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# Thrombopoietic potential and serial repopulating ability of murine hematopoietic stem cells constitutively expressing interleukin 11

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**ABSTRACT** **Based on transplantation studies with bone marrow cultured under various conditions, a role of interleukin 11 (IL-11) in the self-renewal and/or the differentiation commitment of hematopoietic stem cells has been indicated. To better evaluate the *in vivo* effects of IL-11 on stem/progenitor cell biology, lethally irradiated mice were serially transplanted with bone marrow cells transduced with a defective retrovirus, termed MSCV-mIL-11, carrying the murine IL-11 (mIL-11) cDNA and the bacterial neomycin phosphotransferase (*neo*) gene. High serum levels (i.e., >1 ng/ml) of mIL-11 in all (20/20) primary and 86% (12/14) of secondary long-term reconstituted mice, as well as 86% (12/14) of tertiary recipients examined at 6 weeks posttransplant, demonstrated persistence of vector expression subsequent to transduction of bone marrow precursors functionally definable as totipotent hematopoietic stem cells. In agreement with results obtained with human IL-11 in other myeloablation models, ectopic mIL-11 expression accelerated recovery of platelets, neutrophils, and, to some extent, total leukocytes while preferentially increasing peripheral platelet counts in fully reconstituted mice. When analyzed 5 months posttransplant, tertiary MSCV-mIL-11 recipients had a significantly greater percentage of G418-resistant colony-forming cells in their bone marrow compared with control MSCV animals. Collectively, these data show that persistent stimulation of platelet production by IL-11 is not detrimental to stem cell repopulating ability; rather, they suggest that IL-11 expression *in vivo* may have resulted in enhanced maintenance of the most primitive hematopoietic stem cell compartment. The prolonged expression achieved by the MSCV retroviral vector, despite the presence of a selectable marker, contrasts with the frequent transcriptional extinction observed with other retroviral vectors carrying two genes. These findings have potentially important implications for clinical bone marrow transplantation and gene therapy of the hematopoietic system.**

Interleukin 11 (IL-11) is a stromal cell-derived cytokine with potent thrombopoietic properties and activity on a wide spectrum of immature hematopoietic precursors (1–4; reviewed in ref. 5). When combined with IL-3, IL-4, and *kit* ligand (KL, also known as Steel factor), recombinant human IL-11 (rhIL-11) stimulated the growth of multipotential blast colony-forming cells, an effect that was attributed to the shortening of the $G_0$ period of dormant hematopoietic stem cells (6, 7). Several studies have attempted to further define the effects of rhIL-11 on primitive hematopoietic stem cells. In one study, murine bone marrow cultures containing the combination of rhIL-11 and KL had 4-fold greater long-term repopulating ability compared with cultures containing KL alone, suggesting that rhIL-11 could enhance self-renewal of stem cells (8). In contrast, the addition of rhIL-11 to long-term bone marrow cultures decreased the frequency of long-term repopulating cells present at 4 weeks compared with control cultures, consistent with induction of stem cell differentiation (9). In addition to these *in vitro* results, we showed that normal mice treated *in vivo* with the combination of rhIL-11 and KL had increased numbers of both splenic-colony forming units and long-term repopulating cells in the spleen, while mice treated with either factor alone had increased numbers of splenic-colony forming units but not long-term repopulating cells (10). Thus, although it has been demonstrated both *in vitro* and *in vivo* that rhIL-11 can stimulate primitive hematopoietic stem cells, it remains unclear in which physiological situations IL-11 functions as either a regulator of stem cell proliferation (self-renewal) or of stem cell commitment (differentiation).

Bone marrow transplantation reduces primitive stem cell concentrations and decreases the long-term repopulating ability per stem cell, partly because of the excessive differentiative pressure imposed by the necessity to reconstitute an otherwise lethally irradiated recipient (11, 12). Likewise, it has proven difficult to preserve the self-renewal capacity of long-term repopulating cells following *ex vivo* culture, even in the presence of cytokine-containing cocktails, a procedure that is typically used to induce proliferation of hematopoietic stem cells to achieve efficient retroviral transduction (13, 14). These observations have raised questions about the feasibility of *ex vivo* gene transfer approaches using retroviral vectors for long-term gene expression in the bone marrow transplant setting.

We reported previously that mice reconstituted with genetically modified bone marrow cells ectopically expressing a retrovirally introduced hIL-11 cDNA presented with a chronic 1.5-fold increase in peripheral platelet levels (15). In that study, we also demonstrated that hIL-11-expressing precursor cells from primary transplant mice retained the ability to repopulate lethally irradiated secondary hosts (15). However, a detailed analysis of the consequences of sustained hIL-11 expression on the hematopoietic stem pool was not conducted. The recent availability of the murine IL-11 (mIL-11) cDNA has now permitted this issue to be addressed in a homologous model system (16). Accordingly, we have used this cDNA to construct a retroviral vector expressing mIL-11. As a stringent assay of stem cell function, bone marrow cells transduced with the vector were subjected to serial transplantation in lethally irradiated syngeneic recipients, at intervals of 3 to 4 months.

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Abbreviations: IL, interleukin; m, murine; h, human; r, recombinant; LTR, long terminal repeat; MSCV, murine stem cell virus.

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Our results indicate that retrovirally transduced mIL-11-producing bone marrow precursors that have been compromised by two previous cycles of transplantation can regenerate hematopoiesis in the majority of tertiary hosts for at least 5 months posttransplant.

## MATERIALS AND METHODS

**Murine Stem Cell Virus (MSCV)-mIL-11 and Control MSCV Retroviruses.** The mIL-11 cDNA was molecularly cloned from mRNA of a lipopolysaccharide-induced fetal thymic cell line, T2 (16). To construct the MSCV-mIL-11 retroviral vector (see Fig. 3*A*), the mIL-11 coding region minus the 3′ untranslated region and polyadenylylation signal was excised as a 0.72-kb *Eco*RI–*Bgl*II fragment from clone 31A (mIL-11 cDNA subcloned into the *Not*I site of pBluescript II SK+) by complete digestion with *Eco*RI and partial digestion with *Bgl*II, and inserted into the corresponding *Eco*RI and *Bgl*II sites of the polylinker in the MSCV v2.1 retroviral vector which carries the bacterial neomycin phosphotransferase (*neo*) gene under the transcriptional control of an internal murine phosphoglycerate kinase (*pgk*) promoter as dominant selectable marker (17). The MSCV-mIL-11 plasmid was linearized by digestion with *Nde*I and electroporated into GP+E-86 ecotropic helper-free packaging cells (18). Virus-containing supernatant was collected 24 hr later and used to transduce tunicamycin-treated (0.1 μg/ml for 16 hr) GP+E-86 cells. Pooled populations of stable GP+E-86 transductants (GP+E-86/MSCV-mIL-11 cells) produced recombinant MSCV-mIL-11 virus at a titer of $5 \times 10^6$ G418-resistant colony-forming units/ml when assayed on NIH 3T3 fibroblasts. GP+E-86/MSCVv2.1 cells (19) exporting *neo* virus with a comparable titer were used to generate control transplant mice. Virus-producing cells were maintained in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose supplemented with 10% (vol/vol) calf serum in a humidified atmosphere containing 5% $CO_2$/95% air at 37°C.

**Retrovirus Transduction and Transplantation of Bone Marrow.** Female C57BL/6 mice were used at 8 weeks of age as bone marrow donors and recipients. Bone marrow processing, transduction, and transplantation were carried out essentially as described (15, 20–22) with minor modifications. In brief, bone marrow was flushed from hind limbs of donors injected 4 days previously with 150 mg/kg 5-fluorouracil with ice-cold Iscove's modified Dulbecco's medium containing 50 μM 2-mercaptoethanol and 10% (vol/vol) heat-inactivated fetal bovine serum. Following erythrocyte lysis in 0.17 M ammonium chloride, nucleated cells were divided into two equal portions and added to 100 mm Petri dishes at a density of $5 \times 10^5$ cells/ml in Iscove's modified Dulbecco's medium supplemented with 50 μM 2-mercaptoethanol, 10% (vol/vol) heat-inactivated fetal bovine serum, and either 10% (vol/vol) conditioned medium from X630-rIL3 cells (a source of recombinant murine IL-3; a gift of F. Melchers, Basel Institute, Basil, Switzerland) (23) and 10% (vol/vol) conditioned medium from Sp2/mIL-6 cells (a source of recombinant mIL-6) (24) or 10% (vol/vol) conditioned medium from Chinese hamster ovary cells producing soluble KL (Genetics Institute) and 10% (vol/vol) conditioned medium from B9/hIL-11 cells (a source of rhIL-11) (15) throughout the entire procedure. After 48 hr of prestimulation, the bone marrow cells were collected and added to subconfluent monolayers of GP+E-86/MSCV-mIL-11 or GP+E-86/MSCVv2.1 cells in 100-mm tissue culture dishes at a density of $5 \times 10^5$ cells/ml in fresh medium supplemented with 8 μg/ml polybrene. Following a 48-hr coculture period, nonadherent bone marrow cells were harvested and transferred to fresh virus-producing monolayers in medium containing 0.75 mg/ml G418 (active drug). The cells were collected 24 hr later, pooled, and injected into the tail vein of recipients that had received 1050 cGy of irradiation (split dose with 3 hr between doses from a $^{137}Cs$ source). Each mouse received $0.5–1.0 \times 10^6$ cells (≈7 donor equivalents per recipient). Altogether, bone marrow extracted from 168 5-fluorouracil-treated donors in 9 separate experiments was used to generate 24 primary transplant recipients (20 MSCV-mIL-11 experimental and 4 MSCV control animals). For serial transplantations, $2 \times 10^6$ pooled bone marrow cells from mice reconstituted for 14–16 weeks were injected i.v. into lethally irradiated (1050 cGy) recipients.

**Hematologic Analysis.** Blood was collected from the retroorbital sinus at weekly intervals following transplant and immediately before sacrifice. Total leukocytes, total erythroid cells, hemoglobin, hematocrit, and total platelets were determined on a System 9000 Hematology Series Cell Counter (Serono-Baker Instruments, Allentown, PA) using mouse-specific discriminator settings. Blood smears were prepared and stained with Diff-Qwik and manual differential cell counts were performed on 100 cells. The absolute neutrophil count was calculated by multiplying the total leukocyte count by the percentage of neutrophils in the differential analysis. The probability of significant differences between experimental MSCV-mIL-11 and control MSCV groups was determined using the two-tailed Student's *t* test.

**Measurement of Serum IL-11 Levels.** An enzyme-linked immunosorbent assay employing two murine monoclonal antibodies directed against hIL-11 that cross-react with mIL-11 (Genetics Institute) was used to estimate the levels of mIL-11 in the serum of bone marrow-reconstituted mice. Purified rhIL-11 (Genetics Institute) used as a standard was produced in *Escherichia coli* and had a specific activity of $2.5 \times 10^6$ units/mg protein. The detectable limit of the assay was 1 ng/ml rhIL-11.

**Progenitor Assay.** The number of colony-forming cells was determined as described by using single cell suspensions from pooled bone marrow samples (4, 15, 20). Colonies, defined as discrete clusters containing >50 cells, were enumerated on day 7. Where indicated, G418 was added to a final active concentration of 1 mg/ml.

**Molecular Analyses.** The procedures and probes used for Southern and Northern blot analyses have been detailed in prior publications (15, 19–22). Relative proviral copy number was determined by scanning densitometry after normalization to the endogenous c-*myc* gene, detected as a 10.5-kb band following digestion with *Kpn*I (22).

## RESULTS

**Engraftment of Lethally Irradiated Mice with Genetically Modified Bone Marrow Cells Constitutively Expressing Biologically Active mIL-11.** Cell lines exporting helper-free recombinant virus with ecotropic host range were generated from GP+E-86 packaging cells (see *Materials and Methods*). Conditioned medium prepared from GP+E-86/MSCV-mIL-11 cells, but not that from control GP+E-86/MSCVv2.1 cells, strongly stimulated the proliferation of IL-11-dependent B9E cells (25), indicating that the MSCV-mIL-11 virus directed synthesis of functional mIL-11.

Lethally irradiated mice reconstituted for 10–15 weeks with MSCV-mIL-11-transduced bone marrow displayed elevated peripheral platelet counts ($1301 \pm 162 \times 10^3/mm^3$ compared with $753 \pm 96 \times 10^3/mm^3$ in control MSCV mice). These results were similar to those obtained previously in mice persistently expressing hIL-11 (1.7-fold increase in platelets in mice expressing mIL-11 versus 1.5-fold increase in platelets in mice expressing hIL-11) (15). Measurement of serum mIL-11 levels by enzyme linked immunosorbent assay revealed concentrations ranging from 3 to 60 ng/ml (mean, 19 ± 12 ng/ml; $n = 20$). Notably, despite the high systemic mIL-11 levels in completely reconstituted mice, no significant changes in peripheral leukocyte counts were detected (see below). These

latter observations corroborate and thus validate the earlier studies with hIL-11 (3, 4, 15).

**Influence of Constitutive mIL-11 Expression on Hematopoietic Recovery of Secondary Transplant Recipients.** Three representative primary recipients of MSCV-mIL-11-transduced bone marrow were sacrificed 3 months posttransplant and their bone marrow was pooled and transplanted to 15 lethally irradiated secondary recipients. Similarly, bone marrow was pooled from 3 primary recipients of MSCV-transduced bone marrow for generation of 15 control secondary transplants. As shown in Fig. 1*A*, secondary MSCV-mIL-11 recipients had accelerated peripheral platelet recoveries compared with secondary recipients transplanted with control MSCV-transduced bone marrow. The secondary MSCV-mIL-11 transplants also displayed accelerated recovery of peripheral leukocytes (Fig. 1*B*) but not erythroid cells (data not shown) 4 to 6 weeks posttransplant compared with control secondary MSCV recipients. Sampling of peripheral blood at biweekly intervals during an observation period of 3 months revealed that the secondary MSCV-mIL-11 recipients with elevated platelet counts had circulating mIL-11 levels of up to ≈90 ng/ml (Table 1). Even when examined at times greater than 6 months posttransplant, several mice still had serum IL-11 concentrations >10 ng/ml. During the early stages of hematopoietic reconstitution, there was a tendency for mice with higher platelet counts to have higher circulating concentrations of mIL-11, as reported previously in preliminary analyses of primary transplant recipients at early times posttransplant (5). However, after complete reconstitution, a strict correlation between circulating mIL-11 levels and platelet counts could not be demonstrated (data not shown).

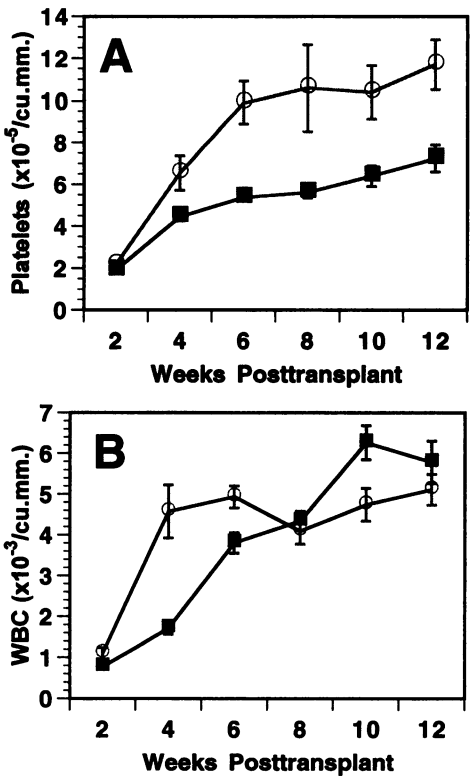


FIG. 1. Effects of overexpressing mIL-11 on hematopoietic recovery in secondary MSCV-mIL-11 transplant recipients. Peripheral platelet counts (*A*) and peripheral leukocyte (white blood cells, WBC) counts (*B*) from MSCV-mIL-11 (○) and MSCV control (■) mice. The data represent the mean ± standard error, with a minimum of 13 mice for each time point. Platelet counts were significantly elevated in MSCV-mIL-11 mice compared with MSCV control mice on weeks 4–12 ($P < 0.01$), and leukocyte counts were significantly elevated on weeks 4 and 6 ($P < 0.01$).

Table 1. Serum mIL-11 concentrations in secondary and tertiary MSCV-mIL-11 transplant recipients

| Time posttransplant, week | Mean,* ng/ml | Range, ng/ml | No. of IL-11 serum positive mice/total no. of mice |
|---|---|---|---|
| | Secondary recipients | | |
| 2 | 4.5 | 2.5–6.0 | 6/15 |
| 4 | 10.8 | 1.0–28.5 | 14/15 |
| 6 | 14.1 | 4.8–56.7 | 12/15 |
| 8 | 16.4 | 3.4–55.1 | 9/15 |
| 10 | 12.4 | 1.9–62.3 | 11/14 |
| 12 | 16.1 | 1.7–89.4 | 12/14 |
| | Tertiary recipients | | |
| 2 | 1.8 | 1.3–2.3 | 4/14 |
| 4 | 5.0 | 2.7–6.8 | 8/14 |
| 6 | 5.3 | 1.6–10.9 | 12/14 |
| 8 | 4.3 | 2.8–6.7 | 7/14 |
| 10 | 4.1 | 1.6–6.3 | 6/14 |
| 12 | 5.5 | 3.6–7.9 | 4/12 |

*Determined by an enzyme-linked immunosorbent assay as described. When tested, serum mIL-11 concentrations in control MSCV and naive mice were found to be below the level of detection (ref. 5, and data not shown).

**Successful Transfer of mIL-11-Producing Hematopoietic Precursors to Lethally Irradiated Tertiary Recipients.** Sustained high-level mIL-11 expression in secondary MSCV-mIL-11 recipients argued that integration of functional MSCV-mIL-11 proviruses had occurred at the level of long-term hematopoietic repopulating cells. To obtain evidence in support of this hypothesis, five secondary MSCV-mIL-11 recipient mice and five control secondary MSCV mice were sacrificed 4 months posttransplant, and their bone marrow pooled and transferred to 14 lethally irradiated tertiary recipients per group. Similar to the findings in the secondary transplants, recovery of peripheral platelets and leukocytes, but not erythroid cells, was accelerated in tertiary recipients of MSCV-mIL-11-bone marrow compared with tertiary controls (Fig. 2); in general, the differences were not as pronounced as those obtained for the secondary transplants (Fig. 1). Differential leukocyte counts revealed that neutrophil recovery was accelerated in MSCV-mIL-11 tertiary recipient mice compared with control MSCV recipient mice (Fig. 2*B*). Even in these tertiary hosts, high concentrations of mIL-11 could be readily detected in the serum of 12 of 14 MSCV-mIL-11 recipient mice 6 weeks posttransplant and in 4 of 12 surviving animals 3 months posttransplant (Table 1).

Five to 6 months posttransplant, when all tertiary MSCV-mIL-11 recipients were sacrificed, serum mIL-11 activities equivalent to rhIL-11 of 3.1, 2.4, and 7.4 ng/ml, could still be detected in the case of mice B1, C2, and D0, respectively. At this time, a high percentage (≈44%) of G418-resistant clonogenic progenitors were present in the bone marrow of the tertiary MSCV-mIL-11 recipients tested (Table 2). By comparison, less than 1% of the colony-forming cells detected in the bone marrow from control tertiary MSCV recipients were resistant to G418. This result is noteworthy since the clonogenic cell populations in bone marrow samples from both experimental MSCV-mIL-11 and control MSCV secondary recipients exhibited similarly high frequencies of G418 resistance at time of sacrifice (Table 2).

The Northern blot in Fig. 3*B* documents vector expression in spleen RNA from five tertiary MSCV-mIL-11 recipients. As can be seen, hybridization with a *neo* probe demonstrated the expected transcripts initiating from the long terminal repeat

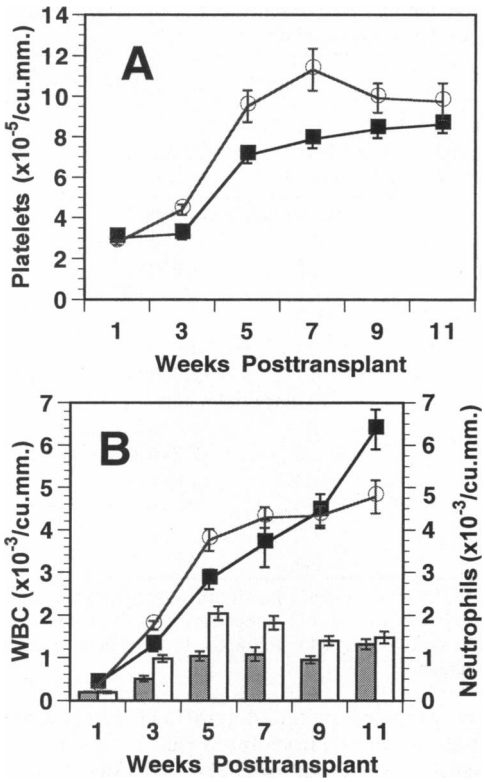


FIG. 2. Accelerated recovery of platelets and neutrophilic leukocytes in tertiary MSCV-mIL-11 transplant recipients. Peripheral platelet counts (*A*) and peripheral leukocyte (white blood cells, WBC) counts (*B*) from MSCV-mIL-11 (○) and MSCV control (■) mice. In *B*, the open and solid bars represent absolute neutrophil counts of MSCV-mIL-11 mice and MSCV control mice, respectively. The data represent the mean ± standard error, with a minimum of 13 mice for each time point. The differences in platelet counts between MSCV-mIL-11 mice and MSCV control mice were statistically significant on weeks 3–7 ($P < 0.01$), and in absolute neutrophil counts, on weeks 3–9 ($P < 0.01$).

(LTR) and internal *pgk* promoters; on average, LTR-directed mIL-11 transcripts were more abundant than *pgk*-directed *neo* transcripts.

The clonal composition of the reconstituted hematopoietic system of a tertiary MSCV-mIL-11 recipient (mouse B1) for which DNA was available from several hematopoietic tissues was examined by Southern blot analysis with a *neo* probe. As illustrated in Fig. 3*C*, digestion of DNA prepared from myeloid (bone marrow) and lymphoid (lymph node, spleen, and thymus) tissues of mouse B1 with *Bam*HI, which cleaves the MSCV-mIL-11 provirus once (Fig. 3*A*) yielding unique integration sites, revealed one prominent band (≈4.6 kb) and two other bands of varying intensity common to all lanes, plus a fourth band which was present only in the lanes containing DNA from lymphoid tissues. Southern blot analysis of spleen DNA from three other tertiary MSCV-mIL-11 recipients that received aliquots of the same pooled bone marrow revealed different hybridization patterns (Fig. 3*D*, and data not shown), indicating that the hematopoietic systems of these animals had been reconstituted with distinct MSCV-mIL-11-marked cells. Southern blot analysis with a *neo* probe revealed the presence of nonrearranged MSCV-mIL-11 proviruses in *Kpn*I-digested spleen DNA (*Kpn*I cleaves once within each LTR) of all samples tested (Fig. 3*E*). Serum from mouse B1 and another tertiary MSCV-mIL-11 transplant recipient (mouse D4) plus serum from two representative control tertiary MSCV transplant recipients (mice B0 and D1) tested negative for the presence of replication-competent "helper" viruses in a sensitive marker virus mobilization assay (26), indicating that the multiple proviral integrants observed in the tertiary MSCV-mIL-11 recipients as well as sustained MSCV-mIL-11 expression was not a consequence of vector spread by a transmissible virus. These findings established that a number of long-term repopulating cells originally transduced with the MSCV-mIL-11 virus *in vitro* survived transfer through primary and secondary recipients to regenerate the hematopoietic systems of tertiary recipients.

Table 2. Frequency of G418-resistant progenitors in bone marrow from secondary and tertiary MSCV-mIL-11 transplant recipients

| Transplant recipients | Colony forming cells per femur | | Percent G418 resistance |
|---|---|---|---|
| | − G418 | + G418 | |
| Secondary MSCV-mIL-11 | 18,622 ± 1250 | 12,937 ± 949 | 70 ± 7 |
| Secondary MSCV | 16,192 ± 536 | 13,504 ± 797 | 84 ± 6 |
| Tertiary MSCV-mIL-11 | 26,040 ± 449 | 11,400 ± 1113 | 44 ± 10 |
| Tertiary MSCV | 31,417 ± 2082 | 120 ± 170 | <1 |

Bone marrow was isolated and pooled from secondary ($n = 6$) or tertiary ($n = 5$) recipients at 16 or 20 weeks posttransplant, respectively, and assayed for colony-forming cells as described. Bone marrow from naive mice was assayed in parallel and found in all cases to be 100% sensitive to G418 at the concentration used (1 mg/ml).

## DISCUSSION

At the present time, the only definitive measure of the stem cell content of a particular hematopoietic population is to assess self-renewal and long-term hematopoietic function by serial transfer in myeloablated or genetically disadvantaged hosts (27, 28). Here we have demonstrated successful transduction of long-term hematopoietic repopulating cells, including totipotent hematopoietic stem cells—primitive hematopoietic precursors which, after having been subjected to two previous cycles of bone marrow transplantation, retained the capacity to reconstitute steady-state lympho-myelopoiesis in lethally irradiated tertiary recipients. The ability of the MSCV vector to stably express the mIL-11 cDNA throughout the duration of the experimental procedure has allowed us to evaluate the consequences of persistent mIL-11 expression on the stem cell compartment as well as on peripheral hematology.

Accumulated data has provided evidence that the MSCV retroviral vector constructed in our laboratory functions efficiently in immature hematopoietic cells (17), including myeloid progenitors (21), B- and T-lymphoid precursors (19), as well as short- and long-term hematopoietic repopulating cells (15, 20, 29–31). Of particular relevance to the present study is a previous report where we demonstrated transduction of hematopoietic stem cells capable of long-term multilineage reconstitution of lethally irradiated primary and secondary recipients by functional MSCV viruses harboring the hIL-11 cDNA (15). Use of the homologous murine system, as reported in this paper, obviates the question of whether the lack of adverse effects associated with dysregulated overexpression of hIL-11, observed in the previous study, was due to suboptimal cross-species activity or the mounting of an immune response to the human protein.

In this regard, other studies have examined the effects of hIL-11 on hematopoietic recovery following bone marrow transplantation (3, 32). In one study, mice treated with rhIL-11 had accelerated recovery of platelets and neutrophils compared with control transplant mice (3). In a second report, in which the hIL-11 cDNA was expressed in reconstituting bone marrow from a different retroviral vector, accelerated recovery of platelets and erythroid cells was observed (32). In the current experiments, no effects of mIL-11 on the recovery of erythroid cells was noted but accelerated recovery of platelets and total leukocytes was observed in secondary transplant recipients, and accelerated recovery of platelets as well as

neutrophilic and total leukocytes was observed in tertiary transplant recipients receiving mIL-11-expressing bone marrow. In steady-state hematopoiesis (i.e., >12 weeks posttransplant), only the thrombopoietic activity of mIL-11 was maintained, with continued high level mIL-11 expression having little impact on peripheral leukocyte counts. Since *in vitro* data have revealed that IL-11 generally functions in synergy with other factors (2, 6, 7), these *in vivo* results suggest that during bone marrow reconstitution sufficient quantities of the complementary synergistic factor(s) were available to cooperate with mIL-11, evoking accelerated recovery of neutrophils in addition to platelets. The synergistic cytokine(s) that cooperates with mIL-11 to stimulate granulopoiesis at early times after bone marrow transplantation is not known, but the findings from this and our previous study overexpressing the hIL-11 gene allow us to rule out both species-specific effects of IL-11 (as noted above) as well as strain-specific differences in IL-11 responsiveness (i.e., BALB/c mice were used as recipients in the previous study while C57BL/6 mice were used in the current experiments) as possible explanations for the lack of a granulopoietic effect in the steady state (15).

The highly deleterious effects of transplantation on hematopoietic stem cell repopulating ability have been well documented (11, 12). Transplantation of $10^6$ bone marrow cells from normal donors has permitted three serial transfers in lethally irradiated hosts (11, 12), but there have been few examples reporting successful long-term reconstitution of tertiary recipients with retrovirally-transduced bone marrow cells serially passaged in this manner (33). Here, $2 \times 10^6$ MSCV-mIL-11-transduced marrow cells from primary transplants were successfully serially transplanted to secondary and tertiary recipient mice. Presumably, the ≈10-fold enrichment in long-term repopulating cells achieved with a 4-day 5-fluorouracil pretreatment (34) and the fact that 7 donor equivalents were infused into each primary lethally irradiated recipient (see *Materials and Methods*) compensated to some degree for the engraftment defect associated with *ex vivo* expansion (35). Nonetheless, it is significant that bone marrow precursors constitutively expressing mIL-11 were not overtly compromised in their long-term repopulating ability in that chronic exposure to mIL-11 did not rapidly induce stem cell "burnout." Such a result would have been expected if IL-11 were to function solely *in vivo* as a differentiative factor enhancing stem cell lineage commitment (9).

Conversely, a contribution of mIL-11 to the maintenance or expansion of hematopoietic stem cells was suggested by the results of the *in vitro* progenitor assays of bone marrow cells, which revealed a much higher frequency of G418-resistant colony-forming cells in the case of the tertiary MSCV-mIL-11 recipients than in control mice that received serially-transplanted MSCV-transduced marrow (Table 2). Although variations in proviral copy number were observed from mouse to mouse in both the experimental and control groups, more extensive contribution overall to the reconstituted hematopoietic systems of tertiary recipients by bone marrow cells transduced with the MSCV-mIL-11 virus was substantiated by Southern blot analysis (Fig. 3*E*, and data not shown). We cannot, however, exclude the possibility that more efficient transduction of the most primitive hematopoietic stem cell subset at the outset by the MSCV-mIL-11 virus than by the parental MSCV virus—e.g., because of more frequent entry of these cells into cycle owing to microenvironmental presentation of mIL-11 during coculture with the GP+E-86/MSCV-mIL-11 producer line (36)—contributes to the "enhanced" proliferative longevity of mIL-11-producing cells compared with control MSCV-transduced cells. Others have used the competitive repopulation technique, which directly compares long-term repopulating potential of two populations of donor hematopoietic cells in lethally irradiated recipients, as a quantitative assay of stem cell content (37). This assay was not employed in the present study because of potential complications resulting from the presumed paracrine action of constitutively secreted mIL-11 on competitor normal or MSCV-

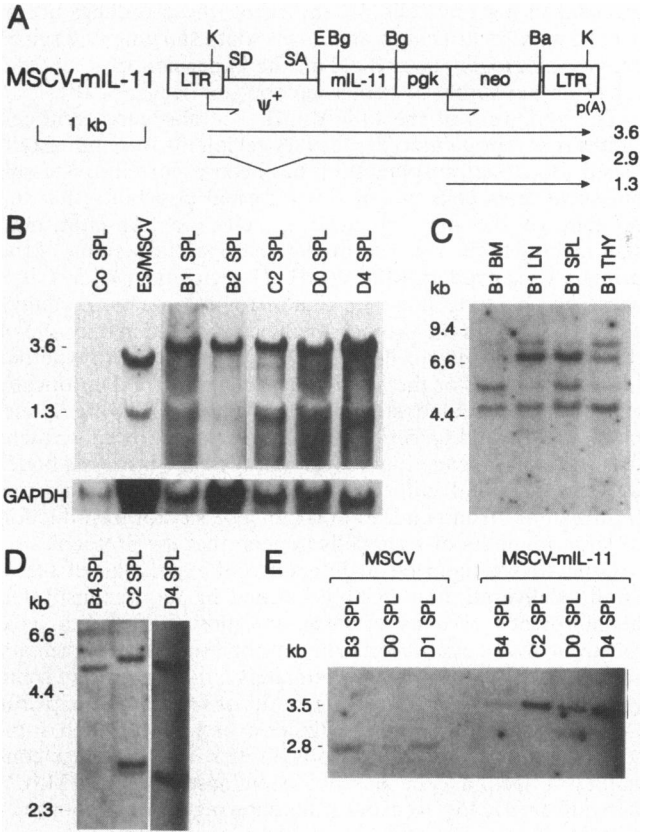


FIG. 3. Expression and distribution of MSCV-mIL-11 proviruses in hematopoietic tissues of long-term reconstituted tertiary bone marrow transplant recipients. (*A*) Structure of the MSCV-mIL-11 retroviral vector. The mIL-11 cDNA is translated from retroviral LTR-directed 3.6-kb (full-length viral RNA) and 2.9-kb (spliced) transcripts which also contain *neo* sequences. The 2.9-kb spliced mIL-11 mRNA is normally present as a minor species. The *neo* gene is transcribed from the murine *pgk* promoter as a 1.3-kb mRNA; p(A) indicates the polyadenylylation site for all transcripts. SD, splice donor; SA, splice acceptor; $\psi^+$, extended packaging region. Shown are the cleavage sites for the *Bam*HI (Ba), *Bgl*II (Bg), *Eco*RI (E), and *Kpn*I (K) restriction endonucleases. (*B*) Northern blot analysis of total cellular RNA (10 μg) prepared from spleens (SPL) of tertiary MSCV-mIL-11 recipients B1, B2, C2, D0, and D4, 5 months posttransplant. The blot was sequentially hybridized with a *neo* probe, a mIL-11 probe (data not shown), and a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences. The expected mIL-11 and *neo* mRNAs are indicated (3.6 kb and 1.3 kb, respectively). Co SPL, spleen RNA from a naive mouse. ES/MSCV, RNA from embryonic stem cells transduced with the parental MSCV virus. (*C*) Southern blot analysis of *Bam*HI-digested DNA (10 μg) from bone marrow (BM), lymph node (LN), spleen (SPL), and thymus (THY) of tertiary MSCV-mIL-11 recipient, B1, with a *neo* probe. The sizes of the molecular weight standards (*Hin*dIII-digested λ phage DNA) are indicated on the left. (*D*) Southern blot analysis of *Eco*RI-digested DNA (10 μg) from spleens of tertiary MSCV-mIL-11 recipients, B4, C2, and D4, with a *neo* probe. The sizes of the molecular weight standards (*Hin*dIII-digested λ phage DNA) are indicated on the left. (*E*) Southern blot analysis of *Kpn*I-digested DNA (10 μg) from spleens of tertiary MSCV and MSCV-mIL-11 recipients with a *neo* probe. Structurally intact MSCV and MSCV-mIL-11 proviruses are indicated (2.8 and 3.5 kb, respectively). Note that of the tertiary recipients examined at the time of sacrifice, proviral sequences could be detected in a greater proportion of the animals reconstituted with MSCV-mIL-11-transduced cells (7/10) than in those reconstituted with MSCV-transduced cells (2/9). Moreover, in the mice evaluated, MSCV-mIL-11 proviruses were present at a higher (≈6-fold) copy number on average than MSCV proviruses.

transduced marrow cells. In any event, these findings are in accord with other *in vitro* and *in vivo* data showing synergistic activity of exogenous rhIL-11 in the expansion of long-term repopulating ability of hematopoietic stem cells (8, 10).

The pedigrees of the MSCV-mIL-11-transduced stem cell clones that repopulated the tertiary recipients were not established. Because transplantation has been reported to activate quiescent stem cells, it remains a formal possibility that the progeny of the marked ancestor cells did not contribute significantly to the reconstituted hematopoietic systems of the primary or secondary MSCV-mIL-11 recipients (38, 39). It is worth emphasizing, however, that retroviral infection requires cell division, with the provirus being established in only one of the pair of daughter cells (40). Thus, irrespective of whether the ancestor cells or their clonal progeny remained mitotically active the entire time, stem cell totipotency following *ex vivo* manipulation and successive serial transplantation subsequent to retroviral transduction was demonstrated. This result bodes well for potential clinical use of rhIL-11 in bone marrow transplantation and underscores the necessity for transduction of large numbers of stem cells in gene therapy protocols.

Others have reported maintenance of expression of retrovirally delivered nonselectable genes in the reconstituted hematopoietic systems of mice; but previous studies have mostly analyzed expression in hematopoietic tissues of primary recipients for periods of approximately 6 months and, in some instances, spleen colony-forming units of secondary recipients (41–44). Interestingly, the extinction of LTR-directed transcription routinely seen with other retroviral constructs containing a second gene has not been observed with MSCV derivatives (41, 45). Recent results showing efficient transduction of primitive human hematopoietic precursors, including those having a $CD34^{+}CD38^{low/-}$ phenotype, with MSCV-based recombinant retroviruses suggest that the MSCV design might also prove useful in human stem cell gene transfer applications (46).

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