Distinct role of gp130 activation in promoting self-renewal divisions by mitogenically stimulated murine hematopoietic stem cells

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Previous studies have demonstrated hematopoietic stem cell amplification *in vitro* after the activation of three cell-surface receptors: flt3/flk2, c-kit, and gp130. We now show flt3-ligand and Steel factor alone will stimulate >85% of c-kit$^+$Sca-1$^+$lin$^-$ adult mouse bone marrow cells to proliferate in single-cell serum-free cultures, but concomitant retention of their stem cell activity requires additional exposure to a ligand that will activate gp130. Moreover, this response is restricted to a narrow range of gp130-activating ligand concentrations, above and below which hematopoietic stem cell activity is lost. These findings indicate a unique contribution of gp130 signaling to the maintenance of hematopoietic stem cell function when these cells are stimulated to divide with additional differential effects dictated by the intensity of gp130 activation.

IL-6 | serum-free culture

Over the last few years, an increasing body of evidence has accumulated to suggest an important role of the gp130 signaling receptor in the amplification of hematopoietic stem cells. This role was first suggested by the finding of reduced numbers of primitive hematopoietic cells in gp130 null mice (1) and the concomitant identification of IL-11 as a potent cooperating factor with Steel factor (SF) and flt3-ligand (FL) in stimulating the expansion of murine hematopoietic stem cells *in vitro* (2–4). A large family of cytokines is now known to use gp130 as a signaling receptor. These include IL-6, leukemia inhibitory factor, and oncostatin M, as well as IL-11 (5, 6). gp130 is ubiquitously expressed but to be activated requires ligand binding of specific proteins (α receptors) whose cellular expression is more restricted, thus explaining the cell-specific responses elicited by different members of this cytokine family (6). Soluble forms of many of these α receptors retain their ligand-binding specificity and gp130-activating capacity (7, 8). This activity was first shown in studies with the soluble IL-6 receptor (sIL-6R), which enabled cells that do not express IL-6Rα (6, 7, 9, 10), including primitive human hematopoietic cells (11–15), to become IL-6-responsive. Subsequent experiments demonstrated that IL-6Rα^- cells could also be activated following exposure to a recombinant fusion protein composed of IL-6 joined to the sIL-6R by a flexible linker (16). This latter molecule was named hyperIL-6 (H-IL-6) because it displays a higher specific activity than the molar equivalent of IL-6 plus sIL-6R (16–18). Additional studies have suggested that this increased specific activity of H-IL-6 is because of its unique gp130 binding properties that result in decreased gp130 internalization (19).

We recently reported that H-IL-6 could substitute for IL-11 in stimulating normal adult murine bone marrow (BM) stem cell self-renewal divisions in short-term serum-free cultures containing SF and FL (20). In the present study, we have used H-IL-6 either alone or in combination with IL-11 to investigate the dependence of this stem cell response on the intensity of gp130 activation.

Methods

Mice. Breeding pairs of C57BL/6J-*Ly5.2* (B6), B6-Pep3b-*Ly5.1* (Pep), and C3H/HeJ-*Ly5.2* (C3) mice (originally obtained from commercial stocks of The Jackson Laboratory) and breeding pairs of W^{41}/W^{41}-Ly5.2 (W^{41}) (originally from Dr. J. Barker, The Jackson Laboratory) and derivative B6C3F1-Ly5.2/Ly5.2 (B6C3) and PepC3 F1-Ly5.1/Ly5.2 (PepC3) mice were generated at the BC Cancer Agency Joint Animal Facility (Vancouver) and maintained in microisolator cages with sterile water, food, and bedding. Animals received acidified water (pH 3.0) for the first 4–6 wk after irradiation.

Cells. Lineage marker negative (lin$^-$) BM cells from adult Pep or PepC3 donors were removed immunomagnetically (using a murine StemSep kit provided by StemCell Technologies) (21). Lin$^-$ or freshly isolated BM cells were further enriched for competitive repopulation units (CRU) by isolation of propidium iodide (PI)$^-$ Sca-1$^+$lin$^-$ or c-kit$^+$Sca-1$^+$lin$^-$ cells using a fluorescence-activated cell sorter (FACStar Plus; Becton Dickinson) after sequential incubations with 6 μg/ml of 2.4G2 [an anti-Fc receptor antibody (22)], a mixture of the same lin$^+$ antibodies listed above (all labeled with biotin), anti-Sca-1-FITC (PharMingen), anti-c-kit-APC and streptavidin-PE (PharMingen), and 1 μg/ml PI (Sigma) added to the final wash as described (20). Sca-1$^+$lin$^-$ or c-kit$^+$Sca-1$^+$lin$^-$ cells were collected in bulk in Hanks' balanced salt solution with 2% FBS (HF). Single c-kit$^+$Sca-1$^+$lin$^-$ cells were sorted directly using the FACS single cell deposition unit into the individual wells of a 96-well plate, each of which had been preloaded with 100 μl of serum-free medium plus cytokines (see below).

Cultures. A total of 1000 or 1500 Sca-1$^+$lin$^-$ or c-kit$^+$Sca-1$^+$lin$^-$ BM cells were cultured for the period indicated at 33°C in a humidified atmosphere of 5% CO_2 in air in 35-mm Petri dishes (StemCell Technologies) containing 2 ml of Iscove's medium supplemented with 1% BSA, 10 μg/ml bovine pancreatic insulin, and 200 μg/ml human transferrin (BIT; StemCell Technologies), 40 μg/ml low-density lipoproteins (Sigma), 10^{-4} M 2-mercaptoethanol, 2 mM glutamine, 100 ng/ml human FL (Immunex, Seattle, WA), 50 ng/ml murine SF (expressed in COS cells and purified at the Terry Fox Laboratory), and other cytokines, as indicated. The latter included IL-11 (Genetics Institute, Cambridge, MA), human IL-6 (Cangene, Mississauga,

Abbreviations: BM, bone marrow; CFC, colony-forming cells; CRU, competitive repopulating unit; FL, flt3-ligand; HF, Hanks' balanced salt solution with 2% FBS; sIL-6R, soluble IL-6 receptor; H-IL-6, hyperIL-6; SF, Steel factor; PI, propidium iodide.

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ON) with or without sIL-6R (R & D Systems), and H-IL-6 [expressed in *Pichia pastoris* and purified as described (16)]. At the end of the incubation period, the cells in suspension were removed and additional adherent cells, if present, detached by addition of 0.5 ml trypsin-EDTA (GIBCO/BRL) for 4 min at room temperature followed by ≥2 rinses with Iscove's medium containing 5% FBS. The combined cells were then centrifuged at 350 × *g* for 7 min, cell counts performed on the resuspended cells, and colony-forming cells (CFC) and CRU assays (see below) undertaken using appropriate aliquots. CFC numbers were determined by plating suitable aliquots of test cells in methycellulose medium (Methocult; StemCell Technologies) supplemented with 10 ng/ml mIL-3, 10 ng/ml hIL-6, 50 ng/ml mSF, and 3 units/ml human erythropoietin and scoring the colonies (containing ≥30 cells each) present after 12 days of incubation at 37°C (23).

Single c-kit$^+$Sca-1$^+$lin$^-$ cell cultures were examined after 2 days to confirm the presence of a single viable (refractile) cell in each well. After 10 days, clone sizes were determined by counting the total number of viable cells present in each well using an inverted microscope.

Transplantation Procedure and Assessment of Engrafted Mice. B6C3 (Ly5.2) recipients were given 950 cGy ^{137}Cs γ-rays, and W^{41} (Ly5.2) mice were given a sublethal dose of 400 cGy (23) before IV injection with freshly isolated purified Ly5.1 BM cells (50 to 8000/mouse) or one-tenth of the cells harvested from 10- or 11-day-old cultures initiated with 1000 to 1500 purified Ly5.1 BM cells plus (for the B6C3F$_1$ recipients only) 10^5 normal Ly5.2 BM cells as described (24). Usually 8–12 mice were injected for each cell suspension assessed. To determine the levels of repopulation obtained after transplantation of increasing numbers of CRU, larger doses of purified fresh BM cells (up to 8×10^4 lin$^-$ or 3×10^4 Sca-1$^+$lin$^-$ cells per mouse) or cultured c-kit$^+$Sca-1$^+$lin$^-$ cells (up to 5×10^5 cells per mouse) were injected. Engraftment was assessed 4 mo posttransplant after lysis of the red cells in recipient peripheral blood samples with NH_4Cl and staining of the leukocytes with biotinylated anti-Gr-1 and anti-Mac-1 (prepared in the Terry Fox Laboratory) for 30 min on ice, followed by a single wash in HF, and further incubation of the cells for 30 min with anti-CD45.1 (anti Ly5.1, clone A20-FITC prepared in the Terry Fox Laboratory) and SA-RPE, with addition of 1 μg/ml of PI to the second of two washes in HF. A minimum of 5000 cells were then analyzed on a FACSort (Becton Dickinson). Recipients were considered to be engrafted with at least 1 CRU only if all of the following three conditions were met: ≥1% of PI$^-$ cells were Ly 5.1$^+$, ≥0.4% of PI$^-$ Ly 5.1$^+$ cells were also Gr-1$^+$ and/or Mac-1$^+$, and ≥6% of PI$^-$ Ly5.1$^+$ cells were also lymphoid, as defined by their low orthogonal light scattering characteristics.

To assess the numbers of CRU regenerated in primary recipients, four to six mice (from original groups of 8–12 B6C3 or W^{41} mice) were randomly selected (including negative animals) and their BM cells transplanted into irradiated secondary recipients of the same strain (i.e., cells from primary B6C3 recipients were transplanted into secondary B6C3 mice given 950 cGy and cells from primary W^{41} recipients were transplanted into secondary W^{41} recipients given 400 cGy). Secondary B6C3 recipients were not cotransplanted with normal B6C3 BM cells because the doses of test cells from the primary mice were sufficient to ensure their survival.

CRU frequencies in the test cell suspensions were calculated from the proportions of negative mice in each group using the method of maximum likelihood (25) available in the L-CALC software (StemCell Technologies).

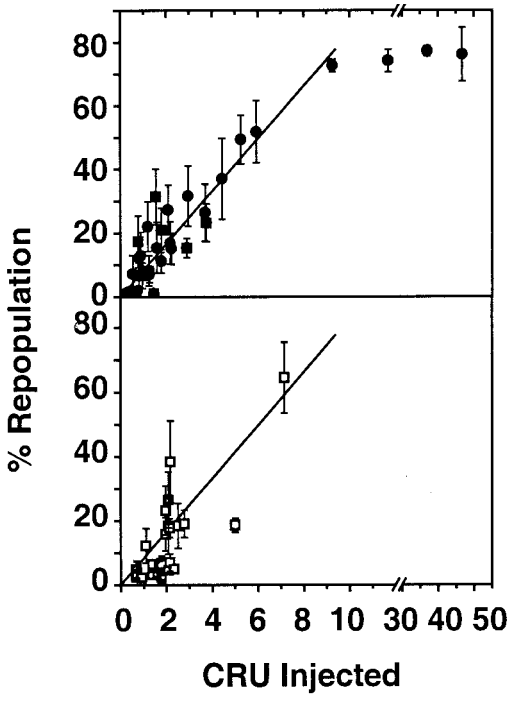

Fig. 1. Percent repopulation is linearly related to the number of CRU injected. Percent repopulation is shown as a function of the number of CRU injected into W^{41} recipients given a sublethal dose of radiation of 400 cGy (●) or B6C3 recipients given a lethal dose of 950 cGy and cotransplanted with 10^5 syngeneic BM cells (■). Levels of repopulation were assessed 4 mo after the transplant of either fresh (*Top*) or cultured (*Bottom*) BM cells from congenic Ly5.1 donors and are expressed as the proportion of lymphoid and myeloid donor-derived (Ly5.1$^+$) cells present in the peripheral blood. The regression line shown in the *Top* was determined by the method of least squares and has been redrawn in the *Bottom* to show its closeness of fit to the cultured cell data.

Statistical Analyses. Significance of differences between values was determined by either a one or two-tailed Student's *t* test, as appropriate. All error bars represent the SEM.

Results

Lympho-Myeloid Engraftment Levels Can Be Used to Infer the Number of Normal and Cultured Stem Cells Transplanted Over a Defined Range. To facilitate studies of the effect of different cytokine combinations and concentrations on stem cell expansion in 10-day cultures, we first explored the validity of using recipient lympho-myeloid engraftment levels to infer quantitative changes in the number of stem cells present in a given transplant inoculum. We have previously described a robust and specific assay for quantitating murine stem cells with long-term *in vivo* lympho-myeloid repopulating activity using a limiting dilution analysis procedure (3, 26, 27). The cells thus identified are referred to operationally as CRU. As shown in Fig. 1, the average level of donor-derived cells detected in the circulation of a large number of mice injected with independently quantitated CRU numbers was found to increase linearly (by 8.3 ± 0.4% per CRU) as a function of the number of test CRU initially injected, for transplant doses of up to ≈8 test CRU per recipient. Importantly, in the linear range, the average number of cells produced by each CRU was the same for both freshly isolated CRU (Fig. 1, *Top*) and CRU that had been amplified *in vitro* (Fig. 1, *Bottom*), independent of the purity of the test CRU injected (data not shown) or the types of recipients used. We also assessed the number of CRU generated in primary recipients of cells that had been previously stimulated with different cytokines

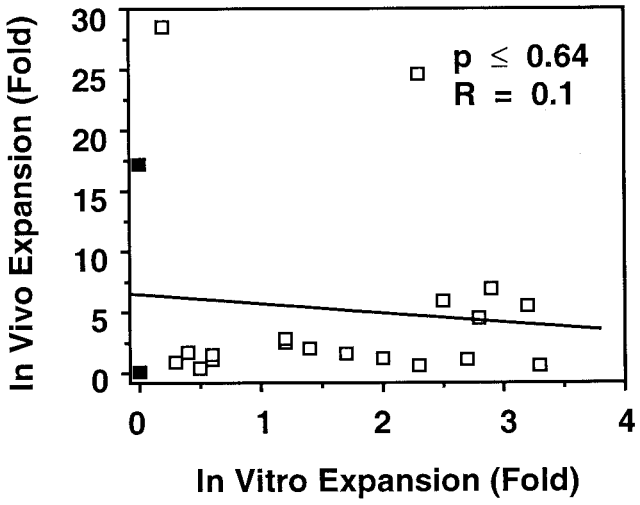

Fig. 2. Expansion of CRU in primary recipients is independent of the extent of prior CRU expansion *in vitro*. Primary recipients of fresh (■) or cultured BM cells (□) containing limiting numbers of CRU were killed 4 mo posttransplant in 21 experiments. Similar numbers of their BM cells were then assayed in secondary recipients to determine the frequency and hence the number of CRU regenerated from the original fresh or cultured CRU injected into the primary recipients. Fold expansions *in vivo* were calculated by dividing the number of CRU detected 4 mo posttransplant in primary recipients by the corresponding number of CRU calculated to be present initially in the cell suspension injected. Cultured cells were obtained by incubating Sca-1$^+$lin$^-$ mouse BM cells for 11 days with SF (50 ng/ml) and FL (100 ng/ml) and different combinations and concentrations of gp130-activating cytokines. Fold expansions *in vitro* were calculated by dividing the number of CRU detected in the 11-day cultures by the corresponding number of CRU calculated to be present in the cells used to initiate each culture. The regression line was obtained by the method of least squares.

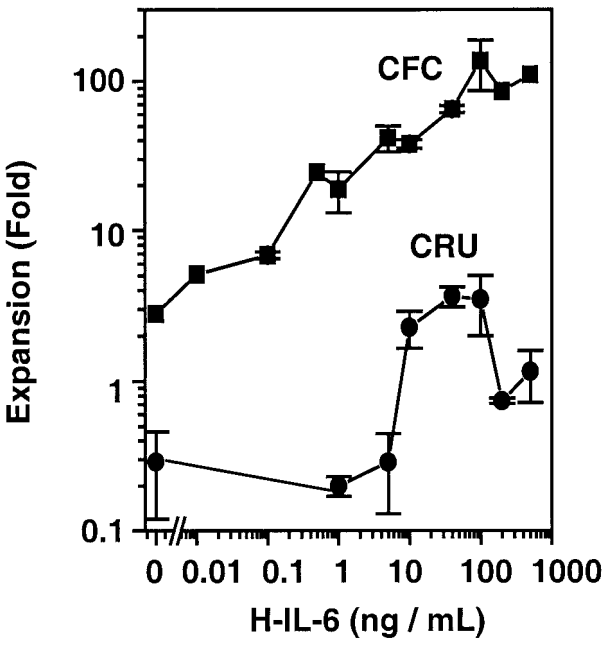

Fig. 3. Dependence of CRU and CFC expansion *in vitro* on the concentration of H-IL-6 present. Sca-1$^+$lin$^-$ mouse BM cells were cultured in serum-free medium containing SF (50 ng/ml) and FL (100 ng/ml) and varying concentrations of H-IL-6. The fold increase in CRU and CFC (±SEM, n = 2–4) was calculated by dividing the number of these cells detected after 10 days by the corresponding numbers present in the number of Sca-1$^+$lin$^-$ cells used to initiate each culture.

in vitro. This measurement involved sacrificing the primary recipients in 21 different experiments 4 mo posttransplant and quantitating the number of test cell-derived CRU present in their marrow by transplantation of limiting numbers of CRU into appropriate secondary recipients. The extent of expansion of both fresh and cultured CRU posttransplant was highly variable in individual experiments (ranging from 0.1- to 18-fold and 0.1- to 28-fold, respectively). However, as shown in Fig. 2, on average, there was no evidence that this *in vivo* behavior was affected either by the extent to which the CRU had been previously amplified *in vitro* (Fig. 2, $r = 0.1$), or by the particular cytokine mixture to which the cells had been exposed (data not shown). Based on these findings, average levels of donor-derived lymphoid and myeloid blood cells were used to assess changes in CRU numbers in the experiments described below.

Dose–Response Effects of gp130-Activating Ligands on CRU Amplification *in Vitro*. As shown in Fig. 3, the addition of 40–100 ng/ml H-IL-6 (to a base mixture of 50 ng/ml SF and 100 ng/ml FL) gave near maximal expansions of both CRU and CFC (4-fold and 100-fold, respectively). However, a significantly ($P < 0.05$) enhanced expansion of the CFC population was already achieved with H-IL-6 concentrations of <1 ng/ml (5-fold greater expansion of CFC numbers than in cultures containing SF and FL only), and the H-IL-6 dose–response curve showed a continuous and linear increase in log CFC expansion as a function of the log H-IL-6 concentration over the full 10^5-fold range of H-IL-6 concentrations tested. In contrast, there was no net expansion in CRU numbers in the same cultures until at least 5 ng/ml H-IL-6 was added. Very high concentrations of H-IL-6 (>100 ng/ml) also proved inhibitory to CRU but not to CFC expansion. Thus, when 200–500 ng/ml H-IL-6 was added, the yield of CRU after 10 days was no longer expanded above the input value but the number of CFC generated in the same cultures was even higher than that obtained when 40–100 ng/ml H-IL-6 was present. The ability of an optimal concentration of H-IL-6 (40 ng/ml) to amplify CRU numbers was then compared with a slightly lower molar equivalent concentration of sIL-6R + IL-6 (400 ng/ml + 20 ng/ml, respectively), or IL-6 alone (20 ng/ml) or IL-11 (at a dose subsequently determined to be on the plateau of the IL-11 dose–response curve for CRU amplification; unpublished observations) in the same type of 10-day serum-free cultures, all of which contained 50 ng/ml SF and 100 ng/ml FL. As shown in Fig. 4, all gp130-activating cytokines tested in these latter experiments cooperated with SF and FL to maintain or expand the input CRU population and to enhance the expansion of the CFC population (relative to that obtained with SF and FL alone). Addition of sIL-6R on its own had no significant effect on CRU yields ($P > 0.2$), and IL-6 appeared slightly (but not significantly, $P < 0.14$) less effective than H-IL-6. Addition of IL-6 plus sIL-6R or IL-11 gave results equivalent to H-IL-6 ($P > 0.2$ and $P > 0.3$, respectively). Addition of IL-11 + IL-6 + sIL-6R + H-IL-6 resulted in a net loss of CRU, mirroring the decreased CRU numbers obtained in the presence of excess concentrations of H-IL-6 alone (Fig. 3). For CFC, the effects obtained with these different cytokine combinations also paralleled those predicted by the H-IL-6 dose–response curve, although the combination of sIL-6R and IL-6 in this case was less potent than H-IL-6 ($P < 0.03$). Further evidence of the ability of H-IL-6 and IL-11 to substitute for one another in terms of their effect on very primitive mouse BM cells was obtained from a time course study of CRU expansion in serum-free cultures of Sca-1$^+$lin$^-$ cells. As can be seen in Fig. 5, neither the addition of 40 ng/ml H-IL-6 nor of 100 ng/ml IL-11 to 50 ng/ml SF and 100 ng/ml FL was able to stimulate a significant increase in CRU numbers until after the cells had been in culture for at least 5 days and a

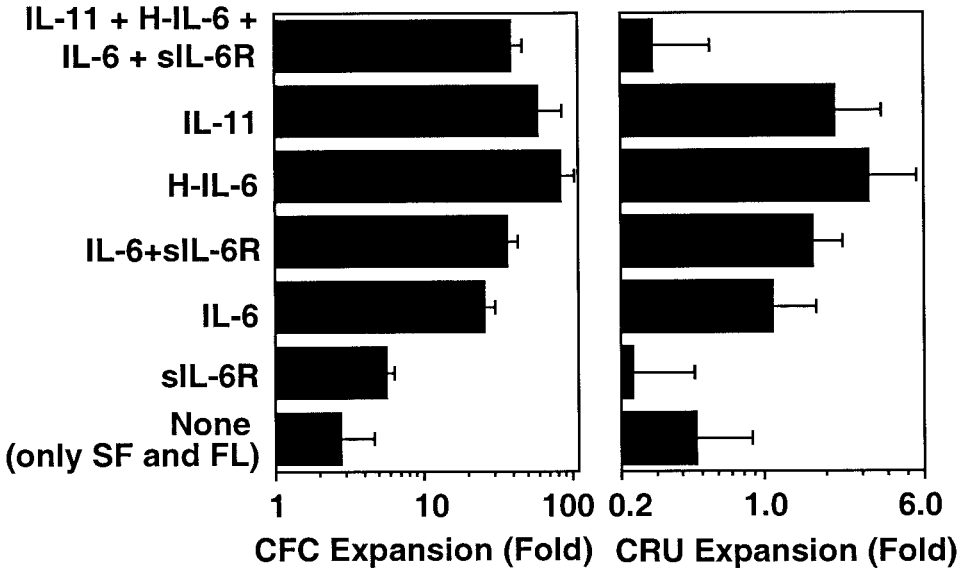

Fig. 4. Similar dose effects of different gp130-activating cytokines on CRU and CFC expansion. Sca-1$^+$lin$^-$ mouse BM cells were cultured in serum-free medium containing SF (50 ng/ml) and FL (100 ng/ml) and various gp130-activating cytokines at the following concentrations: IL-6 at 20 ng/ml, sIL-6R at 400 ng/ml, IL-11 at 100 ng/ml, H-IL-6 at 40 ng/ml when used alone, and H-IL-6 at 10 ng/ml when used in the four-factor combination. The fold increase in CRU and CFC (±SEM, $n = 4–8$) was calculated by dividing the number of these cells detected after 11 days by the corresponding numbers present in the number of Sca-1$^+$lin$^-$ cells used to initiate each culture.

similar maximal increase was attained by day 11 with either H-IL-6 or IL-11 present.

Lack of Requirement of gp130 Activation to Stimulate c-kit$^+$Sca-1$^+$lin$^-$ Cell Proliferation. The results of the H-IL-6 dose–response experiments indicated a requirement for intense gp130 activation to obtain a net increase in CRU numbers. To determine whether this reflected a dependence of CRU viability or mitogenesis on gp130 activation or a separate effect on maintenance of stem cell competence, the effect of different cytokine combinations on the proliferation of highly enriched CRU-containing c-kit$^+$Sca-1$^+$lin$^-$ BM cells was examined in single cell serum-free liquid cultures. Fig. 6 presents the results of two representative experiments in which it can be seen that SF and

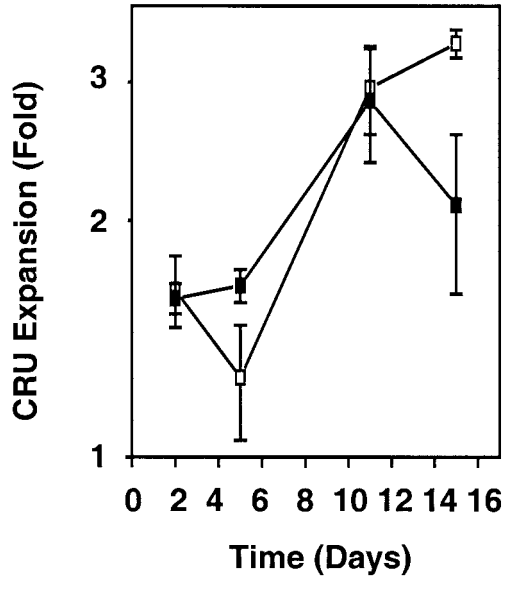

Fig. 5. The kinetics of CRU expansion in cultures supplemented with IL-11 or H-IL-6 is similar. Changes in CRU numbers are shown as a function of time when Sca-1$^+$lin$^-$ mouse BM cells were cultured in serum-free medium containing SF at 50 ng/ml and FL at 100 ng/ml and either IL-11 at 100 ng/ml (□) or H-IL-6 at 40 ng/ml (■). The fold increase (±SEM, $n = 2–4$) was calculated by dividing the number of CRU detected in the cultured populations by the number of CRU present in the number of cells used to initiate each culture.

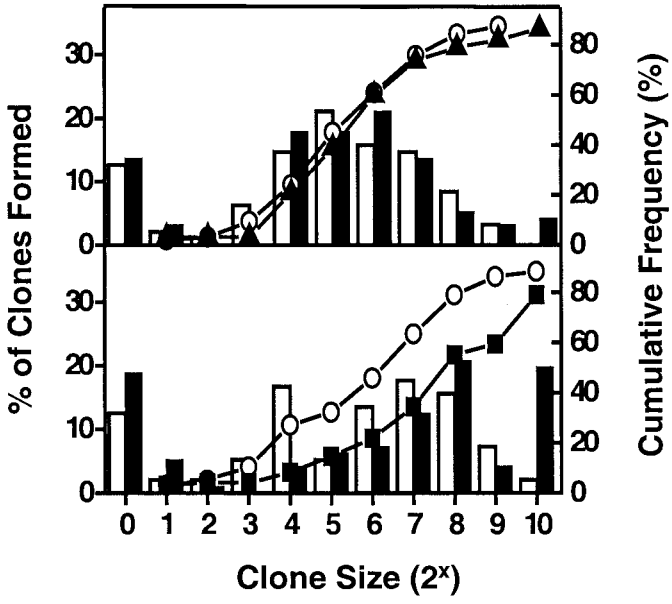

Fig. 6. Presence of gp130-activating cytokines has no effect on the survival or initial mitogenesis c-kit$^+$Sca-1$^+$lin$^-$ cells also stimulated by SF and FL. Clone size distribution (bars) and cumulative frequency of clones (symbols) generated in 10 days by single PI$^-$ c-kit$^+$Sca-1$^+$lin$^-$ mouse BM cells incubated in serum-free medium supplemented with SF (50 ng/ml) and FL (100 ng/ml) in combination with (*Top*) IL-6 at 50 ng/ml (closed bars and ▲) or no other cytokine (open bars and ○, $n = 95$ wells); (*Bottom*) H-IL-6 at 40 ng/ml (closed bars and ■, $n = 96$ wells) or no other cytokine (open bars and ○, $n = 96$ wells).

FL alone were able to stimulate most of the cells (87%, *Top*; 88%, *Bottom*) to divide and that the additional presence of either 50 ng/ml IL-6 (*Top*) or 40 ng/ml H-IL-6 (*Bottom*) did not increase the number of clones obtained, although there was a obvious increase in the maximum size of clones obtained within 10 days.

Discussion

The present studies reveal an important role of gp130 activation in promoting hematopoietic stem cell self-renewal divisions, independent of the particular ligand used to stimulate this signaling receptor. Thus, at optimal concentrations, H-IL-6, a fusion protein of IL-6 and sIL-6R (16), IL-6 plus sIL-6R, and IL-11 were all able to ensure equivalent maximal amplification (4-fold within 10–14 days) of murine hematopoietic stem cells costimulated with SF and FL. Recently, hematopoietic tissue has been found to contain rare cells that do not immediately display transplantable stem cell activity but may acquire this potential following appropriate cytokine stimulation *in vitro* before injection (28–32). It is thus possible that recruitment of cells into the detectable CRU pool, as well as amplification of preexisting CRU, may be regulated by gp130 activation. Future studies with differently purified subsets of lin^- cells should make it possible to address this question. Similarly, it is possible that concomitant activation of other pathways might further enhance short-term CRU self-renewal. For example, recently it has been reported that thrombopoietin might promote the amplification of human "CRU" assayed in NOD/SCID mice (33). However, in previous experiments, we were unable to demonstrate a similar effect on cultured CRU from adult mouse bone marrow (3).

An unexpected finding was the different dose–response relationship found between the concentration of H-IL-6 added and the number of CFC and CRU obtained within a 14-day period of culture. This difference was manifested in three ways: (*i*) a 1000-fold higher H-IL-6 concentration was required to produce a net increase in CRU vs. CFC, (*ii*) the range of H-IL-6 concentrations over which a net increase in CRU could be achieved was much narrower than that able to stimulate CFC expansion (10 vs. 10^5-fold, respectively), and (*iii*) exposure to very high doses of H-IL-6 (>100 ng/ml) resulted in a loss of CRU but not CFC activity. Because these effects were also obtained using other gp130-activating ligands, they are most likely indicative of differences in signaling events that have unique biological consequences in the most primitive subsets of hematopoietic cells when the intensity of gp130 activation is varied.

In the present study, analyses of single cell cultures demonstrated that gp130 activation was not required for either the survival or initial mitogenesis of c-kit^+Sca-1^+lin^- cells because >85% of these proliferated, even in the absence of gp130-activating cytokines when adequate concentrations SF and FL were present. The measured frequency of CRU in the c-kit^+Sca-1^+lin^- population is ≈1 in 50 (34) (also confirmed here; data not shown), but the absolute frequency of cells with this potential is likely to be 3- to 10-fold higher, assuming a seeding efficiency of approximately 20% (35). Thus, numerically, it can be assumed that very few CRU would be accounted for in the c-kit^+Sca-1^+lin^- cells not responding to SF and FL. We have previously shown that, in the presence of SF, FL, and either IL-11 or H-IL-6, no CRU remain quiescent beyond 3 days *in vitro* (20). The H-IL-6 dose-dependent changes in CRU yields seen must therefore, at least at the level of the input cells, reflect a signaling intensity-mediated control of the intracellular mechanisms that promote or block CRU differentiation. A number of examples where receptor-activated signaling intensity can regulate such processes within (36–42) as well as outside the hematopoietic system (43–46) have been reported. These suggest a generalized mechanism of stem cell differentiation control in which a threshold effect of ligand-receptor signaling is important (47).

The high-dose gp130-mediated inhibition of CRU activity that we documented here is reminiscent of the decreased biological response observed by others in similar studies of hyperstimulated cell lines (48, 49). It has been suggested that the mode of assembly of the IL-6 receptor complex could constitute one element of regulation by which organisms can respond to surplus IL-6 (50). A similar self-antagonizing capacity is already known for insulin, which also signals by means of a homodimeric receptor (51, 52). Thus, according to the assumption of a tetramer-hexamer shift (53), lower concentrations of IL-6/IL-6R would lead to the formation of active tetramers, whereas higher concentrations would shift the tetramers into inactive hexamers. It is not known, however, if a tetramer–hexamer shift exists for H-IL-6, and we can only speculate that such a mechanism might explain the apparent self-antagonization seen when hematopoietic stem cells are exposed to very high concentrations of gp130-activating cytokines.

We also investigated the *in vivo* differentiation and self-renewal activity of CRU that had been expanded *in vitro*. CRU differentiation activity was inferred from the numbers of progeny lymphoid and myeloid cells detected in the blood of recipient mice 4 mo posttransplant. The outputs observed are consistent with those reported previously (54), but the more extensive data obtained in the present study allowed a significant regression ($P \leq 0.0001$) relating percent repopulation to the number of CRU transplanted (for up to 8 CRU per recipient) to be revealed. Assessment of the extent of further CRU amplification after their transplantation into primary recipients also demonstrated a lack of effect of their prior *in vitro* manipulation on subsequent *in vivo* self-renewal activity. Because expression of this activity is strongly influenced by the number of CRU transplanted (per recipient) (55), particular care was taken here to use a restricted range of CRU numbers in the inocula injected into the primary mice. We were also able to confirm that both types of recipient mice used to test for CRU function (W^{41} mice given a sublethal dose of radiation and +/+ mice given a lethal dose of radiation plus an accompanying graft of 10^5 normal BM cells) give similar results. Taken together, these findings validate the use of average engraftment levels (below 60–70%) to measure injected CRU numbers in a variety of experimental settings, which should facilitate further investigation of the mechanisms that regulate their activity both *in vitro* and *in vivo*.

In summary, we have demonstrated an important role of gp130-activating cytokines in amplifying stem cell yields from populations that can be independently stimulated to survive and proliferate by exposure to other cytokines (SF and FL). This finding points to a separate molecular mechanism regulating the ability of hematopoietic stem cells to divide and the subsequent retention or loss of their stem cell potential by alterations in the level and/or intracellular distribution of signaling intermediates that can be triggered by gp130 activation. One candidate for such a role might be STAT3. STAT3 is known to be phosphorylated by cytokines that activate gp130 (5, 56–58), and its functional blockade can affect stem cell differentiation in cell line models (59, 60). Further pursuit of this hypothesis is ongoing in our laboratory.

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