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Hematologic effects of stem cell factor (SCF) and leukemia inhibitory factor (LIF) *in vivo*: LIF-induced thrombocytosis in SCF-primed mice

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Abstract: Stem cell factor (SCF) administered as daily bolus injections in dose-response experiments in mice causes a progressive and dramatic dose-dependent panleukocytosis characterized by neutrophilia, eosinophilia, monocytosis, and lymphocytosis. SCF causes circulating platelet numbers to be dose-dependently increased after 2 weeks of daily injections. Leukemia inhibitory factor (LIF) administered as daily bolus injections in mice causes a peripheral leukopenia that is largely due to peripheral lymphopenia. LIF causes thrombocytosis peaking after approximately 1 w. Coinjection of SCF and LIF for 1 to 2 wk in mice does not cause a much greater thrombocytosis than the maximum thrombocytosis achievable with SCF or LIF alone. On the other hand, daily injection of SCF for 5 days followed by daily injection of LIF for 5 to 6 d in mice causes a very substantial increase in platelets that was lineage-specific in terms of not being accompanied by a generalized leukocytosis. In contrast, only a very modest thrombocytosis was noted in SCF-primed LIF-treated rats. LIF causes a large increase in the cytoplasmic volume of splenic megakaryocytes in mice, but not in rats. In conclusion, SCF-induced priming followed by LIF-induced maturation of megakaryocytes causes a substantial selective increase in the numbers of circulating platelets in mice.

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Stem cell factor (SCF) is a growth factor that is the product of the steel (*Sl*) locus of the mouse and is a ligand for the c-kit proto-oncogene receptor encoded by the white spotting (*W*) locus of the mouse (1–3). Mice with mutations at *Sl* or *W* display a variety of similar developmental disturbances and hematologic defects, including macrocytic anemia and mast cell deficiency. SCF administered to rats at doses of up to 100 μg/kg causes mild increases in myelopoiesis and large increases in mast cells (4). SCF coinjected with G-CSF causes a synergistic myeloid hyperplasia in rats (5) and mice (6) and significantly blunts the mast cell growth seen in rats treated with SCF alone (5), suggesting that lineage-specific growth factors such as G-CSF can be used to direct the *in vivo* differentiation and maturation of a pool of SCF-responsive hematopoietic precursors. The *in vivo* synergism of SCF with a lineage-specific factor such as G-CSF is in accordance with the *in vitro* synergism between SCF and several colony-stimulating factors *in vitro* (2).

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that binds to a cellular transmembrane receptor that is structurally similar to one of the molecules involved in IL-6 signal transduction (7). LIF shares many biological effects with IL-6 (8). LIF (9–10) and IL-6 (11) both cause thrombocytosis. In addition to stimulating thrombocytosis, IL-6 promotes megakaryocytic differentiation (11, 12) as well as proliferation of other hematopoietic lineages (13, 14). Among hematopoietic cells, those of monocyte/macrophage lineage primarily express LIF receptors (15–17). Hepatocytes express very high levels of LIF receptors (15).

The purpose of the present *in vivo* study in mice and rats is to 1) examine the hematologic effects of daily administration of SCF at higher doses than previously reported, 2) examine the hematologic

effects of daily administration of LIF, 3) examine the thrombopoietic effects of daily coinjection of SCF and LIF, and 4) examine the thrombopoietic effects of LIF after priming with SCF.

Material and methods

Adult BALB/c mice weighing approximately 20 g and 8 wk of age (experiments performed at Amgen) or Lewis rats (experiments performed at University of California) were injected intraperitoneally with varying amounts of endotoxin-free polyethylene-glycol-modified recombinant *E. coli*-derived rat SCF, recombinant *E. coli*-derived human LIF, or carrier consisting of 1% normal mouse or rat serum in sterile saline. Peripheral whole blood (20 µl) obtained by tail bleeding of mice and rats was immediately transferred into 10 ml EDTA-containing Cell-Pack diluent (TOA Medical Electronics, Los Alamitos, CA) for the quantitation of absolute numbers of circulating leukocytes and platelets with an automated cell counter (Sysmex Cell Analyser, TOA Medical Electronics, Kobe, Japan). Blood smear differentials were stained with a modified Wrights-Giemsa

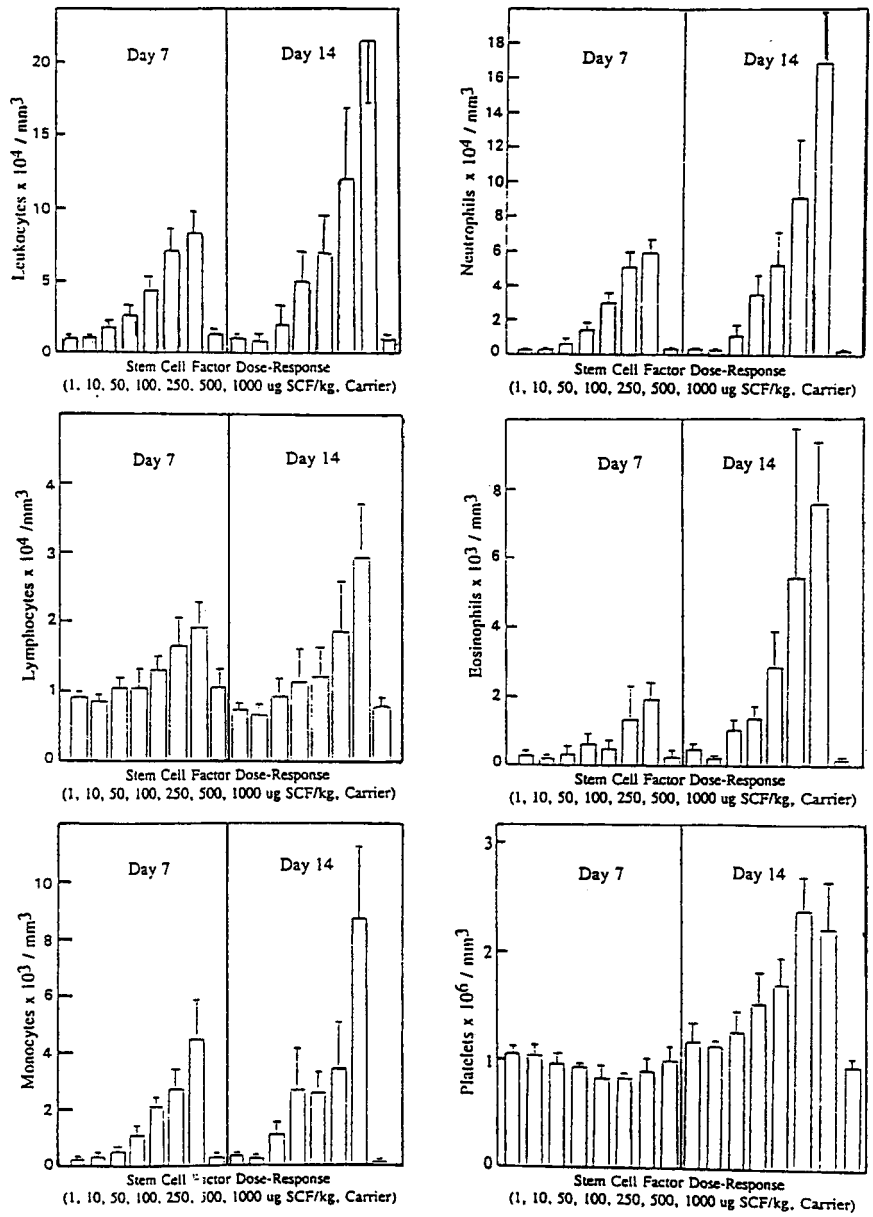

Fig. 1. Daily injection of SCF at increasing doses of 1, 10, 50, 100, 250, 500, and 1000 µg/kg causes a dose-dependent peripheral leukocytosis, neutrophilia, lymphocytosis, eosinophilia, and monocytosis increasing between 1 and 2 weeks (n = 5 in each group). SCF causes a dose-dependent decrease in circulating platelets at 1 week followed by a dose-dependent increase in platelets at 2 weeks. The values for carrier control mice (n = 5 in each group) are shown in the last bar.

method. Differentials were performed by identifying at least 100 cells per peripheral blood smear under oil immersion using previously described criteria for the identification of leukocytes (18). Bouin's-fixed paraffin-embedded histologic sections of spleens were stained with hematoxylin and eosin or the Congo red-acidified toluidine blue method (19), and extramedullary hematopoiesis was evaluated in double-blind fashion. Splenic megakaryocytopoiesis was assessed by counting the number of megakaryocytes per high power (× 400) microscopic field. Erythropoiesis and myelopoiesis were semiquantitatively graded as 0, 1+, 2+, and 3+ based on the amount of erythroid and myeloid precursors in the red pulp. In additional experiments, male Lewis rat weighing approximately 250 g were injected with LIF via the dorsal vein of the penis. Peripheral blood and bone marrow quantitative leukocyte differentials in these rats were performed as previously described (18). Data are presented as plus-or-minus one standard deviation and statistical analysis is by the independent t-test.

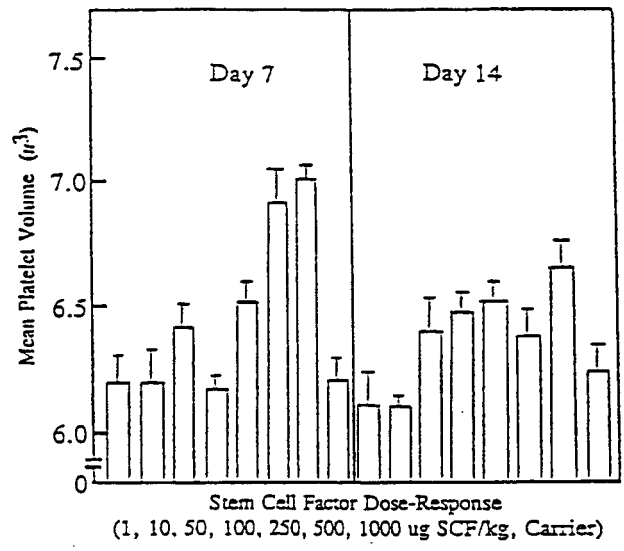

Fig. 2. Daily injection of SCF at increasing doses causes a dose-dependent increase in MPV at 1 and 2 weeks (n = 5 in each group). Note, however, that the increase in MPV is not progressive between 1 and 2 weeks and that the MPV actually decreases between 1 and 2 weeks in mice treated with the two highest doses of SCF. The values for carrier control mice are shown in the last bar.

Results

Dose-response experiments examined the peripheral hematologic effects of increasing doses of SCF in the mouse. SCF injected as daily bolus doses of 1, 10, 50, 100, 250, 500, and 1000 μg/kg for 1 to 2 wk causes a dose-dependent peripheral leukocytosis composed of an increase in leukocytes of all lineages as evidenced by neutrophilia, lymphocytosis, monocytosis, and eosinophilia (Fig. 1A–E). The increase in leukocytes generally becomes apparent at doses of 100 μg/kg and above and progresses between 1 and 2 wk with no evidence of a plateau (Figs. 1A–E). The effect of SCF on circulating platelets is more complex, showing a dose-dependent decrease in platelet numbers at 1 wk after daily injection of SCF followed by a dose-dependent increase in platelets at 2 wk after daily injection of SCF (Fig. 1F). SCF causes a dose-dependent increase in mean platelet volume (MPV) at both 1 and 2 wk (Fig. 2). However, the increase in MPV is not progressive between 1 and 2 wk (Fig. 2). In fact, the MPV decreases between 1 and 2 wk in mice treated with 500 to 1000 μg/mouse/d. The SCF-induced dose-dependent peripheral leukocytosis is accompanied by dose-dependent splenomegaly (data not shown). Histologically, splenomegaly is shown to be due to splenic extramedullary hematopoiesis replacing the red pulp and causing atrophy of the white pulp. The extramedullary hematopoiesis is characterized by increases in myeloid and erythroid precursors as well as in megakaryocytes and mast cells. Myeloid is more prominent than erythroid differentiation.

Dose-response experiments next examined the peripheral hematologic effects of LIF. LIF injected as daily bolus doses of 50, 250, 500, 750, and 1250 μg/kg for 2 wk causes a peripheral leukopenia at doses of 250 to 1250 μg/kg (Fig. 3A). Leukopenia does not occur at the dose of 50 μg/kg/d (data not shown). The leukopenia is of approximately the same magnitude and is thus not dose-dependent in the dose range of 250 to 1250 μg/kg/d (Fig. 3A). The LIF-induced leukopenia is largely due to lymphopenia (Fig. 3B) without significant decreases in the circulating numbers of other leukocyte subsets (data not shown). LIF causes thrombocytosis beginning after approximately 4 d, peaking at approximately 1 wk, and persisting after 2 wk (Fig. 3C). The increase in circulating numbers of platelets occurs at doses of 50 to 1250 μg/kg and is again of approximately the same magnitude at all doses (Fig. 3C). The MPV peaks at 2 to 4 d after injection of LIF and then gradually decreases towards control values by 2 wk (Fig. 3D). A mild, approximately two-fold increase in megakaryocytes was histologically noted in the spleens of mice treated with 25 μg LIF/mouse/d but not in the spleens of mice treated with lower doses of LIF (data not shown). No other evidence of increased splenic extramedullary hematopoiesis was histologically noted in LIF-treated mice. Splenomegaly was not appreciated in LIF-treated mice. LIF-treated mice demonstrated a decrease in body weight in the dose groups of 250 to 1250 μg/kg (data not shown). Total body weight did not change in carrier control mice or in mice treated with 50 μg/kg.

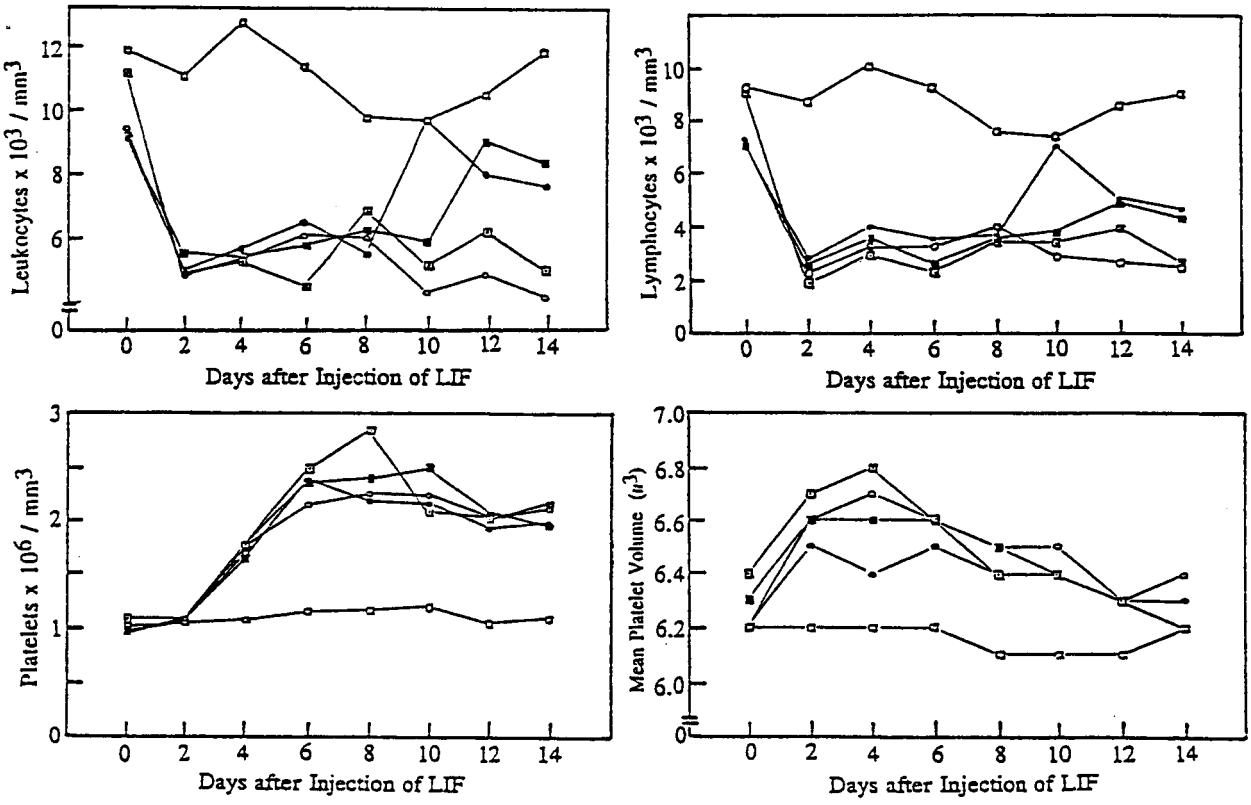

Fig. 3. Daily injection of LIF at doses of 250 to 1250 μg/kg causes peripheral leukopenia due to lymphopenia (□ = carrier, ● = 250, ■ = 500, ○ = 750, ⊡ = 1250 μg/kg, n = 5 mice at each timepoint). LIF causes a thrombocytosis that does not appear to be dose-dependent within the examined dose range. MPV increases rapidly within the first 2 to 4 days and then gradually returns towards control values by 2 weeks.

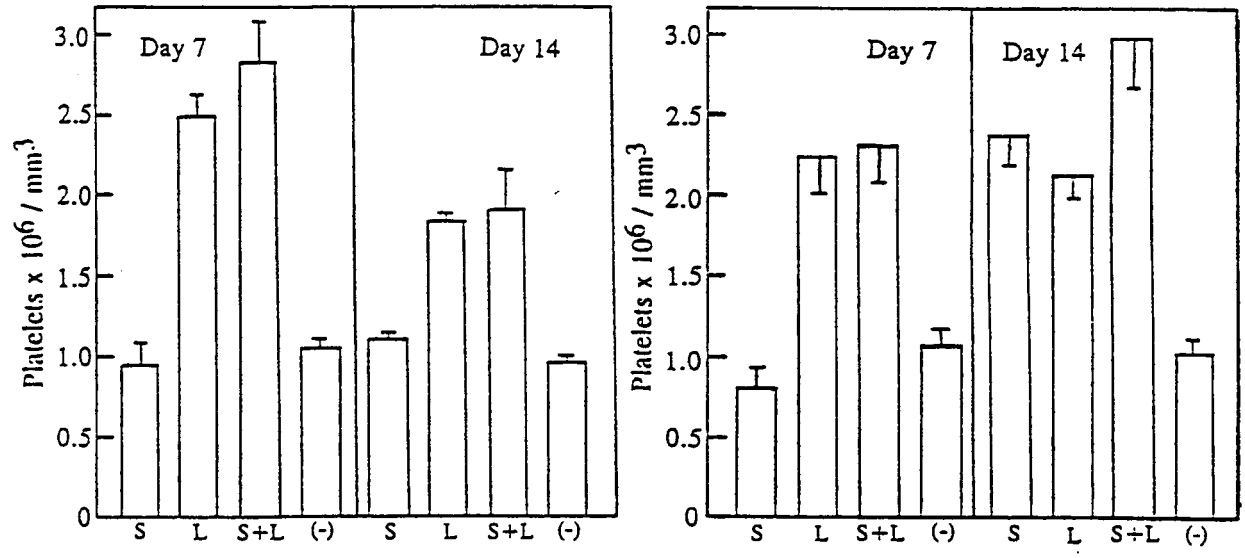

Fig. 4. Daily coinjection of SCF at 100 μg/kg (a dose at which SCF does not cause thrombocytosis) with LIF at 500 μg/kg does not cause an increase in platelets at 1 or 2 weeks that is significantly greater than the thrombocytosis induced by LIF alone (Fig. 4A: S = SCF, L = LIF), (-) = carrier; n = 10 mice/group on day 7; n = 5 mice/group on day 14). Daily coinjection of SCF at 500 μg/kg (a dose at which SCF does cause thrombocytosis by 2 weeks, but not at 1 week) with LIF at 500 μg/kg does not cause an increase in platelets at 1 week as compared to LIF alone, but does cause slightly increased circulating numbers of platelets at 2 weeks (Fig. 4B: S = SCF, L = LIF, (-) = carrier; n = 10 mice/group on day 7; n = 5 mice/group on day 14 in SCF and carrier groups and n = 4 mice/group in LIF and SCF + LIF groups).

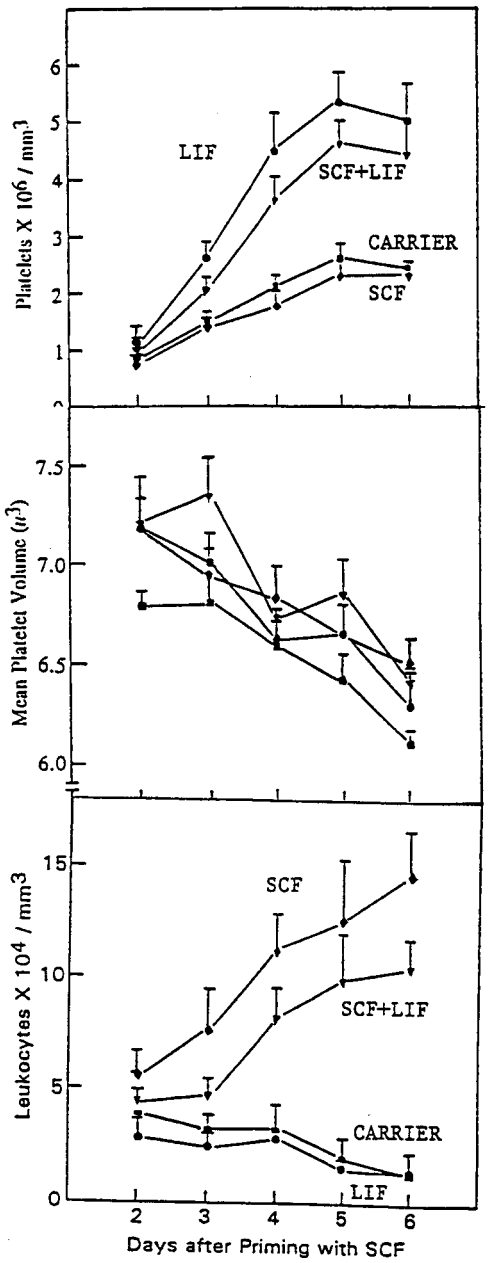

Fig. 5. Daily injection of SCF alone for 5 days (i.e. priming with SCF at a dose of 500 μg/kg that does not cause thrombocytosis after 5 days) followed by injection for 5 to 6 days with either carrier, SCF (500 μg/kg), LIF (500 μg/kg), or SCF plus LIF (n = 5 mice/time point in all groups) shows (A) an approximately 2- to 3-fold ensuing increase in platelets in mice treated with either carrier or SCF and an approximately 4- to 6-fold ensuing increase in platelets in mice treated with either LIF or SCF plus LIF, (B) a progressive decrease in MPV in mice in all experimental groups at the same time as circulating numbers of platelets are on the rise, and (C) the lineage-specific nature of the LIF-induced thrombocytosis since the LIF-treated mice do not show any evidence of leukocytosis whereas the mice treated with continued injections of SCF show the expected peripheral panleukocytosis.

Since both SCF and LIF cause thrombocytosis, the thrombopoietic effects of coinjection of SCF and LIF were investigated at 1 and 2 wk after daily injections. In the first set of experiments (Fig. 4A), SCF was injected at a dose of 100 μg/kg and LIF at a dose of 500 μg/kg. SCF itself does not cause thrombocytosis at either 1 or 2 wk at the dose of 100 μg/kg. LIF causes thrombocytosis at both 1 and 2 wk. The combination of SCF plus LIF does not increase platelet numbers beyond the increase noted with LIF alone. In the second set of experiments (Fig. 4B), SCF was injected at a dose of 500 μg/kg that does cause thrombocytosis at 2 wk, but not after 1 wk of daily injections. LIF was, as previously, injected at 500 μg/kg and caused the expected increase in platelets. The combination of SCF plus LIF again did not cause an increase in platelets beyond that caused by LIF alone at 1 wk. At 2 wk a very modest increase in circulating platelets was noted in the group treated with SCF plus LIF as compared to SCF ($p < 0.01$) or LIF alone.

The effect of "priming" with SCF followed by "lineage-specific differentiation" with LIF was next studied. Mice received daily injections of SCF at 500 μg/kg for 5 d followed by 2, 3, 4, 5, or 6 d of daily injections of either carrier, SCF, LIF, or SCF plus LIF. Although SCF did not cause thrombocytosis after 5 d of injection, thrombocytosis was observed in all groups on d 2 through 6 after priming with SCF (Fig. 5A). A two-fold increase in platelets was noted in mice treated with either continued SCF of with carrier for 6 d after priming with SCF. A four-fold to greater than five-fold increase in platelets, respectively, was noted in mice treated with SCF plus LIF or with LIF alone for 6 d after priming with SCF. A progressive decrease in MPV in all experimental groups is observed at the same time as circulating numbers of platelets are on the rise (Fig. 5B). The LIF-induced thrombocytosis is not accompanied by a panleukocytosis, whereas the mice treated with continued injections of SCF or SCF plus LIF show the remarkable peripheral panleukocytosis associated with high-dose administration of SCF (5C). SCF-primed mice in both the LIF- and SCF + LIF-treated groups lost approximately 20% to 25% of their total body weight on average whereas mice in the SCF- and carrier-treated groups did not lose any weight. The magnitude of thrombocytosis in SCF-primed LIF-treated mice is dependent upon the priming dose of SCF (Fig. 6).

Histologically, the spleens of all SCF-primed mice continued to show extramedullary hematopoiesis (myelopoiesis, erythropoiesis, and megakaryocytopoiesis) at 5 to 6 d after cessation of priming with SCF. Extramedullary hematopoiesis, predominantly myeloid in lineage, is most prominent in the spleens of SCF-primed mice who continue to receive SCF (Fig. 7A) either alone or in combination with LIF. The extent of splenic hematopoiesis thus correlates

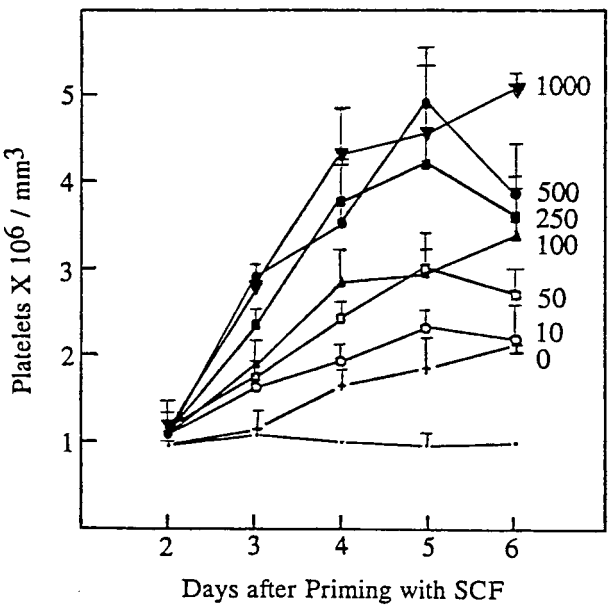

Fig. 6. The increase in platelets in SCF-primed LIF-treated mice is dependent upon the priming dose of SCF as shown by the increase in circulating platelets in mice primed for 5 days with 0, 10, 50, 100, 250, 500 and 1000 μg/kg SCF (n = 5 mice/group at all time points) followed by treatment with 500 μg/kg LIF in all groups (with the exception of the bottom line representing treatment with carrier alone).

with the presence of peripheral panleukocytosis. Extramedullary hematopoiesis occurs in the red pulp of the spleen. The extent of extramedullary hematopoiesis is therefore reflected by the proportion of the spleen occupied by red pulp as compared to the white pulp. The red pulp in normal control mice or in mice treated with carrier alone is approximately 40%. The red pulp in SCF-primed mice increases to around 60% in SCF-primed carrier-treated mice and to around 90% in SCF-primed SCF- or SCF + LIF-treated mice. Erythroid precursors, as contrasted to myeloid precursors, constitute a much more prominent proportion of the hematopoietic activity in the red pulp of SCF-primed mice subsequently treated with carrier or LIF (Fig. 7B) as compared to those subsequently treated with SCF or SCF plus LIF. All SCF-primed mice show an increase in splenic megakaryocytes as compared to normal control mice. The largest number of megakaryocytes per high power microscopic field is noted in SCF-primed LIF-treated mice. The size and cytoplasmic maturity of megakaryocytes is much greater in SCF-primed LIF-treated mice than in SCF-primed SCF- or carrier-treated mice (Fig. 7). The foregoing histologic observations were confirmed to be statistically significant by blinded semiquantitative grading as described in the Methods (data not shown).

The effects of LIF at 40 μg/kg, i.e. at a relatively low dose as compared to the above-described experiments in mice, were also studied in rats in which quantitative marrow differentials were performed. LIF at 40 μg/kg causes an almost two-fold throm-

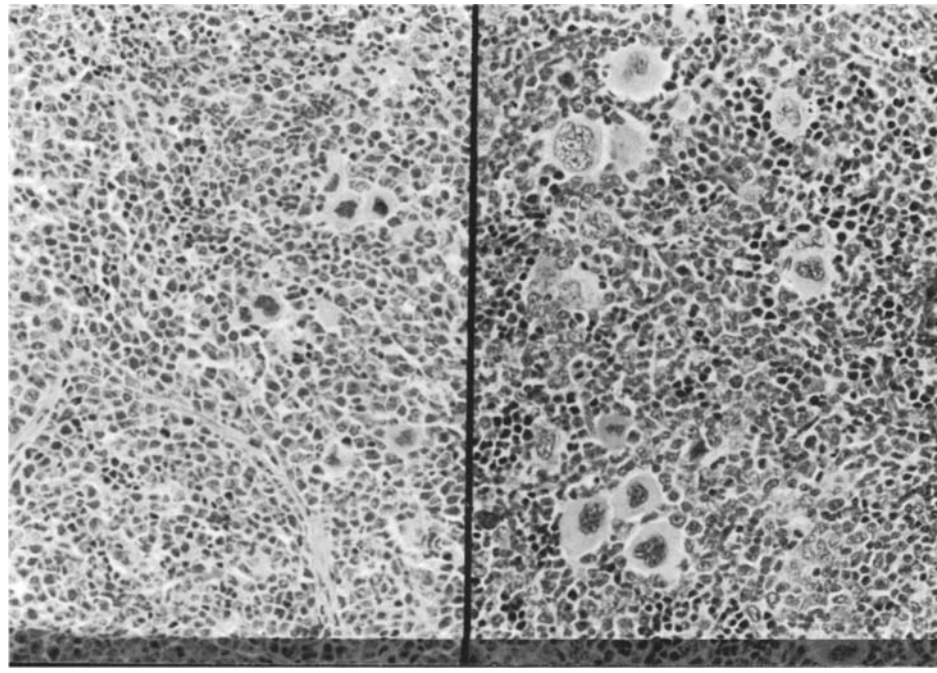

Fig. 7. Histologic examination of the red pulp of spleens of SCF-primed SCF-treated mice shows an increase in predominantly myeloid extramedullary hematopoiesis (16A). SCF-primed LIF-treated mice demonstrate extramedullary hematopoiesis with a greater amount of erythropoiesis and an increase in number and especially in size of megakaryocytes (16B). Compare the size and maturity of the megakaryocytes in the adjacent photomicrographs taken at the same magnification (× 400).

bocytosis after 1 wk of daily injections in rats with a peak thrombocytosis of slightly over two-fold after 10 d of LIF and a return of platelet numbers towards control values at 2 wk (data not shown). As measured at 24 hours after each daily injection of 40 μg/kg in rats, LIF did not cause significant changes in circulating leukocytes (data not shown). Specifically, peripheral lymphopenia was not observed. Bone marrow lymphopenia was also not observed at either 1 day or 2 wk after daily injections of LIF. LIF exerted a mild cachectic effect on the rats whose weight during the 2-wk experiment remained constant while the controls experienced an approximate 10% gain in weight (data not shown). LIF does cause a transient neutrophilia and lymphopenia at 4 to 6 h in both rats and mice after injection with a return towards control values by 12 h (data not shown). In rats primed with SCF (100 μg/kg per d) for 5 d followed by LIF (100 μg/kg per d) for 5 d only a modest increase in circulating platelets was noted (Fig. 8). Rats also did not demonstrate the changes in mean platelet volume noted in mice

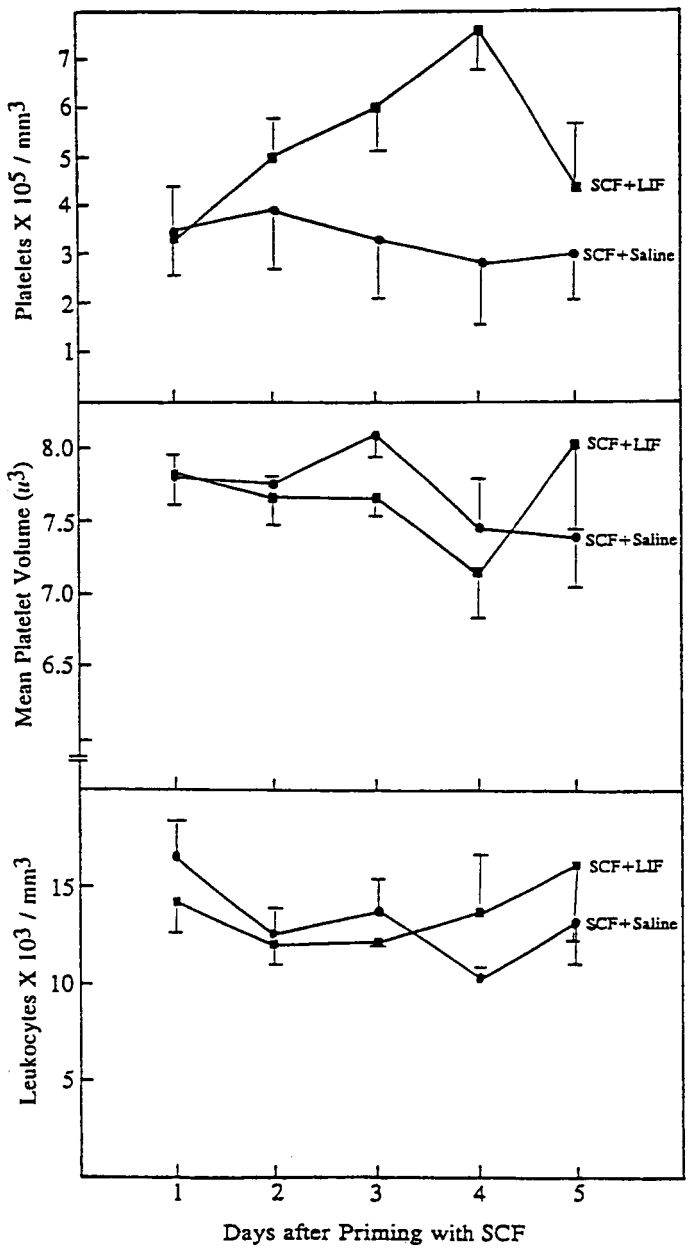

Fig. 8. In rats primed with daily intravenous bolus injections of SCF (100 μg/kg per day) followed by daily intravenous bolus injections of LIF (100 μg/kg per day), only a very modest increase in platelets is noted in LIF-treated rats (n = 3 rats/time point). In contrast to the mouse, significant changes in mean platelet volume are not noted. Priming with SCF actually caused a slight decrease in circulating numbers of platelets after 5 days as compared to day 0 (data not shown).

(Fig. 8). The splenic megakaryocytes of rats did not demonstrate the increase in cytoplasm noted in mice.

Discussion

SCF is shown to stimulate a remarkable dose-dependent peripheral panleukocytosis and thrombocytosis *in vivo* in mice. The ability of SCF by itself to cause panleukocytosis of leukemoid proportions was not appreciated in our previous studies at lower doses (4, 5). LIF is shown to stimulate thrombocytosis as is consistent with previous reports by Metcalf and colleagues (9, 10). The reason for a lack of a dose-response relationship between dose of LIF and platelet number is unclear. The daily coinjection of SCF and LIF for 1 to 2 wk does not result in a much greater increase in circulating platelets than is appreciated after injection of LIF alone or SCF alone at a high dose. Perhaps the most clinically provocative observation is that a dramatic and selective increase in circulating platelets is attainable by priming mice with SCF for 5 d at a dose that does not cause thrombocytosis and then injecting LIF for 5 d. One may postulate that SCF causes an expansion of megakaryocytes and megakaryocyte precursor cells in the marrow and spleen and that LIF either directly or indirectly causes the differentiation of these precursors, resulting in increased numbers of platelets. Murine megakaryocytes have been reported to possess receptors for LIF (20). The splenic megakaryocytes in SCF-primed LIF-treated mice were strikingly larger and more mature than in SCF-primed SCF-treated mice, suggesting that LIF is a differentiation factor for megakaryocytes. LIF's effect on the size of megakaryocytes is thus very similar to that of IL-6 (11). The large majority of lymphocytes are not thought to possess receptors for LIF (15), and LIF-induced lymphopenia may be indirectly mediated via another yet to be identified cytokine or via glucocorticoids, since adrenal cortical cells possess LIF receptors (21).

Mice primed with SCF and subsequently treated with SCF plus LIF exhibit a thrombocytosis almost equal to mice subsequently treated with LIF alone. However, the thrombocytosis in SCF-primed mice treated with SCF plus LIF is accompanied by a peripheral panleukocytosis of leukemoid magnitude that may be undesirable in some clinical situations. SCF-primed mice treated with LIF develop thrombocytosis without a generalized leukocytosis.

The slightly lesser thrombocytosis in SCF-primed SCF plus LIF-treated as compared to LIF alone-treated mice may be due to competition for proliferative factors between platelet and myeloid precursors in the SCF plus LIF-treated mice. Alternatively, SCF may act to downregulate the expression of endogenous hematopoietic differentiation factors. The latter hypothesis is supported by the observation that thrombocytosis occurs in SCF-primed mice subsequently treated with carrier for 5 d. The cessation of SCF treatment may allow the re-expression of SCF-suppressable endogenous platelet growth factors that are able to act upon the SCF-primed platelet precursor pool to cause thrombocytosis. A similar phenomenon may be occurring in the spleens of SCF-primed carrier-treated mice in which an increase in the proportion of erythroid extramedullary hematopoiesis is observed. SCF appears to promote myeloid over erythroid differentiation *in vivo* as seen by the predominantly myeloid nature of extramedullary hematopoiesis in mice treated with SCF alone. The cessation of SCF administration may allow the expression of erythropoietin or other erythroid differentiating factors to act on an erythroid precursor pool expanded by priming with SCF. Another hypothesis to explain the experimental observations would be that SCF causes erythroid and platelet precursors to become less responsive to lineage differentiation factors.

MPV is often considered to represent an index for thrombopoiesis with an increase in MPV signalling increased thrombopoiesis except in megaloblastic anemia (22). As might therefore be anticipated, the MPV increased in mice treated with LIF alone or with SCF alone at doses that cause thrombocytosis. The increase in MPV preceded the observed increase in circulating platelets. The MPV is also increased after 5 d in SCF-primed mice as would be expected given the ensuing thrombocytosis that is known to occur in all SCF-primed mice. On the other hand, a decrease in MPV accompanies the post-priming thrombocytosis noted in all SCF-primed mice. The decrease in MPV might suggest that an *in vivo* activity exists that determines the rate of fragmentation of megakaryocytes into platelets and that this activity is increased after priming with SCF. The increase in platelet numbers in SCF-primed mice is proportionately far greater than the decrease in MPV so that the total platelet mass (the product of platelet numbers and MPV) increases. Also of interest in regard to the MPV is that LIF differs from IL-6 in that the IL-6 induced thrombocytosis in mice (23–25) is not accompanied by an increase in MPV as reported by Hill et al. (24) and McDonald et al. (25). Our own experience has similarly been that recombinant human IL-6 administered up to 7 d by daily intraperitoneal injections at doses of 1 to 800 μg/kg causes a slight thrombocytosis (i.e. less than two-fold increase in circulating platelets) but no change in MPV in mice (Juan del Castillo, unpublished data).

In summary, SCF-induced priming or proliferation of megakaryocytic stem cells followed by LIF-

induced lineage-specific maturation of platelets provides a pharmacologic approach to achieve selective increases in circulating platelets in mice. Evidence that SCF is important in thrombopoiesis *in vivo* has recently been provided by Hunt and colleagues (26), who demonstrated a role for SCF in the rebound thrombocytosis that follows 5-fluorouracil treatment. Substantial species variability appears to exist between mice and rats, and the *in vivo* effects of combined treatment with SCF and LIF in humans would therefore be difficult to predict. IL-6 and LIF have both been reported to cause a significant increase in circulating platelets in primates (27). The present data is consistent with the hypothesis that thrombopoiesis can be regulated by combinations of factors such as SCF, IL-3, LIF, IL-6, and IL-11 (28) acting at various stages of thrombopoiesis.

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