Hematopoietic stem and progenitor cells are differentially mobilized depending on the duration of Flt3-ligand administration

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ABSTRACT

Background
Flt3-ligand is a cytokine that induces relatively slow mobilization of hematopoietic cells in animals and humans *in vivo*. This provides a time-frame to study hematopoietic stem and progenitor cell migration kinetics in detail.

Design and Methods
Mice were injected with Flt3-ligand (10 μg/day, intraperitoneally) for 3, 5, 7 and 10 days. Mobilization of hematopoietic stem and progenitor cells was studied using colony-forming-unit granulocyte/monocyte and cobblestone-area-forming-cell assays. The radioprotective capacity of mobilized peripheral blood mononuclear cells was studied by transplantation of 1.5×10^6 Flt3-ligand-mobilized peripheral blood mononuclear cells into lethally irradiated (9.5 Gy) recipients.

Results
Hematopoietic progenitor cell mobilization was detected from day 3 onwards and prolonged administration of Flt3-ligand produced a steady increase in mobilized progenitor cells. Compared to Flt3-ligand administration for 5 days, the administration of Flt3-ligand for 10 days led to a 5.5-fold increase in cobblestone-area-forming cells at week 4 and a 5.0-fold increase at week 5. Furthermore, transplantation of peripheral blood mononuclear cells mobilized by 5 days of Flt3-ligand administration did not radioprotect lethally irradiated recipients, whereas peripheral blood mononuclear cells mobilized by 10 days of Flt3-Ligand administration did provide 100% radioprotection of the recipients with significant multilineage donor chimerism. Compared to the administration of Flt3-ligand or interleukin-8 alone, co-administration of interleukin-8 and Flt3-ligand led to synergistic enhancement of hematopoietic stem and progenitor cell mobilization on days 3 and 5.

Conclusions
These results indicate that hematopoietic stem and progenitor cells show different mobilization kinetics in response to Flt3-ligand, resulting in preferential mobilization of hematopoietic progenitor cells at day 5, followed by hematopoietic stem cell mobilization at day 10.

Key words: stem cell mobilization, Flt3-ligand, IL-8, animal models.

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Introduction

Hematopoietic stem cells (HSC) are capable of extensive self-renewal and confer long-term multilineage repopulating ability upon transfer into myeloablated recipients. Under steady state conditions, HSC comprise a small proportion (0.01%) of bone marrow cells, where they serve as a reservoir of mainly quiescent cells that have the ability to be recruited into cycle upon hematopoietic stress.[1] The immediate progeny of hematopoietic stem cells are hematopoietic progenitor cells (HPC), which retain full lineage potential but have limited self-renewal capacity.[2] They are relatively more abundant than HSC and able to transiently reconstitute myeloablated recipients. The absolute frequency of HPC/HSC in the peripheral blood is relatively low under normal circumstances, but is considerably increased by mobilizing HPC and HSC from the bone marrow using a variety of chemokines and cytokines.[3,4]

The tyrosine kinase type III receptor flt3/flk2 (fms-like tyrosine kinase receptor-3/ fetal liver kinase-2; CD135), is primarily expressed by hematopoietic progenitor cells, as well as multipotent progenitor cells.[5] Its ligand, Flt3-ligand (FL), is a type 1 transmembrane protein that exists in both a membrane bound and a soluble form. FL is a cytokine that is involved in cell survival, proliferation, and differentiation during early hematopoiesis.[6] In addition, FL is important for lymphocyte (B-cell and T-cell) development, but not for differentiation into myeloid lineages.[7-9] In contrast to the more limited expression of flt3 on hematopoietic cells, FL mRNA has been found in various hematopoietic and non-hematopoietic tissues.[10]

Alone, or in combination with other growth factors, FL stimulates the proliferation of highly enriched human and murine HSC *in vitro* and leads to expansion and mobilization of progenitor cells in animals and humans *in vitro*.[6,11-15] Mice and non-human primates treated with recombinant FL for 3 to 10 days have increased numbers of colony-forming units in bone marrow, spleen and peripheral blood, with the highest frequency after 10 days of administration, showing that FL is a relatively slow mobilizing agent.[6,15-17] In addition, peripheral blood mononuclear cells (PBMC) obtained from mice treated with FL for 10 days also have the capacity to rescue and reconstitute multiple hematopoietic lineages following transplantation in irradiated recipient animals.[6] When administered in combination with granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), FL leads to mobilization of significantly higher numbers of cells with long-term, multilineage reconstitution potential.[15]

Previously, we showed that interleukin-8 (IL-8) significantly increases HPC/HSC mobilization in combination with several other hematopoietic growth factors such as G-CSF, GM-CSF, interleukin-3 and stem cell factor.[18] However, IL-8 has not been studied in combination with FL. Since FL is a slow mobilizing agent, the subsequent addition of a very quick mobilizing agent such as IL-8 (i.e. optimal HSC/HPC mobilization at 20 min after injection) might accelerate HPC/HSC mobilization. In the current study, we analyzed the kinetics of FL-induced HSC/HPC mobilization.

Design and Methods

Animals

Male Balb/c and C57BL/6 mice (8-12 weeks old) were purchased from Charles River (Maastricht, the Netherlands). C57BL/6-Ly5.1 congenic recipients were bred in our own facilities and were used at 6-12 weeks of age. The animals were fed commercial rodent chow and acidified water and were maintained in the animal facility of the Leiden University Medical Center under conventional conditions. Bone marrow cell recipients received sterilized regular chow for a period of 4 weeks directly after bone marrow transplantation. In addition, from week -1 until +4 after bone marrow cell transplantation, drinking water was supplied with antibiotics (polymixin B, Bupha Uitgeest, the Netherlands), ciprofloxacin (Bayer b.v., Mijdrecht, the Netherlands) amphotericin B (Bristol-Myers Squibb, Woerden, the Netherlands) and saccharose. All experimental protocols were approved by the institutional ethics committee on animal experiments.

Growth factors

All growth factors were administered as an intraperitoneal (i.p.) injection in 0.2 mL 0.1% bovine serum albumin/phosphate-buffered saline (0.1 % BSA/PBS). Recombinant human FL (kindly provided by Amgen, Thousand Oaks, California, USA) and recombinant human G-CSF (filgrastim, Amgen) were administered at a dose of 10 μg i.p. per day. IL-8 (Novartis, Vienna, Austria) was administered as a single, 30 μg i.p. injection. Control mice were injected with 0.2 mL 0.1% BSA/PBS alone (referred to as PBS).

Preparation of cell suspensions

Twenty-four hours after the last FL or G-CSF injection or 20 min after IL-8 administration, mice were sacrificed by CO_2 asphyxiation and peripheral blood and bone marrow cells were harvested. Under sterile conditions, PBMC suspensions were obtained by Ficoll (LUMC Pharmacy, Leiden, the Netherlands) density separation. Bone marrow cells were harvested by flushing a femur under sterile conditions with RPMI 1640, containing 500 μg/mL penicillin, 250 μg/mL streptomycin, 10% heparin and 2% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). Spleen cells were obtained by straining the spleen through a 100 μm pore filter (BD Falcon, Franklin Lakes, New Jersey, USA) and washing the cell suspension twice in a RPMI 1640 solution containing penicillin, streptomycin and FBS, as described above.

Monoclonal antibodies

For lineage analysis, cells were stained with fluorescein isothiocyanate-labeled anti-GR-1 (clone RB6-8C5), anti-CD90.2 (Thy-1.2, clone 53-2.1), phycoerythin-labeled anti-B220 (clone RA3-6B2), anti-CD11b (clone M1/70) and anti-CD3 (clone 145-2C11). To detect chimerism in transplanted recipient mice, cells were stained with phycoerythin-labeled anti-CD45.1 (clone A20) and/or fluorescein isothiocyanate-labeled anti-CD45.2 (clone 104). All monoclonal antibodies used in this study were obtained from Pharmingen (San Diego, CA, USA).

Progenitor cell assay

The colony-forming units - granulocyte-macrophage (CFU-GM) assay is specifically designed to quantify the frequency of HPC. CFU-GM were cultured by resuspending 5×10^5 peripheral blood cells, 5×10^4 bone marrow cells or 1×10^6 spleen cells in a 25 mm^2 Petri dish containing 1 mL of a 40% methylcellulose, 30 % IMDM and 20% FBS solution with the addition of 1.25 ng/mL recombinant murine GM-CSF. After 7 days of incubation at a temperature of 37°C in a 5% CO_2 environment, colonies (defined as a cluster of 20 or more cells) were scored using an inverse light microscope.

Cobblestone-area-forming cell assay

The cobblestone-area-forming cell (CAFC) assay measures a spectrum of hematopoietic cells and allows early progenitor cells (arising at day 7–14 of culture) to be separated from more primitive HSC with long-term repopulating ability (day 28–35 CAFC). Confluent stromal layers of FBMD-1 cells in flat-bottomed 96-well plates (Falcon, Etten-Leur, the Netherlands) were overlaid with various dilutions of PBMC to allow limiting dilution analysis of the precursor cells forming hematopoietic clones under the stromal layers. To assay a particular cell suspension, we used eight dilution steps differing by a factor of 2.5, with 15 wells per dilution. The cells were cultured at 33°C, 7% CO_2 and were fed weekly by changing half of the medium. Between 7 and 42 days after overlay, all wells were inspected at weekly intervals and scored positive if at least one phase-dark hematopoietic clone (cobblestone area, at least 5 cells) was observed. The CAFC frequencies were calculated using Poisson statistics.[19]

Peripheral blood mononuclear cell transplantation

Prior to PBMC transplantation, recipients were irradiated in Perspex chambers with a linear accelerator (Philips SL 75-5/6 mV, Philips Medical Systems, Best, the Netherlands) at a dose rate of 98 cGy/min. Total doses of 9.5 Gy were administered. Four to 6 hours following total body irradiation, PBMC were transplanted via caudal vein injection in 0.2 mL of saline, containing 0.2% bovine serum albumin.

Statistical analysis

Statistical differences were determined by the Student's t-test and the log rank test using GraphPadPRISM (GraphPad Software, San Diego, CA, USA). *P* values of less than 0.05 are considered statistically significant.

Results

Prolonged administration of Flt3-ligand leads to increased mobilization of hematopoietic progenitor cells

To determine the mobilizing capacity of FL, Balb/c mice were given a daily dose of 10 μg FL i.p. for 3, 5, 7 or 10 days. Twenty-four hours after the last FL injection, peripheral blood was harvested and CFU-GM colony formation was determined. On day 3 of FL administration, HSC/HPC mobilization was 9.5-fold higher than that in PBS-treated controls. HSC/HPC migration was even further increased on days 5, 7 and 10 following FL administration compared to PBS administration (50-fold, 153-fold and 823-fold; $P<0.001$ for comparisons between all groups; Figure 1A).

Bone marrow CFU-GM was significantly increased after FL administration as compared to PBS (CFUGM/femur ±SEM: PBS 35,839±7,433, FL 3 days 96,532±26,140, 5 days 183,819±41,515, 7 days 82,210±16,963 and 10 days 111,504±59,750, $P<0.02$ for the comparison between PBS and 3, 5, 7 or 10 days of FL, Figure 1B). The highest number of bone marrow CFU-GM was seen on day 5 of FL administration, although no significant difference was found between the FL-treated groups (FL 3 days *versus* FL 5 days, $P=0.12$; FL 5 days *versus* FL 7 days, $P=0.10$; FL 7 days *versus* FL 10 days, $P=0.52$).

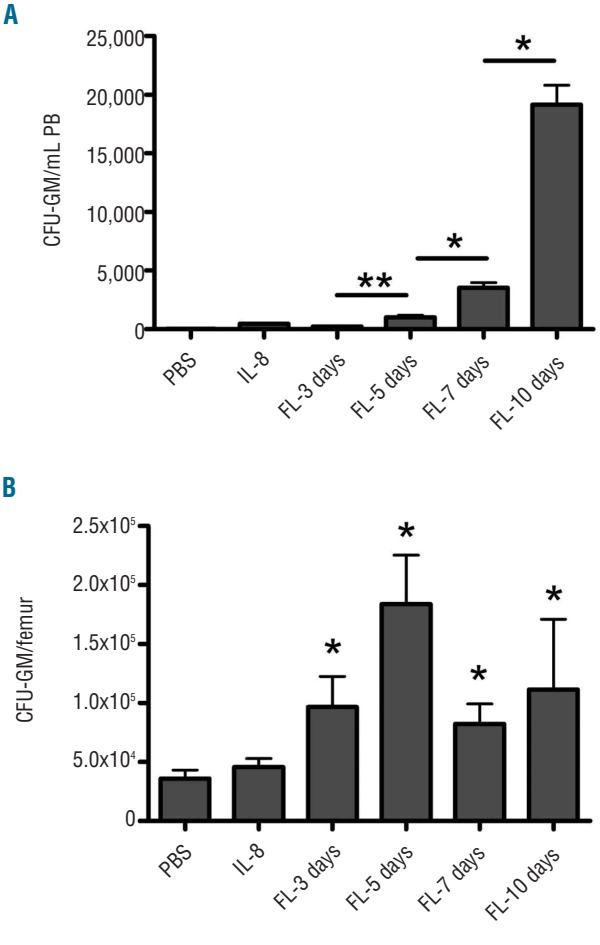

Figure 1. Flt3-ligand administration induces HPC mobilization. FL was administered for 3, 5, 7 or 10 days. (A) Colony-forming cells as determined by CFU-GM assay in peripheral blood (n= 10-20 per group; results are shown as mean±SEM; **P*<0.0001, ***P*=0.0007). (B) Colony-forming cells in bone marrow expressed as CFU-GM/femur (n= 10-20 per group; results are shown as mean ± SEM; **P*<0.02 compared to PBS).

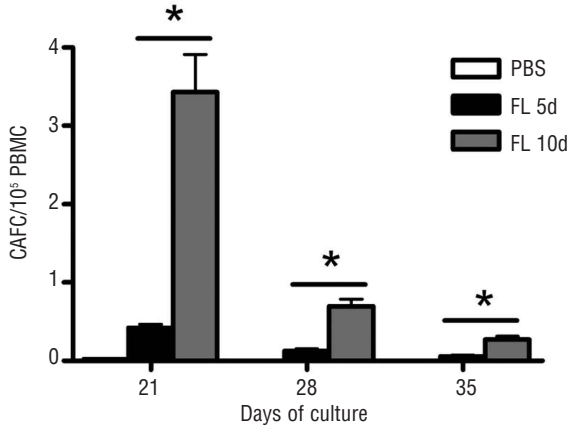

Figure 2. Mobilization of long-term repopulating stem cells is dependent on the duration of FL administration. PBMC mobilized by 5 or 10 days of FL administration to mice (n=12 per group) were analyzed for CAFC frequency. Results of two independent experiments are shown as the mean ± SEM, **P*<0.001.

Migration of long-term repopulating stem cells is dependent on the duration of Flt3-ligand administration

In order to determine the frequency of primitive HSC in the peripheral blood of FL-mobilized mice, CAFC frequency was determined. PBMC obtained from mice that received FL for 5 or 10 days were compared to those from PBS-injected controls. As for the number of CFU-GM, the highest proportion of CAFC-day 7 was found following 10 days of FL administration (*data not shown*). As the duration of the culture increased, the CAFC frequency in both groups steadily decreased. After 28-35 days of culture, the frequency of CAFC was significantly higher in the group that received FL for 10 days than in the group that received FL for 5 days (CAFC on day 28±SEM: FL for 5 days 0.16±0.02, FL for 10 days 0.69±0.10 per 10^5 PBMC; CAFC on day 35±SEM: FL for 5 days 0.096±0.017 and FL for 10 days 0.27±0.04 per 10^5 PBMC; $P<0.001$ for the comparison between FL for 5 days and 10 days at 28 days and 35 days; Figure 2). In PBS-injected controls, the frequency of HSC was below the detection limit of the CAFC assay.

These results indicate that the frequency of primitive HSC in the peripheral blood is dependent on the duration of FL administration and is significantly enhanced after 10 days of FL administration as compared to 5 days of FL administration.

Peripheral blood mononuclear cells mobilized from mice by 10 days of Flt3 ligand administration are radioprotective in vivo

To assess the long-term repopulating ability of FL-mobilized HSC/HPC, radioprotection assays were performed. To this end, 1.5×10^6 PBMC obtained from Balb/c mice that received daily injections of FL for 5 or 10 days were transplanted into lethally irradiated (9.5 Gy) syngeneic recipient mice. Controls received either 1.5×10^6 or 3.0×10^6 PBMC obtained from PBS-injected donors or 1.5×10^6 PBMC obtained from donors that were injected with G-CSF for 4 days.

The mean survival time for recipients of 1.5×10^6 PBS-injected donor cells was 11.5 days whereas that for recipients of 3.0×10^6 PBMC obtained from PBS-injected donors was 13 days. PBMC that were harvested following administration of FL for 5 days were not radioprotective *in vivo*. Although their mean survival time exceeded that of the PBS control group (66 days *versus* 11.5 days, $P=0.001$), all mice had died within 231 days. This was in sharp contrast with the animals in the group given PBMC that were harvested following FL administration for 10 days, which showed 100% radioprotection (mean survival time >260 days, Figure 3A).

Similarly, transplantation of 1.5×10^6 PBMC obtained from G-CSF-mobilized donors showed a radioprotection rate of 100% (mean survival time >260 days). At several time-points following PBMC transplantation and at the end of the experiment at 260 days, multi-lineage reconstitution capacity of peripheral blood cells was analyzed. Granulocyte, monocyte, T-lymphocyte and B-lymphocyte lineages were detected in all recipient mice that received PBMC mobilized by FL administration for 10 days or by G-CSF (*data not shown*).

A second set of transplantation experiments was performed specifically to determine donor chimerism. In these experiments lethally irradiated (9.5 Gy) C57Bl/6 Ly5.1 recipients received 1.5×10^6 PBMC, obtained from C57Bl/6 Ly5.2 donors that had received daily injections of FL for 5 or 10 days or G-CSF for 4 days as a control. The mean survival time for recipients of PBS-injected donor cells was 14 days and one out of the three recipients of PBMC obtained from G-CSF-mobilized donors died on day 14. At 27 weeks after transplantation, one of the five recipients in the FL-5 days group (mean survival time 42 days) and all five recipients in the FL-10 day group were alive (Figure 3B). Long-term donor chimerism was determined 27 weeks after transplantation by FACS analysis. In the groups in which PBMC were mobilized by 10 days of FL administration or G-CSF, donor chimerism was 90.9±1.3% and 91.5±0.51% respectively. However, in the group given PBMC for 5 days, significant donor chimerism (i.e. 84.0%) was seen in only one of the two surviving recipients. Long-term multilineage donor chimerism showed an identical pattern in all lineages that were tested (granulocytes, lymphocytes and monocytes), with significant donor chimerism in all mice in the FL-10 days and G-CSF-groups (Table 1).

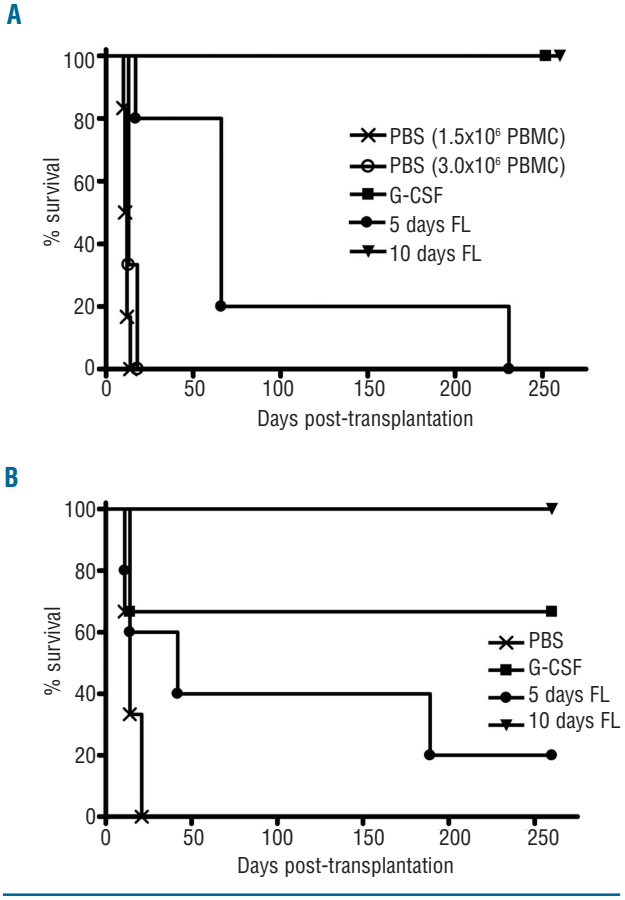

Figure 3. PBMC mobilized by 10 days of FL administration to mice are radioprotective *in vivo*. (A) Lethally irradiated recipient Balb/c mice (9.5 Gy; n=5 per group) were transplanted with 1.5×10^6 PBMC obtained from Balb/c donors treated with 5 or 10 days of FL or 4 days of G-CSF. Control mice received 1.5×10^6 or 3.0×10^6 PBMC obtained from PBS-injected donors. (B) Lethally irradiated C57Bl/6 Ly5.1 recipients (9.5 Gy; n=5 per group) received 1.5×10^6 PBMC obtained from C57Bl/6 Ly5.2 donors treated with 5 or 10 days of FL or 4 days of G-CSF. As a control, 1.5×10^6 PBMC obtained from PBS-injected donors were administered.

Together, these results indicate that FL-induced mobilization for 5 days preferentially mobilizes HPC, whereas 10 days of FL administration induces the mobilization of HSC with long-term repopulating ability *in vivo*.

Flt3-ligand-induced mobilization is synergistically enhanced by combined administration of interleukin-8

It has previously been shown that the mobilizing capacity of FL can be synergistically enhanced by co-administration of G-CSF.[15] To investigate whether co-administration of FL and IL-8 had a synergistic effect on the mobilization of HPC and HSC, FL was administered for 3, 5 and 7 days. Twenty-four hours after the last FL injection, 30 μg IL-8 was administered i.p. Twenty minutes following IL-8 injection, the mice were sacrificed and the frequency of CFU-GM was determined in peripheral blood and bone marrow. Co-administration of IL-8 and FL induced significant increases of mobilization on days 3 and 5, compared to the mobilization achieved by FL or IL-8 alone (5.85-fold and 2.57-fold increases with FL + IL-8 compared to FL alone for 3 and 5 days, respectively; $P<0.001$ and $P=0.003$, respectively, Figure 4A). In contrast, co-administration of FL for 7 days and IL-8 did not further increase the number of CFU-GM (0.95-fold increase of FL + IL-8 compared to FL alone; $P=0.78$, Figure 4A). No difference in CFU-GM/femur was seen between the groups that received FL as a single agent or with subsequent IL-8 administration (Figure 4B).

Transplantation of 1.5×10^6 PBMC obtained from 5-day FL + IL-8-mobilized donors showed a radioprotection rate of 100% (mean survival time >300 days). By comparison, PBMC that were harvested following a single injection of IL-8 showed a 60% long-term radioprotection rate, whereas PBMC that were harvested following FL administration for 5 days did not show long-term radioprotection *in vivo* (Figure 4C).

Collectively, these data indicate that combined administration of FL for 5 days and IL-8 induces the mobilization of radioprotective HSC.

Discussion

It has been shown previously that FL mobilizes large numbers of PBMC into the circulation of mice, with a maximum effect after 10 days of administration.[6,13-15] However, the qualitative content of these mobilized cells with respect to their short- and long-term repopulating ability has not been fully elucidated. In the current study, we addressed this issue.

Long-term HSC, short-term HSC and HPC show similar multilineage capabilities, but differ in their self-renewal and proliferative capacity. When long-term HSC differentiate into short-term HSC and next into HPC, their self-

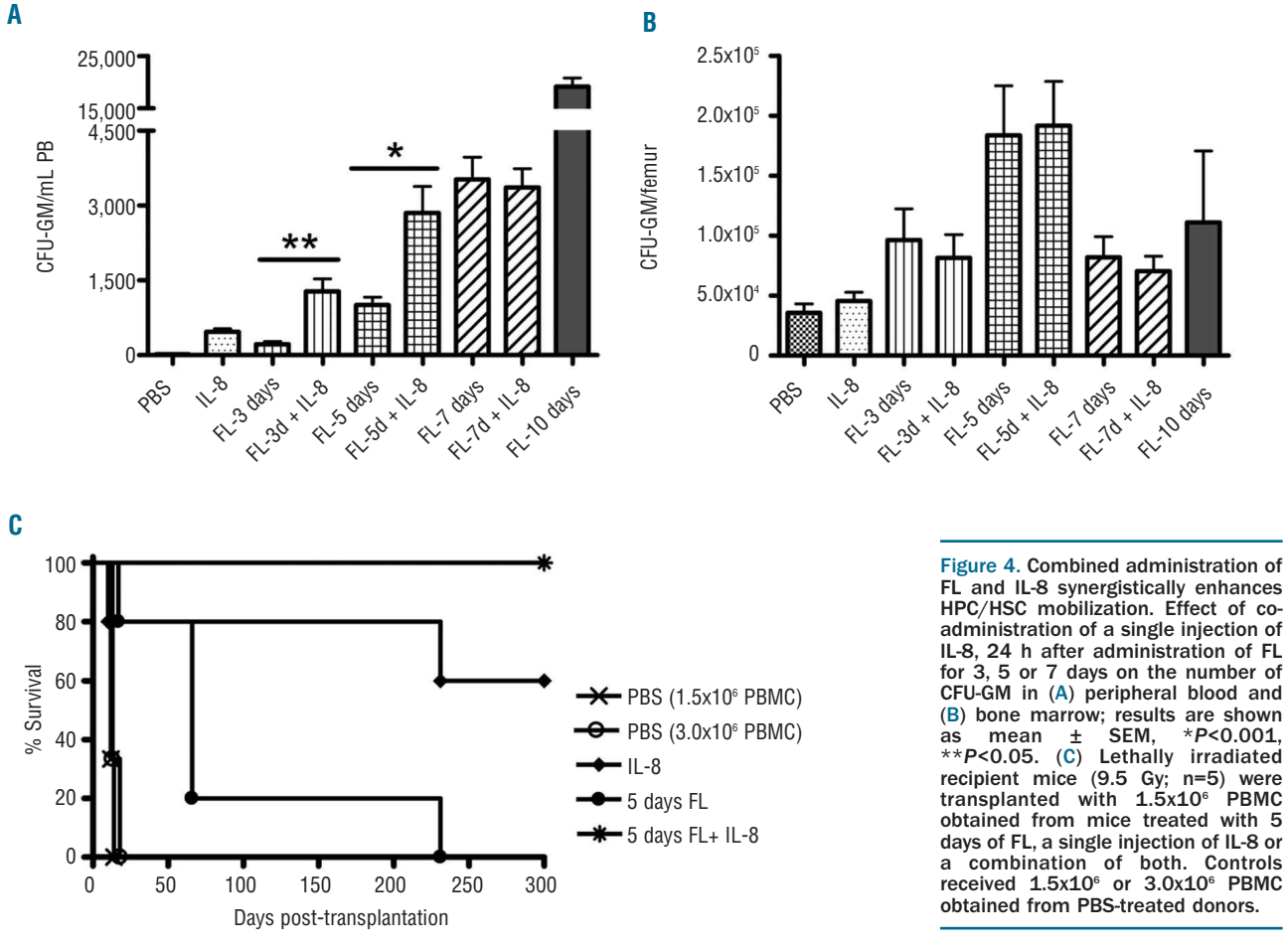

Figure 4. Combined administration of FL and IL-8 synergistically enhances HPC/HSC mobilization. Effect of co-administration of a single injection of IL-8, 24 h after administration of FL for 3, 5 or 7 days on the number of CFU-GM in (A) peripheral blood and (B) bone marrow; results are shown as mean ± SEM, *$P<0.001$, **$P<0.05$. (C) Lethally irradiated recipient mice (9.5 Gy; n=5) were transplanted with 1.5×10^6 PBMC obtained from mice treated with 5 days of FL, a single injection of IL-8 or a combination of both. Controls received 1.5×10^6 or 3.0×10^6 PBMC obtained from PBS-treated donors.

renewal capacity progressively declines.[20] Previous studies have shown that long-term HSC fail to radioprotect conditioned mice and are incapable of rapid reconstitution because of their quiescent state.[21] In lethally irradiated recipients, primitive short-term HSC are necessary for rapid replenishment of myeloid and erythroid progenitors, thereby providing early rescue from lethal myeloablation. However, long-term HSC are required for long-term repopulation. In accordance with others, we found that administration of FL for 10 days leads to significant mobilization of both short-term and long-term HSC, as was shown by the cells' capacity to radioprotect lethally irradiated recipients.[15] By analyzing donor chimerism at several time points following transplantation, we showed that the survival of the mice was indeed due to the engraftment of mobilized donor HSC.

Although a significant number of HPC was mobilized in mice treated with FL for 5 days (represented by CFU-GM and CAFC-day 7 in their respective assays), the frequency of repopulating HSC (represented by CAFC-day 28/35) in the peripheral blood was very low. Furthermore, PBMC obtained from mice that were injected with FL for 5 days did not radioprotect lethally irradiated mice. This indicates that the number of long-term HSC mobilized was below the threshold capable of radioprotecting lethally irradiated recipients. These findings point towards different mobilization kinetics for HPC and HSC in response to FL, resulting in preferential mobilization of HPC at day 5, followed by an increase in HSC mobilization from day 5 to 10. A possible explanation for this could be that HPC are more easily recruited from their bone marrow compartment than are long-term HSC.

It is interesting to note that long-term and short-term HSC activity is correlated with Flt3-expression. In mouse bone marrow, c-Kit^{hi}Lin^{neg}Sca1^{pos} (KLS) that do not express Flt3 provide radioprotection and long-term reconstitution in ablated recipients, whereas purified Flt3-positive KLS cells fail to do so.[22] This would suggest that mobilized long-term HSC are not directly targeted by FL. However, since long-term HSC are indeed mobilized by FL there must be an alternative explanation for this phenomenon. One explanation could be an indirect effect of FL on long-term HSC through an interaction with the CXCL12/CXCR4 signaling pathway.[23] Alternatively, neutrophils may play a role in this process, since neutrophils are increased in the bone marrow upon FL administration[6] and are indispensible for cytokine (IL-8 and G-CSF)-induced HSC mobilization.[16,17]

Previously, it was shown that FL administration leads to an increase in bone marrow-derived CFU-GM, BFU-E and CFU-GEMM, with maximums occurring on day 3 of FL treatment. From day 3 onwards, BM-CFU begin to decrease with concurrent increases in splenic and peripheral blood-CFU which peak on days 8 to 10 of FL treatment.[6] In our study we found a steady increase of bone marrow CFU-GM after FL treatment for 3 and 5 days with a subsequent, although not significant, decrease on days 5 to 10. This suggests that CFU expansion occurs in the bone marrow prior to HPC/HSC mobilization.

The mobilizing capacity of FL was significantly enhanced when IL-8 was given after 3 and 5 days of FL administration, with an increase in peripheral blood CFU-GM and superior long-term survival of lethally irradiated recipients. Our results are in accordance with data from studies in which FL was combined with G-CSF, showing a significant increase of peripheral blood CFU-GM after combined administration of FL and G-CSF as compared to administration of either cytokine alone.[6,15,24,25] More specifically, in the study by Brasel *et al.*, the highest synergy of combined FL and G-CSF administration was seen on day 6, whereas by day 10 the synergistic effect of G-CSF co-administration had disappeared, reflecting our results with co-administration of IL-8.[6] An explanation for the superior overall survival in the group treated with FL + IL-8 could be the higher numbers of mobilized HPC in the FL+IL-8 group in comparison with groups treated with IL-8 or 5 days of FL alone (as shown by the higher frequency of CFU-GM in the FL+IL-8 group in Figure 4A). These HPC are necessary for early survival after lethal irradiation and facilitate engraftment of long-term HSC that sustain long-term survival of the recipient mice. An alternative explanation could be that FL treatment results in neutrophil expansion in the bone marrow, as was shown by Brasel *et al.*, facilitating more efficient stem cell mobilization in response to additional FL treatment or IL-8 injection.[6]

In summary, these results indicate that HPC and HSC show different mobilization kinetics in response to FL, resulting in preferential mobilization of HPC at day 5, followed by HSC mobilization at day 10, allowing for the use of FL as a selective mobilizing agent.

Authorship and Disclosures

EK and MP: conception and design of the study, collection, analysis and interpretation of the data and writing of the paper; HH: collection, analysis and interpretation of the data; GV: conception and design of the study and collection of the data; WF: conception and design of the study and critical revision of the paper. All authors approved the final version of the manuscript.

The authors reported no potential conflicts of interest.

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