFC were induced to enter active phases of cell cycle by activation with cytokines. Here we present data from competitive engraftment studies in which the equivalent of 20 × 10⁶ starting male or female cells were obtained after a culture with the cytokines mSCF, IL-3, IL-6, and IL-11 for 48 hours. Shown is the progenitor cell content present in 10 × 10⁶ noncultured (0 hours) cells and that of culture flasks initiated with 10 × 10⁶ cells after 48 hours of culture. HPP-CFC are represented by dark shaded bars (■) at the bottom of the columns, and LPP-CFC are represented as lighter bars (□) at the top of the columns. (A) Cultured marrow cells from C57BL/6 (Ly5.2) were expanded by 49% for HPP-CFC and 14% for LPP-CFC. (B) Cells from B6.SJL (Ly5.1) were expanded by 26% for HPP-CFC and 166% for LPP-CFC. This expansion of noncultured progenitors by cytokine-treatment was significant for HPP-CFC and LPP-CFC in Ly5.1 marrow (P = .01, Wilcoxon’s rank sum test), and was marginally significant (P = .1) in Ly5.2 marrow.

not challanged with radioactivity. The percentage of HPP-CFCs and LPP-CFCs killed by exposure to ³H-TdR indicate the fraction of HPC that underwent active DNA synthesis in cell-cycle progression. No cell-cycle activity could be detected in noncultured Ly5.2 (C57BL/6) marrow (Fig 2A). The amount of HPP-CFC and LPP-CFC killed in noncultured fresh marrow by thymidine suicide was a mean of 0% indicating that most HPC were in dormancy. After culture with cytokines a mean of 95% of HPP-CFCs and a mean of 86% of LPP-CFCs were killed by exposure to high-activity tritiated thymidine in these Ly5.2 mice (Fig 2A). This indicates that most of the cytokine-treated cells had been induced to enter active phases of the cell cycle.

This change of HPC cycle status induced by culture of marrow cells with cytokines was also observed in marrow from Ly5.1 (B6.SJL) mice (Fig 2B). In this strain 20% of HPP-CFCs and 32% of LPP-CFCs of noncultured marrow were killed by exposure to ³H-TdR. The amount of progenitor cells entering active phases of the cell cycle increased dramatically as shown by HPP-CFC fractions of 98% and LPP-CFC fractions of 86% killed by exposure to high-activity tritiated thymidine in this strain. A standardized-normal test comparing the mean differences of noncultured and cultured progenitors was highly significant (P < .0001) in both strains.

Effect of culture with cytokines on engraftability of BM into lethally irradiated hosts. We have used two competitive murine transplant models in irradiated host mice to assess for the engraftment potential of marrow cells cultured for 48 hours with the cytokines mSCF, IL-3, IL-6, and IL-11. The first model, which uses the Ly-5.1/Ly-5.2 congenic B6.SJL and C57BL/6 strains, allows distinction of donor- and host-derived cells with MoAbs specific for the alleles of Ly-5; the second model uses BALB/c mice, allowing for the detection of male donor cell DNA sequences in female recipients.

The equivalent of 20 × 10⁶ starting cells of either Ly5.1 (B6.SJL) or Ly5.2 (C57BL/6) that were obtained after a culture with the cytokines mSCF, IL-3, IL-6, and IL-11 were mixed and injected together with fresh, noncultured cells of the opposite strain into irradiated (12.0 Gy) recipient mice. The percentages of marrow cells stained with MoAbs specific for the two alleles of Ly-5 were determined 12 and 22 weeks posttransplant by FACS analysis (Fig 3). Noncultured Ly5.2 (C57BL/6) marrow, competing with cytokine-cultured Ly5.1 (B6.SJL) marrow, lead to high engraftment with a mean (±SE) of 70% ± 1% (n = 10) in Ly5.1 (Fig 3A) and of 63% ± 5% (n = 5) in Ly5.2 recipients (Fig 3B) at 12 weeks posttransplantation. Engraftment levels of cultured Ly5.2 marrow cells competing with noncultured Ly5.1 cells were 5% ± 1% (n = 10) in Ly5.1 recipients (Fig 3A) and 7% ± 2% (n = 5) in Ly5.2 recipients (Fig 3B) at 12 weeks posttransplantation. Differences in engraftment between noncultured and cytokine-treated populations were significant (P < .01, Wilcoxon’s rank sum test) in the experiment in Ly5.2-recipients and was marginally significant (P = .1) in the experiment in Ly5.1-recipients. This engraftment defect of cytokine-treated marrow was even more pronounced when analyzed 22 weeks after transplantation (Fig 3C). Mean engraftment of cultured Ly5.2 donor marrow competing with fresh Ly5.1 cells was only 4% ± 2% (n = 10) at 22 weeks, six individual mice showing no engrafting cells. Fresh noncultured Ly5.2 marrow competed with cultured Ly5.1-cells gave a mean level of 93% ± 2% (n = 10) engraftment with three mice showing all Ly5.2 cells. Differences in engraftment between noncultured and cytokine-treated populations were significant at 22 weeks posttransplantation (P < .01).

A similar engraftment defect of expanded progenitor cells in active phases of the cell cycle could be shown in the other transplant model that uses male and female BALB/c donor populations into irradiated female BALB/c marrow recipients (Fig 4). We have previously shown a 2.3-fold increase of LPP-CFCs and maintenance of HPP-CFCs after culture with cytokines for 48 hours. Cell-cycle analysis had indicated that 90% of HPP-CFC were induced to enter active phases of cell cycle by activation with cytokines. Here we present data from competitive engraftment studies in which the equivalent of 20 × 10⁶ starting male or female cells were obtained after a culture with the cytokines mSCF, IL-3, IL-
engraftment levels of cultured male marrow cells competing with noncultured female cells were 13% ± 9% (n = 5) in the marrow of female recipients at 10 weeks. This difference in engraftment of noncultured and cytokine-treated populations is statistically marginally significant (P = .5, Wilcoxon’s rank sum test). The engraftment defect was more impressive in animals analyzed 14 weeks after transplantation (Fig 4A, bottom row). Fresh noncultured male BALB/c marrow cells competing with cytokine-treated female cells contributed to 95% ± 9% (n = 10) marrow engraftment while cytokine-treated male cells competing with noncultured female cells gave a mean level of only 2% ± 1% (n = 10). Differences in engraftment between non-cultured and cytokine-treated populations were highly significant (P < .01). In these BALB/c transplants the engraftment defect was also apparent when spleen and thymus were analyzed, indicating that this is a general engraftment defect (Fig 4B and C).

**DISCUSSION**

These data show that in vitro exposure to a cytokine cocktail of IL-3, IL-6, IL-11, and SCF expands surrogate progenitors/stem cells (LPP-CFC and HPP-CFC) in vitro, induces them to progress through cell cycle, and concomitantly produces a profound engraftment defect in lethally irradiated myeloablated hosts. Studies in both engraftment models show a more profound engraftment defect at longer time intervals posttransplantation, suggesting that the most striking effect may be on the long-term repopulating population.

Cytokine expanded progenitor cells may not be truly renewal stem cells or may retain renewal potential but exist in a nonengrafting state. A number of studies in murine species have shown that multipotential CFCs like GM-CFC, CFU-GEMM, HPP-CFC, and LTC-IC can be expanded in vitro after exposure to a wide variety of cytokine mixtures including SCF, CSF-1, G-CSF, M-CSF, GM-CSF, IL-1α, IL-6, IL-11, erythropoietin, PIXY321, interferon-γ, and leukemia inhibitory factor. Functional aspects such as increased capacity to form CFU-S and augmented capacity for short- and long-term renewal (<35 and >35 days, respectively) have been assessed for fresh cells competing with ‘compromised’ marrow, for fresh cells in noncompetitive transplant studies and for cells obtained after treatment with 5-FU expanded with cytokines in vivo in anemic and/or irradiated mice. This is the first study in which cytokine-treated normal cells, as opposed to cells harvested after chemotherapy, were competed with normal cells and analyzed for their contribution for engraftment. We show that primitive hematopoietic cells induced to divide and proliferate by cytokines in culture acquire an engraftment defect that is related to cell cycle activation. This interpretation is supported by data obtained from competitive transplant experiments in normal (nonlymphomyeloablated) hosts, showing that functional engraftment defect occurs in marrow cells harvested 6 days after treatment of mice with 5-FU; the defect is no longer present 35 days after treatment with 5-FU. Using the same model a cytokine induced engraftment defect was shown. In both cases the engraftment defect correlated with cell-cycle activation. Differentiation
Fig 3. Assessment of BM engraftment after competitive transplantation of noncultured with cultured marrow cells by FACS analysis for donor-derived cells stained with MoAbs specific for the two alleles of Ly-5. Columns represent the marrow fraction of individual mice positive for anti-Ly5.2 antibody staining. Analysis of anti-Ly5.1 antibody staining gave reciprocal results and are not shown here. (A) Analysis at 12 weeks posttransplantation in Ly5.1 (B6.SJL-Ly5a) recipients: noncultured Ly5.2 (C57BL/6) marrow competed with Ly5.1 marrow led to mean (±SE) engraftment levels of 70% ± 1% for the Ly5.2 cells; cytokine-cultured Ly5.2 marrow cells competing with Ly5.1 cells contributed to 5% ± 1% engraftment. Differences in engraftment between noncultured and cytokine-treated populations were significant (P < .01, Wilcoxon's rank sum test). (B) Analysis at 12 weeks posttransplantation in recipient mice of Ly5.2 background: noncultured Ly5.2 cells contributed a mean of 63% ± 5% engraftment for the Ly5.2 cells; cultured Ly5.2 cells competing with Ly5.1 cells contributed to 7% ± 2% engraftment. Differences in engraftment were marginally significant (P = .1). (C) Analysis at 22 weeks posttransplantation in recipient mice of Ly5.1 background: noncultured Ly5.2 marrow cells contributed a mean of 93% ± 2% to engraftment whereas cytokine-treated Ly5.2 cells gave a mean engraftment of 4% ± 2% when competed with Ly5.1 marrow cells. Differences in engraftment between noncultured and cytokine-treated populations were significant at 22 weeks posttransplantation (P < .01).
Fig 4. Percentage of male DNA in BM, spleen, and thymus of individual irradiated recipient female mice given transplants of noncultured male or female BALB/c marrow competed with cytokine-cultured marrow cells of the opposite gender at 10 and 14 weeks posttransplantation. Southern blots were probed with pY2 and the percentage engraftment was calculated using phosphorimage analysis, taking a male control as 100% and a female control as 0%. Samples were corrected for loading with an 11-3 probe. (A) Noncultured male BALB/c marrow cells, competing female marrow, led to mean engraftment levels of 70% ± 2% in marrow of irradiated female BALB/c recipients at 10 weeks posttransplantation (top row). Mean engraftment levels of cultured male marrow cells competing with female cells was 13% ± 9% in marrow of female recipients at 10 weeks. The difference in engraftment of noncultured and cytokine-treated populations is statistically marginally significant \( P = .5, \) Wilcoxon's rank sum test). At 14 weeks after competitive transplantation (bottom row) noncultured male BALB/c marrow cells contributed to 95% ± 9% marrow engraftment whereas cytokine-treated male BALB/c cells gave a mean level of only 2% ± 1%. Differences in engraftment between noncultured and cytokine-treated populations were highly significant \( P < .01.\) (B and C) The engraftment defect was also apparent in spleen and thymus, indicating that this is a general engraftment defect.
in culture could alternatively explain this engraftment defect, but it seems unlikely that all of the hematopoietic stem cells, which are constantly exposed to cytokines in vivo, would differentiate out of the stem cell pool. Findings of altered integrin molecule expression related to cytokine-induced cell-cycle transit suggests a possible mechanism for the cytokine-induced engraftment defect related to modulation of homing factors.10

Engraftable states of cultured hematopoietic progenitors may be maintained by cytokines that spare cell cycle activation. This is suggested by data from Li and Johnson4 showing maintained long-term repopulating ability of rhodamine 123 low, lineage-negative Ly6a/E- cells for as long as 7 days in culture with SCF when injected with compromised marrow. Protocols designed to promote mitosis of hematopoietic progenitors for retroviral gene transfer frequently contain cytokine combinations including SCF, IL-3, IL-6, and IL-11, rendering them unlikely to engraft. Attempts to alter engraftment efficacy of cytokine treated stem cells by reversing cell-cycle–related defects may be indicated both to improve the clinical potential for cytokine-expanded stem cells and for gene therapy approaches.

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