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# The CXCR4 antagonist 4F-benzoyl-TN14003 stimulates the recovery of the bone marrow after transplantation

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**Cytopenia represents a significant complication after chemotherapy, irradiation before bone marrow (BM) transplantation or as a therapy for cancer. The mechanisms that determine the pace of BM recovery are not fully understood. During the recovery phase after chemotherapy or irradiation, the signals for retention of white blood cells within the BM increase significantly. This leads to a delay in the release of WBC, which can be overcome by targeting the CXCR4 axis with the antagonist 4F-benzoyl-TN14003 (T140). The delay in the release of WBC is also accompanied by suppression in the production of progenitor cells and mature cells by the BM stroma. Administration of T140 to mice transplanted with BM cells stimulates the production of all types of progenitors and mature cells, and increases the exit of mature cells to the periphery. Moreover, addition of T140, but not AMD3100, to BM stromal cultures stimulates the production of mature cells and progenitors from all lineages. The unique ability of the CXCR4 antagonist, T140 to stimulate the production and exit of WBC cells may be used as a novel therapeutic approach to overcome cytopenia associated with treatments for cancer and BM transplantation.**
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## Introduction

The trafficking of hematopoietic cells between the bone marrow (BM) and the blood is a physiological process.[1,2] The entrance and exit of these cells to or from the BM takes place during transplantation or mobilization of progenitor and stem cells, two procedures routinely used today in the clinic.[3] Circulating stem cells are capable of homing to the BM and regenerating normal hematopoiesis after myeloablative conditioning with high doses of chemotherapy or radiation. The balance between retention and exit signals determines the rate at which cells are mobilized from the BM. Retention and maintenance of the hematopoietic stem cell pool are regulated by balanced interactions between the undifferentiated cells, maturing cells and stromal cells within the BM. Hematopoietic progenitor cells (HPC) in the BM are thought to be located within specific stroma niches.[4] These specific microenvironments provide soluble factors and cellular interactions required for HPC proliferation and differentiation.[5,6] Whether HPC circulate across, exit or remain sessile within a BM niche may ultimately depend on the type and function of the stromal cell and different molecules such as adhesion or chemokine receptors.

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Over the recent years, it has become apparent that the interactions between stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, play a pivotal role in hematopoietic cell mobilization and engraftment.[7–9] The CXCR4 receptor is widely expressed on many cell types, including HSCs and HPCs, and the interaction with its ligand was found to be involved in chemotaxis, homing and survival. The SDF-1/CXCR4 axis is also involved in the retention of hematopoietic cells within the BM microenvironment;[10] consequently, the disruption of SDF-1/CXCR4 interactions results in the mobilization of hematopoietic cells. Today, there are several known CXCR4 antagonists that have been described as having different levels of efficiency. 4F-benzoyl-TN14003 (T140), a short modified peptide, as well as AMD3100, which is a bicyclam, are highly selective CXCR4 antagonists, which bind with high affinity (1 nM) to CXCR4 and inhibit the binding of HIV and SDF-1$\alpha$. Furthermore, both antagonists inhibit $Ca^{2+}$ influx, as well as the migration of a variety of cells in response to SDF-1$\alpha$. Interestingly, Trent *et al.*[11], found that the structural basis for the interaction of T140 and AMD3100 with CXCR4 confirms that the mechanisms used by these agents are different. Furthermore, it was suggested that T140 acts as an inverse agonist, and AMD3100 acts as a weak partial agonist when the coupling of CXCR4 to G$\alpha$ subunits in mammalian cells was assayed by [35S]GTP$\gamma$S binding.[12]

Indeed, blocking the CXCR4 receptor with antagonists, such as T140 or AMD3100, results in the mobilization of HPCs, including neutrophils.[13,14] Moreover, combination of these two antagonists with granulocyte colony-stimulating factor (G-CSF) has an additive effect.

In this work, we studied the role of CXCR4 in BM recovery after chemotherapy administration or irradiation by using the antagonist T140. Surprisingly, we found that after damage the BM stromal cells suppress the production and exit of mature WBC. Interestingly, we found that CXCR4 is involved in determining both the rate of production and exit of WBC in and from the BM after transplantation.

## Materials and methods

### *Reagents and mice*

4F-benzoyl-TN14003 (T140) was kindly provided by Biokine Therapeutics, Ltd., Building 13AWeizmann Science ParkNess Ziona, Israel. AMD3100 was purchased from Sigma, Rehovot, Israel. G-CSF (Filgrastim Neupogen, Amgen Manufacturing, Limited, a subsidiary of Amgen, Inc. One Amgen Center Drive Thousand Oaks, CA, U.S.A.) was kindly provided by Professor Arnon Nagler. AMD3100 was purchased from Sigma. Cyclophosphamide (CPM) was purchased from Baxter Oncology GmbH, Frankfurt, Germany. GM-6001 was purchased

from CALBIOCHEM, San Diego, CA, USA. Recombinant human SDF-1α was purchased from Peprotec (London, UK).

Female C57BL/6 mice (7–8 weeks old) were purchased from Harlan, Rehovot, Israel and maintained under specific pathogen-free conditions at the Hebrew University Animal Facility (Jerusalem, Israel). All experiments were approved by the Animal Care and Use Committee of the Hebrew University.

### *Mobilization with T140 and Cyclophosphamide treatment*

Mice were injected subcutaneously with T140 (1, 2.5, 5 and 10 mg/kg) in a total volume of 200 μl, 2 h before sacrifice. In some experiments, mice were killed at different time points—1/2, 1, 2, 4 and 24 h after injection. Control mice were injected with phosphate-buffered solution (PBS) at the appropriate volume. Mice received a total dose of 250 mg/kg CPM by two 0.25 ml intraperitoneal injections scheduled on day 0 (150 mg/kg) and day 3 (100 mg/kg).[15] On day 3, some mice were subcutaneously treated with 0.2 ml of T140 (5 mg/kg), G-CSF (5 μg/mouse) or their combination once a day for five constitutive days (day 3–7). BM and blood samples were taken on days 0, 3, 4, 5, 6 and 7 (Figure 2a).

### *Transplantation model*

Mice were lethally irradiated with 900 rad (day 0). After 24 h, normal BM cells ($5 \times 10^6$ cells/mouse) were i.v. transplanted in a total volume of 200 μl PBS (day 1). Mice were treated with T140 or AMD3100 on day 2 till day 9. T140 and AMD3100 were subcutaneously injected (5 mg/kg) once a day. Mice were bled on days 0, 2, 4, 7 and 9 1 h after treatments and BM was harvested on day 10 (Figure 5a).

### *Cell isolation*

Peripheral blood cells were collected from mice by cardiac puncture into heparin-containing tubes followed by lysis of the erythrocyte population using an RBC lysis solution (0.155 M $NH_4Cl$, 0.01 M $KHCO_3$ and 0.01 mM EDTA (pH 7.4)). BM cells were obtained from femurs and tibias and splashed with 1 ml of cold PBS. Cells were counted using a hemocytometer or FACScalibur.

### *Culture medium*

For the *in vitro* studies, cells were cultured in a complete culture medium. The complete culture medium contained either RPMI-1640 or Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, penicillin 50 units/ml and streptomycin 50 Ag/ml (Biological Industries, Bet Haemek, Israel).

### *Flow cytometry*

Isolated cells were washed, and re-suspended with PBS containing 0.1% BSA (Biological Industries) and 0.01% sodium azide (FACS buffer). Cells were stained in 0.1 ml FACS buffer with fluorescence antibodies directed against GR-1, Ter-119, Mac-1 and B-220 molecules or matched isotype controls (all from eBioscience, San Diego, CA, USA) for 30 min and washed with FACS buffer. Immunostained cells were analyzed by flow cytometry using the FACS Caliber Flow Cytometer (BD Biosciences, San Jose, CA, USA); the data were analyzed using the software from CellQuest (version 3.3; BD Biosciences).

### *Hematopoietic progenitor cells assay*

To evaluate the number of progenitor cells, a colony-forming cell assay was used. Burst-forming units (erythrocyte) (BFU-E), colony-forming units granulocyte–macrophage (CFU-GM), colony-forming units macrophage (CFU-M), and colony-forming units granulocyte–erythrocyte–macrophage–megakaryocyte (CFU-GEMM) were assayed by plating the cells in Iscove's-modified Dulbecco's Medium containing 1% methylcellulose, 15% FBS, 1% bovine serum albumin (BSA), 3 U/ml rh EPO, $10^4$ M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml rmSCF, 10 ng/ml rmIL-3, 10 μg/ml rh Insulin, 10 ng/ml rh IL-6 and 200 μg/ml human transferrin (Methocult GF M3434; StemCell Technologies Inc., Vancouver, BC, Canada). The cultures were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. Five days later, typical colonies were visually scored by morphological criteria using a light microscope and the frequency of CFU was calculated.

### *SDF-1 ELISA*

BM cells were isolated from femurs and tibias on days 0, 3, 4, 5, 6 and 7 after CPM treatment (as described above). BM was flashed with 300 μl of PBS containing a protease inhibitor mixture (Roche Diagnostics, Palo Alto (Pharma), CA, USA). After centrifugation, the supernatant was kept and frozen for the detection of SDF-1 protein by ELISA. ELISA was performed using the Quantikine kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### *Isolation of mobilized cells for* in-vitro *characterization*

Mice were subcutaneously injected with 0.2 ml of T140 (5 mg/kg), G-CSF (2.5 μg/mouse) or their combination, twice a day for 2 days. Another injection was performed on day 3, and 1 h later blood was collected and cells were isolated as described above. Control mice were injected with PBS at the appropriate volume.

### *Proliferation of mobilized cells*

To check the proliferation of mobilized cells, mice were treated with T140 with or without G-CSF at the same time while being injected intraperitoneally with 1 mg/mouse of BrdU re-suspended in 300 μl of PBS. After 16 h, a second injection of T140, GCSF and BrdU was performed. At 2 h after the last injections, peripheral blood cells were collected as described above. Proliferation of mobilized cells was measured by the BrdU flow kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions.

### *Expression of CXCR4 and VLA4 on mobilized cells*

Expression of CXCR4 and VLA4 on mobilized cells was detected by flow cytometry after staining with fluorescein isothiocyanate (FITC)-anti-CXCR4 and FITC-anti-VLA4 (BD Pharmingen).

### *Motility assay*

Motility of mobilized cells was measured using transendothelial migration assays by using Costar (Corning, NY, USA) 6.5 mm/diameter, 5 μm/pore wells (Cambridge, MA, USA). Upper chambers were pre-coated with BM endothelial cell line $3 \times 10^4$ cells/well/100 μl with complete DMEM containing 10% FCS, whereas 0.6 ml of complete DMEM was added to the lower chambers. After 24 h, the DMEM was removed and 0.6 ml RPMI 1640 + 10% FCS medium was added to the lower chamber, and $3 \times 10^5$ normal BM cells were placed in the upper chamber and kept at 37 °C in 5% $CO_2$. In some experiments, GM-6100 (pan-MMPs inhibitor) was added to the culture at a

final concentration of 10 μM/ml. Cells migrating within 4 h to the bottom chamber of the Transwell were counted using FACScalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA). To measure the motility of progenitor motility, cells migrating within 6 h to the bottom chamber of the Transwell were cultured for colony assay (as described above) and the percentage of migrating progenitors was calculated.

### *Gelatin zymography*

Equal numbers of mobilized cells ($0.25 \times 10^6$/well) were cultured in 200 μl of serum-free medium (complete RPMI medium with 0.1% BSA) in a 96-U shape plate. Secretion and activity of matrix metalloproteinase-9 (MMP-9) from mobilized cells were determined in cell-culture supernatants after 24 h by SDS-PAGE. Proteins in supernatant were separated on gels containing 0.1% gelatin (Sigma, St Louis, MO, USA). Gels were then re-natured by incubation in 2.5% Triton X-100 for 30 min, and incubated overnight in a substrate buffer (50 mM Tris-HCl, pH 7.5) containing 10 mM $CaCl_2$ and 0.05% Brij (Sigma) at 37 °C, then stained with Coomassie brilliant blue (0.5%). Clear areas in the blue background of gels showed the presence of MMP-9 activity. Molecular weight markers (Bio-Rad, Hercules, CA, USA) were run with each gel. Computerized densitometry was used to evaluate relative enzymatic activity (BioImaging Gel Documentation System, Dinco and Renium, Jerusalem, Israel) and TINA software (Raytest, Straubenhardt, Germany). Number of cells in each well was counted before zymography and at the end of experiments.

### *Proliferation of BM cells in methylcellulose medium*

Normal mouse BM cells were isolated and re-suspended in $1 \times 10^6$ cells/ml of DMEM. 0.3 ml of the cells was cultured with 3 ml of methylcellulose without the addition of cytokines and growth factors. (HSC001; StemCell Technologies Inc.). G-CSF (50 ng/ml), T140 (10, 50 and 500 ng/ml) and their combination were added to the cultures. The cells were cultured at 37 °C in 5% $CO_2$ and the numbers of colonies were counted after 5 days.

### *Proliferation of BM cells in the presence of stromal cells*

Stromal cell lines 14F 1.1 were cultured in 24-well plate in 0.5 ml of complete DMEM containing 10% FCS. When the cells became confluent in the wells, the medium was removed and normal mouse BM cells ($0.5 \times 10^6$/well) were then re-suspended in RPMI medium supplemented with complete 10% FCS at a concentration of 0.75 ml and added on top of the stromal cells. AMD3100 (20 μg/ml), T140 (20 μg/ml), G-CSF (50 ng/ml) or their combination were added to the culture every second day. SDF-1α was added to the culture at a concentration of 5 μg/ml. Six days later, cells were collected after trypsinization and the numbers of Gr-1$^+$ cells or Ter-119$^+$ cells were counted by FACS after staining with fluorescence antibodies (as described above). To count the number of progenitors, 0.3 ml of the cells were cultured with 1.5 ml of methylcellulose and the number of colonies was counted 5 days later, as described above in the HPCs assay.

### *Statistical analysis*

Results are expressed as average ± s.d. Statistical differences were determined by an analysis of two-tailed Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

## Results

### *CXCR4 suppresses the exit of neutrophils to the periphery after chemotherapy*

The CXCR4 antagonist, T140, as well as AMD3100 induce the mobilization of hematopoietic cells, including neutrophils and progenitors, into the blood (Figure 1).[13,14] This effect was dose-dependent with a peak after 1–2 h. Surprisingly, after treatments with both CXCR4 antagonists, the number of neutrophils and progenitors within the BM was not changed (Figure 1). These findings raised questions about the balance between exit and retention signals of hematopoietic cells within the BM and the contribution of CXCR4 to this process.

To study the balance between hematopoietic cell exit and retention signals within the BM, we first used a model in which severe neutropenia and a recovery phase in the blood and BM are observed (Figures 1a–d). In this model, mice received a low dose of chemotherapy using CPM[15] (Figure 2a). Treatment with CPM induced neutropenia, which lasted 5 days (day 3 to day 7) (Figure 2b); however, when T140 was administered to mice once a day, from day 3 to day 7, the duration of the neutropenia was significantly shortened to only 2 days (day 4 and day 5) (Figure 2b). During the decline phase on day 3, T140 successfully released cells from the BM into the circulation. Moreover, on days 4–7 after chemotherapy, we observed a significant elevation in the number of neutrophils in the blood after treatment with T140, as compared with the control mice (Figure 2b). However, the number of neutrophils within the BM did not change significantly after T140 treatment (Figure 2c). To evaluate the mobilization potential of neutrophils within the BM, we calculated the ratio of neutrophil numbers in the BM and blood. We found that under steady state conditions, the ratio of neutrophils between BM and blood was 5 (Figure 2d, arrow). After chemotherapy, this ratio rose up to 50 on day 3 and reached more than 20 on days 5 and 6 (Figure 2d). These findings indicate that although the mice were neutropenic, they had a reservoir of neutrophils within the BM, which failed to mobilize to the periphery. After treatment with T140, neutrophils were released to the periphery and ratio of distribution between BM and blood dropped to its normal levels (Figure 2d). These results suggest that during the recovery phase, strong retention signals prevent the release of neutrophils from the BM to the periphery and that CXCR4 is predominantly controlling this phenomenon.

T140 and G-CSF can act synergistically to mobilize hematopoietic cells.[14] We found that T140, G-CSF or their combination stimulated the release of neutrophils from recovering BM into the blood (Figure 3a). Treatment with T140 and G-CSF together induced either an additive or a synergistic neutrophil mobilization (Figure 3a). It is important to note that the ability of G-CSF to release neutrophils into the periphery in the early days after recovery from chemotherapy was compromised as compared with T140 (Figure 3b).

Granulocyte colony-stimulating factor was found to induce stem cell mobilization by stimulating neutrophils to secrete proteases that cleave SDF-1 and decrease its BM concentration.[16] To evaluate the potential role of SDF-1 in the retention and exit of cells from chemotherapy-treated BM, SDF-1 levels in the BM were measured by ELISA. During the decline (day 3) and until day 6, the levels of SDF-1 remained high. On day 7, when the number of neutrophils increased, the amount of SDF-1 started to drop (Figure 3c). High levels of SDF-1 during the decline and recovery phases, together with the ability of T140 to release cells in the early days after recovery, suggest a critical role for CXCR4 and its ligand in the retention of cells.

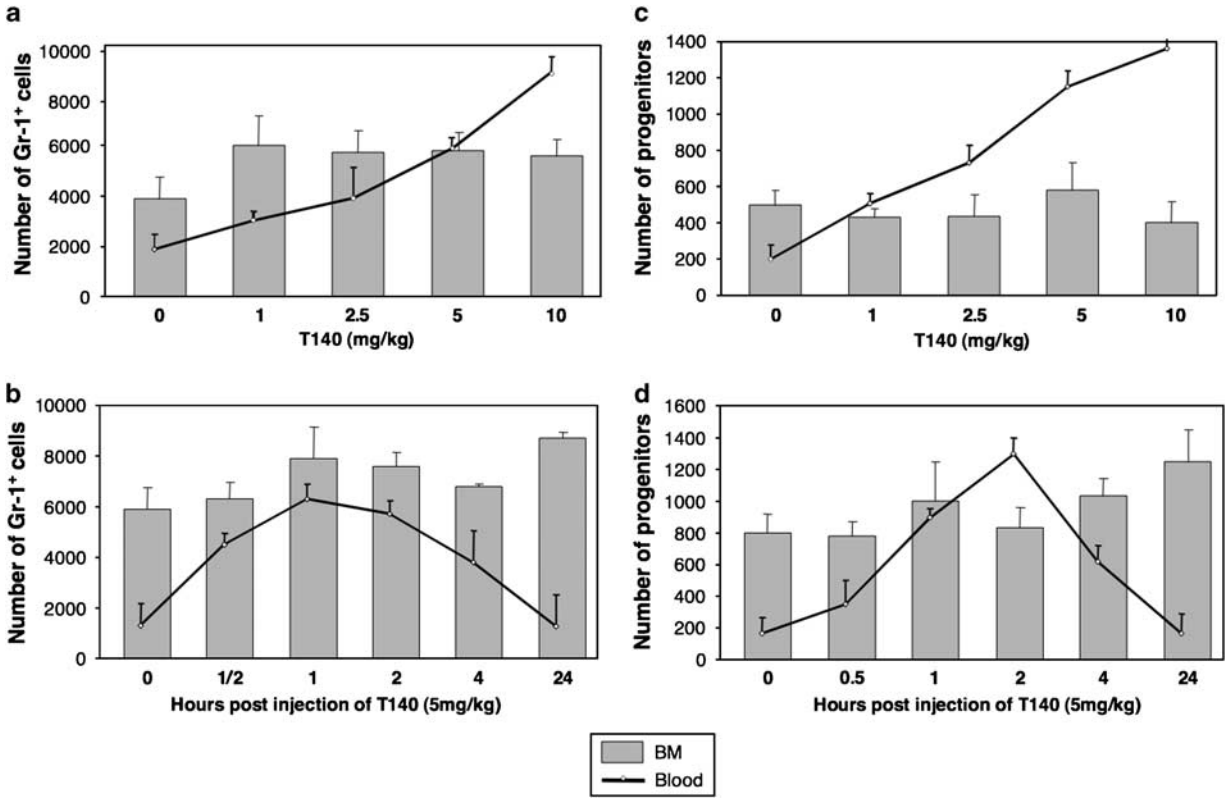


**Figure 1** The effect of T140 on the numbers of neutrophils and progenitors in the blood and bone marrow (BM). (**a** and **c**) Mice were subcutaneously injected with T140 (1, 2.5, 5 and 10 mg/kg) in a total volume of 200 μl. BM and blood were collected 2 h after the injection. (**b** and **d**) Mice, which were injected with 5 mg/kg of T140 were killed at different time points—1/2, 1, 2, 4 and 24 h after injection. Control mice were injected with phosphate-buffered solution (PBS). Number of neutrophils in the blood or the BM was evaluated by flow cytometry after staining with PE-anti-Gr-1 antibody (**a** and **b**) and the number of progenitors was evaluated using colony assay (**c** and **d**). The data shown are the average of 10 mice/group from a total of four separate experiments performed. The data shown are the average ± s.d. of four separate experiments performed (*$P<0.05$).

Furthermore, the results suggest a differential mechanism of action for G-CSF and T140 and a potent role for the use of combination therapy in stimulating the recovery of blood counts after chemotherapy.

### *T140 stimulates the production and mobilization of mature cells from the BM of transplanted mice*

Chemotherapy or irradiation therapy followed by BM transplantation is widely used to treat hematological malignancies. To study the role of CXCR4 in the recovery of the BM after transplantation, mice were treated with lethal total body irradiations and transplanted with normal BM cells. At 1 day after transplantation, mice were treated with T140 and a control group was left untreated (Figure 4a). As a negative control, a group of mice were irradiated and were not transplanted with BM cells. As shown in Figure 4b, mice that were not transplanted with BM cells did not recover and died on day 9 after irradiation, whereas the transplanted mice suffered from 5–6 days of blood neutropenia and a slow recovery of these cells in the blood. Similarly to chemotherapy-induced neutropenia, treatment with T140 at the decline phase (days 0–4) delayed the neutropenia at this phase by releasing a pool of neutrophils from the BM (Figure 4b). At the recovery phase (days 4–9), treatment with T140 significantly increased the number of Gr-1$^+$ neutrophils in the blood and reduced the duration of neutropenia. It is interesting to note that when we isolated cells from the BM of transplanted mice, we found that treatment with T140 stimulated the production of Gr-1$^+$ cells and mononuclear cells (MNCs) in the BM (Figure 4c). Moreover, treatment with T140 significantly increased the production of progenitors from all lineages, that is, CFU-GM, CFU-M, BFU-E and CFU-GEMM (Figure 4d). Interestingly, as opposed to T140, treatment with AMD3100 did not affect the number of progenitor cells within the BM (Figure 4d).

To further understand the unique role of T140 in stimulating the production of mature and immature hematopoietic cells within the BM, we tested the ability of T140 to stimulate directly and indirectly the proliferation and differentiation of hematopoietic progenitors. We tested the ability of T140 to stimulate colony formation in methylcellulose alone or together with G-CSF. T140 alone or in combination with the colony-stimulating factor G-CSF[17,18] could not stimulate the production of hematopoietic colonies (Figure 5a). Same results were observed when we added AMD3100 alone or in combination with G-CSF (data not shown).

The proliferation and differentiation of hematopoietic stem progenitors is tightly regulated by signals provided by stromal cells in the BM and in tissue culture. 14F1.1 mouse stromal cells were earlier shown to support hematopoiesis *in vitro*.[19–21]

To test the effect of T140 or AMD3100 on these cultures, BM cells ($0.5\times10^6$/well) were seeded on top of confluent cultures of 14F1.1 cells and T140, AMD3100 or G-CSF alone or in

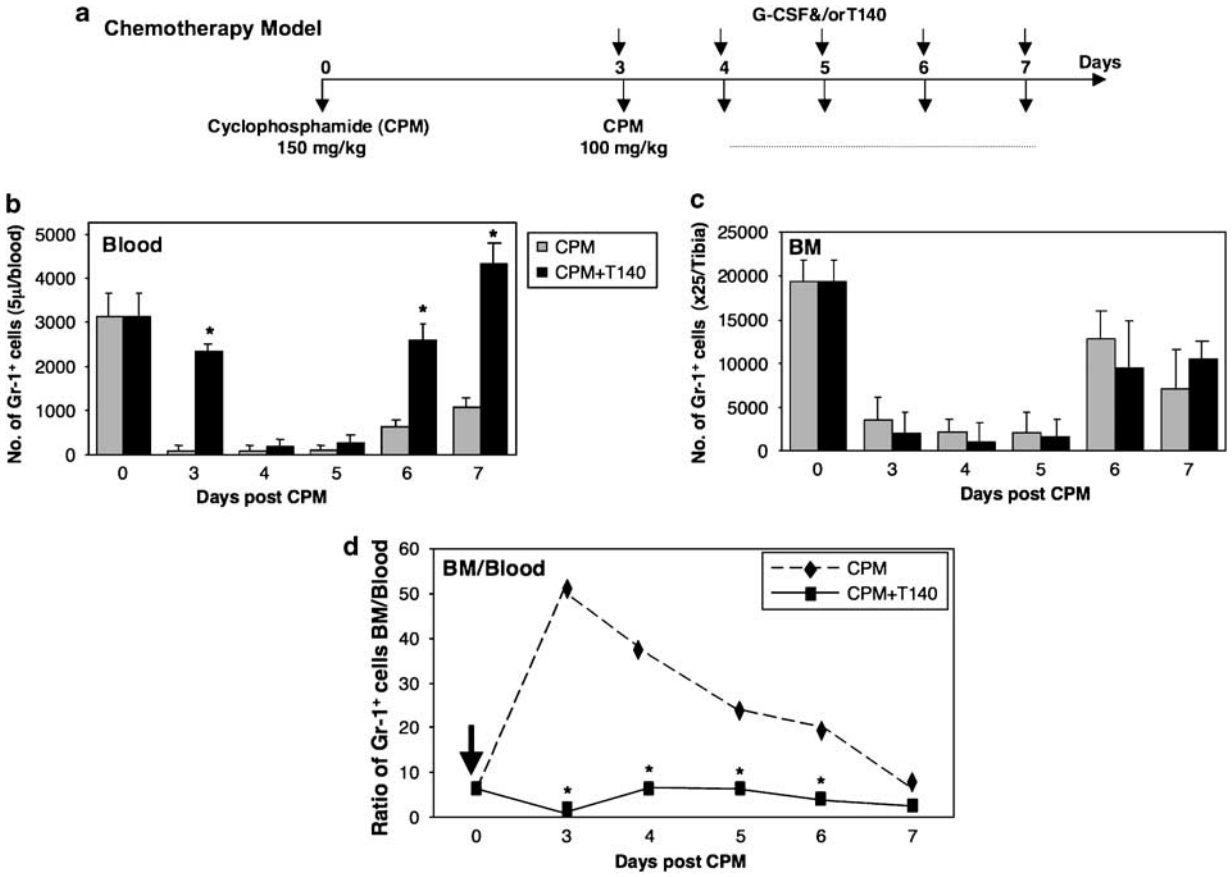


**Figure 2** The role of CXCR4 in the recovery phase after chemotherapy. (**a**) Chemotherapy model—mice received a total dose of 250 mg/kg cyclophosphamide (CPM) by two 0.25 ml intraperitoneal injections scheduled on day 0 (150 mg/kg) and day 3 (100 mg/kg). Mice were subcutaneously treated with 0.2 ml of T140 (5 mg/kg). Flow cytometry was used to assess the number of cells in blood (no. of Gr-1$^+$ cells (in 5 ml of blood)) and the bone marrow (BM) (no. of Gr-1$^+$ cells $\times 25$ in the Tibia). Cells were stained with PE-conjugated Gr-1 antibody and were gated according to forward scatter and side scatter to exclude dead cells and to determine granulocytes (Gr-1$^+$ cells). The ability of T140 to mobilize Gr-1$^+$ cells to the blood and reduce neutropenia is shown in (**b**). Numbers of Gr-1$^+$ cells in the BM are shown in (**c**). The ratio between relative numbers of Gr-1$^+$ cells in the blood and BM are shown in (**d**). The data shown are a representative of the average of 10 mice/group from a total of three separate experiments performed. The data shown are the average ± s.d. of three separate experiments performed (*$P<0.05$).

combination were added to the cultures. At 1 week after seeding, the cells were collected and the number of colony-forming cells was determined and the phenotype of the cells was tested by FACS analysis (Figures 5b and c). In the absence of the above factors, BM cells formed typical cobblestone areas within the stromal layer (Figure 5e). In the presence of G-CSF, but more significantly with T140, the number of cobblestone areas reduced significantly (Figure 5e). Concomitantly, in the presence of G-CSF and T140, or their combination, granulocyte differentiation was accelerated (Figure 5b). It is interesting to note that this effect could not be observed when AMD3100 was added to the culture. Furthermore, addition of AMD3100 together with G-CSF even abrogated the elevation induced by G-CSF alone (Figure 5b). However, only T140, but not G-CSF or AMD3100, stimulated Ter-119$^+$ erythroblasts production (Figure 5c). When we stained the cells with antibodies against Mac-1 or B-220, we did not observe any significant changes. Moreover, both T140 and G-CSF stimulated progenitor proliferation and their combination synergized in accelerating the expansion of the number of hematopoietic progenitors. However, AMD3100 did not show any affect on the proliferation of progenitor cells and was even found to abolish the effect induced by G-CSF alone (Figure 5d). To further test the effect of the natural CXCR4 ligand on the proliferation and differentiation of cells, SDF-1α was added into the cultures. Similarly to T140, SDF-1α stimulated the production of both Gr-1$^+$ cells (Figure 5b) and Ter-119$^+$ erythroblasts (Figure 5c) and hematopoietic progenitors (Figure 5d). These results suggest a role for CXCR4 in keeping the progenitor pool in close contact with the stroma and allowing these cells to suppress differentiation, as well as progenitor cell proliferation. The addition of high levels of SDF-1α may stimulate desensitization and internalization of the receptor and release the cells from the inhibitory effect of the stroma. Indeed, it was reported by Hattori *et al.*[22], that plasma elevation of SDF-1α achieved by adenoviral infection of the liver using adenoviral vector expressing SDF-1α induced the mobilization of mature and immature hematopoietic progenitor and stem cells.

### *Cells mobilized by the CXCR4 antagonist are characterized by high spontaneous motility rate and MMP-9 secretion*

To further understand the role of CXCR4 in the mobilization of hematopoietic cells, after irradiation and chemotherapy, we characterized the mobile cells after CXCR4 blockage. Mobilized cells were isolated from the blood after treatments with T140, G-CSF or their combination (as described in Materials and

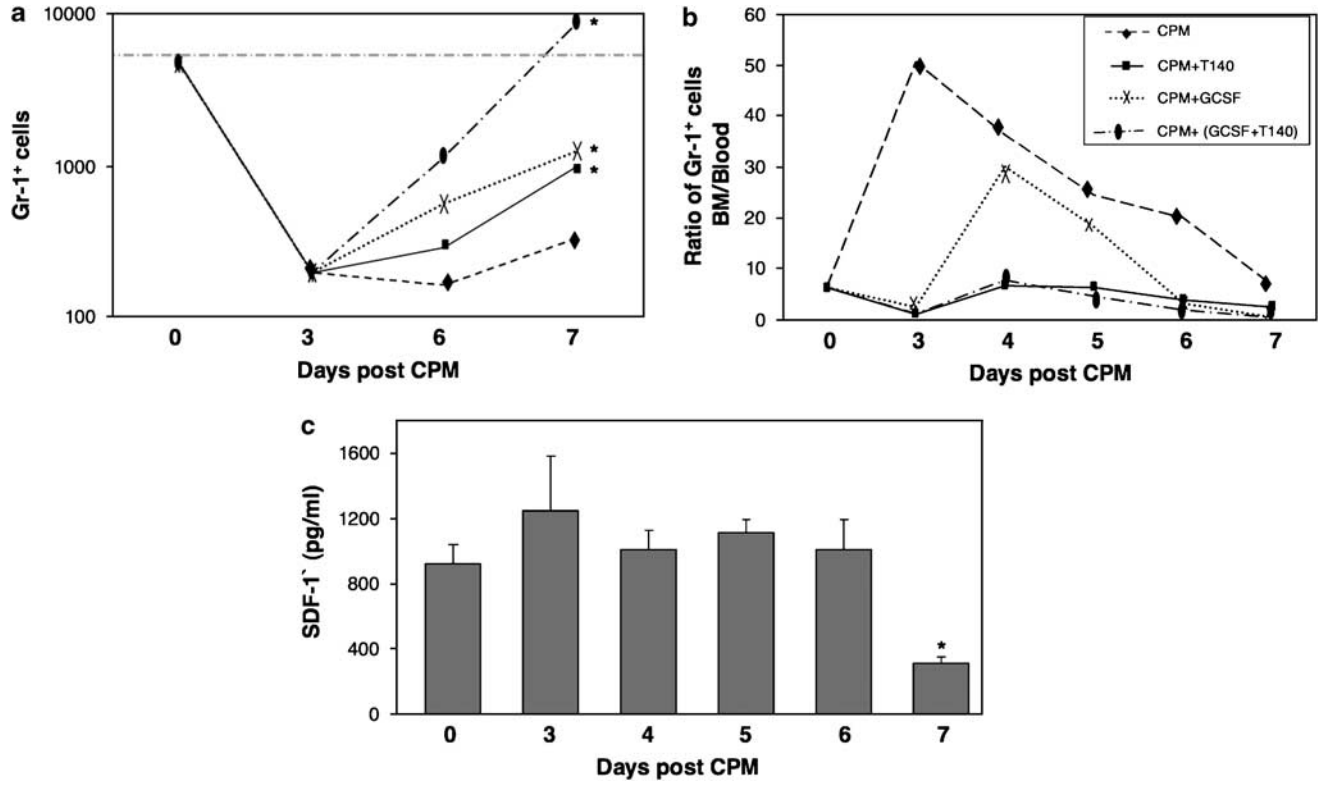


**Figure 3** The role of granulocyte colony-stimulating factor (G-CSF) and CXCR4 in the recovery phase after chemotherapy. Mice received a total dose of 250 mg/kg cyclophosphamide (CPM) by two 0.25 ml intraperitoneal injections scheduled on day 0 (150 mg/kg) and day 3 (100 mg/kg). Mice were subcutaneously treated with 0.2 ml of T140 (5 mg/kg) or G-CSF (5 μg/mouse) or the combination of both. Flow cytometry was used to assess the number of cells in blood and bone marrow (BM). Cells were stained with PE-conjugated Gr-1 antibody and were gated according to forward scatter and side scatter to exclude dead cells and to determine granulocytes (Gr-1$^+$ cells). The ability of T140 and G-CSF by themselves or in combination to mobilize Gr-1$^+$ cells and reduce neutropenia is shown in (**a**). The ratio between relative numbers of Gr-1$^+$ cells in the blood and BM is shown in (**b**). (**c**) The level of stromal cell-derived factor-1 (SDF-1) (pg/ml) within the BM after treatment with CPM was determined using ELISA. The data shown are the average of 10 mice/group from a total of two separate experiments performed. The data shown are the average ± s.d. of two separate experiments performed (*$P<0.05$).

methods). Blocking CXCR4 with T140 stimulated the proliferation of progenitor cells within the BM (Figure 5d). To find out whether these proliferating cells were those, which were mobilized into the blood, we injected BrdU *in vivo* to the mice after treatment with T140 or G-CSF. We found that T140, but not G-CSF, increased the number of BrdU$^+$ cells by 3.5-fold, and when we combined T140 with G-CSF, the number of these cells increased by eightfold (Figure 6a). These results suggest a role for T140 in stimulating the mobilization of cells that have been recently proliferating and differentiating.

We next hypothesized that the motility of the mobilized cells plays a role in the process of cell mobilization induced by T140 and G-CSF. To assess the motility potential of cells, mobilized cells were isolated from the blood and seeded on transwells coated with BM endothelial cells. To study the ability of mobilized cells to spontaneously migrate across the BM, transendothelial migration was evaluated after a 3 h incubation of the mobilized cells on the coated transwells by counting the migrated cells using the FACS. Cells isolated from the blood of control non-mobilized mice had a low rate (7%) of spontaneous migration (SPM) (Figure 6b). Cells isolated from the blood of mice treated with either T140 or G-CSF had an increased SPM, 15 and 20%, respectively. Furthermore, 60% of the cells isolated from the blood of mice treated with both T140 and G-CSF increased their SPM (Figure 6b). These results showed that the cells, which were mobilized by either T140 or G-CSF, or their combination, were more motile.

To test the SPM of mobilized progenitors, we calculated the percentage of motile progenitors by dividing the number of CFC that transmigrates through the endothelial cells by the number of seeded CFC, at the beginning of the migration assay. When we evaluated the motility of progenitors, we found that blood-derived progenitors from control and T140-treated mice had no motility, whereas treatment with G-CSF alone mobilized a low percentage of progenitors with motility (2%, Figure 6c). It is interesting to note that 20% of the progenitor cells isolated from the blood of mice treated with both T140 and G-CSF had increased their SPM (Figure 6c). All motile progenitors belonged to the CFU-GM lineage. These results showed that growth factor such as G-CSF can stimulate the motility of progenitor cells and that these progenitors can be further released from retention in the BM by the CXCR4 antagonist.

One of the factors involved in the motility and mobilization of hematopoietic cells was the secretion of MMP-9.[23–25] To test the role of MMP-9 in T140 and G-CSF-induced mobilization, we cultured the mobilized cells in serum-free medium and measured the activity and secretion of MMP-9 by zymography. As shown in Figure 6d, cells which were mobilized by T140, G-CSF, or the combination of both factors, secreted 8-, 3.5- and 13-fold higher MMP-9 levels, respectively, than cells from the

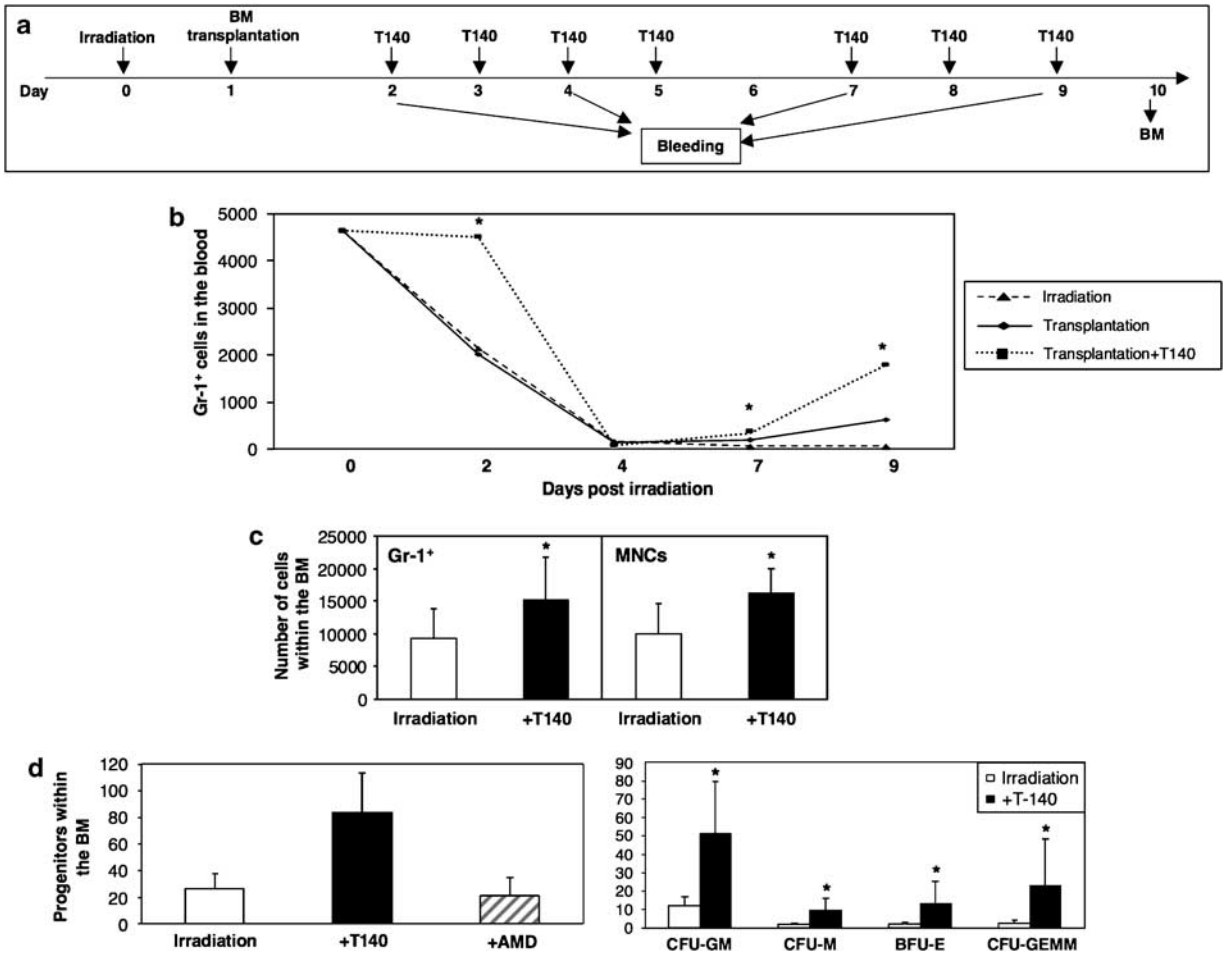


**Figure 4** The role of CXCR4 in the recovery phase after irradiation and bone marrow (BM) transplantation. (**a**) The BM transplantation model—mice received lethal total body irradiations of 900 rad (day 0). After 24 h, normal BM cells ($5 \times 10^6$ cells/mouse) were i.v. transplanted in a total volume of 200 µl phosphate-buffered solution (PBS) day 1. Mice were subcutaneously treated daily with T140 or AMD3100 (5 mg/kg) from day 2 till day 9. Mice were bled on days 0, 2, 4, 7 and 9, 11 h after treatments and BMs were harvested on day 10. Flow cytometry was used to assess the number of cells in blood and BM. Cells were gated according to forward scatter and side scatter to exclude dead cells and to determine granulocytes (Gr-$1^+$ cells) and mononuclear cells (MNC). The ability of T140 to mobilize Gr-$1^+$ cells and reduce neutropenia is shown in (**b**). The numbers of Gr-$1^+$ cells and MNC within the BM with or without treatment of T140 is shown in (**c**). (**d**) The ability of T140 versus AMD3100 to stimulate production of various progenitors in the BM is assayed after isolation of BM on day 10 and cultured for colony assay. The data shown are the average ± s.d. of 6 mice/group from a total of five separate experiments performed (*$P<0.05$).

blood of control mice (Figure 6d). To further study the role of MMP-9 in the motility of the mobilized cells, we used the pan-MMPs inhibitor GM-6100 and added it to the transendothelial motility assay. The transendothelial migration of neutrophils derived from mice treated with T140, G-CSF, or the combination of both factors, was significantly more inhibited than cells derived from control mice, 60, 65, 80 and 30%, respectively (Figure 6e). These results strongly correlate with the increased amount of MMP-9 released by these cells (Figure 6d). It is interesting to note that the MMPs inhibitor had no effect on the transendothelial migration of MNCs (Figure 6f). These results showed the role of MMP-9 in the motility of mobilized granulocytes, but not MNCs.

Both CXCR4, as well as VLA-4 play a critical role in the homing, retention and mobilization of hematopoietic cells.[26–29] We therefore tested the expression pattern of these cell surface receptors on the cells mobilized to the periphery by T140 or G-CSF or their combination. Treatment of mice with T140 decreased the percentage of mobilized neutrophils that expressed CXCR4 by almost twofold while increasing its expression on MNCs (Figure 6g). As shown in Figure 6h more neutrophils (twofold), which were mobilized by T140, but not G-CSF, expressed VLA-4, whereas the expression of VLA4 on MNCs did not show any significant change (Figure 6h). Reduced levels of CXCR4 may be a result of T140-dependent internalization of the receptors. Increased levels of VLA-4 on neutrophils may also be the outcome of increased stimulation of cells with T140. The opposite mild effects observed in MNCs may result from the low number of cells expressing CXCR4.

## Discussion

Cancer patients usually develop neutropenia as a result of chemotherapy or radiation therapy for cancer or before and after BM transplantation and are extremely susceptible and vulnerable to various germal infections, sometimes even life-threatening.[30,31] In this work, we studied the role of CXCR4 in the process of BM recovery after chemotherapy and transplantation by using the CXCR4 antagonist T140.[11,12]

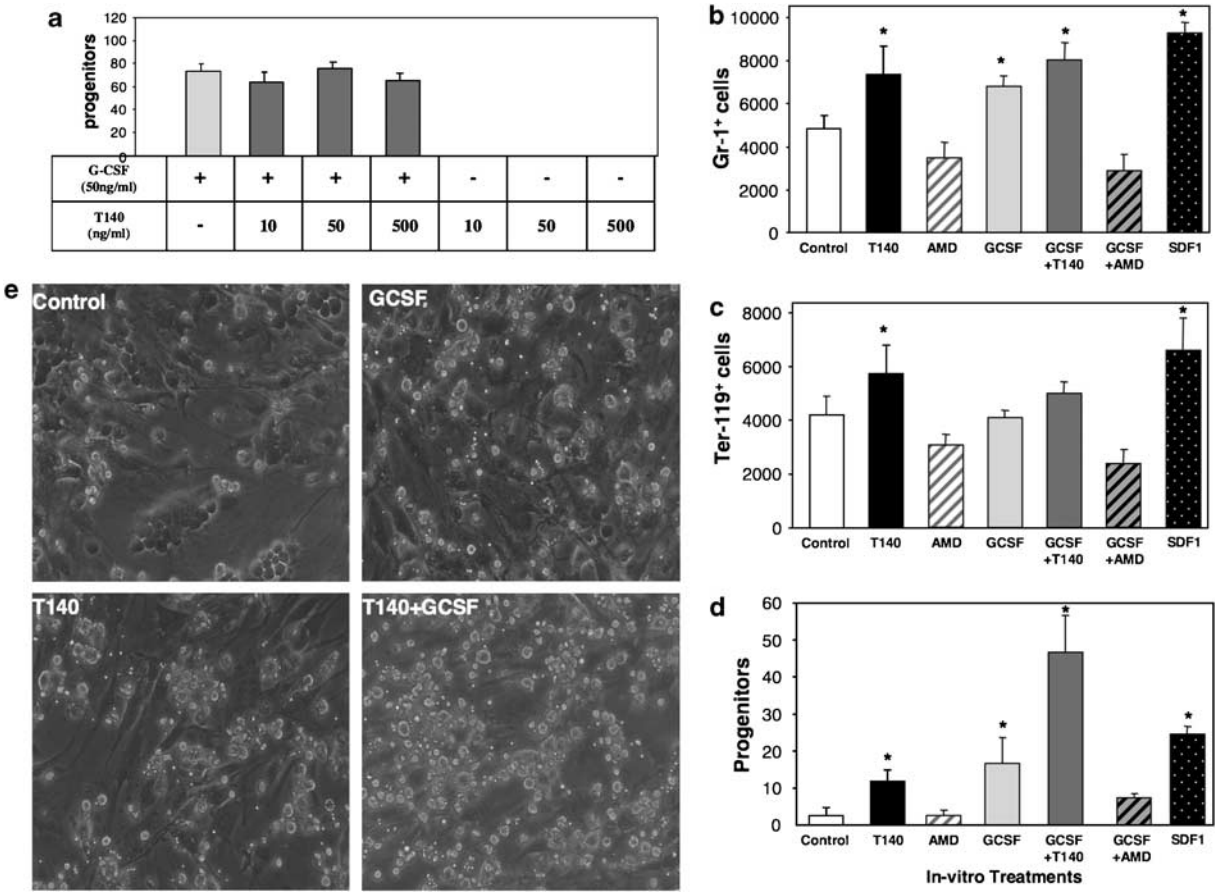


**Figure 5** The role of granulocyte colony-stimulating factor (G-CSF) and CXCR4 in the interaction between hematopoietic progenitors and mature cells and stromal cells. (**a**) Normal bone marrow (BM) cells ($0.3 \times 10^6$) were cultured with 3 ml of methylcellulose in the presence of G-CSF (50 ng/ml), T140 (10, 50 and 500 ng/ml), AMD3100 (10, 50 and 500 ng/ml) and their combinations. Numbers of colonies were counted after 5 days. Normal BM cells ($0.5 \times 10^6$/well) were cultured on top of the stromal cell line 14F1.1. T140 (20 μg/ml), AMD3100 (20 μg/ml) with or without (G-CSF (50 ng/ml) or stromal cell-derived factor-1 (SDF-1)α (5 μg/ml) were added to the culture every second day. After 6 days, cells were collected and the numbers of Gr-$1^+$ cells (**b**) or Ter-$119^+$ cells (**c**) were counted by FACS. (**d**) The number of progenitors was evaluated using the colony assay. The data shown are the average ± s.d. of four separate experiments performed (*$P<0.05$). (**e**) Representative picture of BM cells after 6 days of culture on top of stromal cells with the different treatments.

SDF-1 and CXCR4 are involved in the homing, retention and mobilization of hematopoietic stem cells, progenitors and mature cells during development and under physiological conditions. In contrast, the role of SDF-1 and CXCR4 in the proliferation and differentiation of hematopoietic stem and progenitors is less clear.

*In vitro*, SDF-1 enhanced the expansion and differentiation of primitive cord blood (CB) cells in the presence of specific cytokines. Without cytokine addition, cultures seeded with CD34+CD38−Lin− cells showed substantial cell death; however, the addition of SDF-1 alone preferentially increased progenitor cell frequency.[32] This study was supported by additional reports, indicating that SDF-1 alone can promote the survival of purified CD34+ cells by participating in the autonomous survival and cycling of progenitors under physiological conditions by counteracting apoptosis.[33] The effect of SDF-1 on the differentiation of progenitor cells was also tested and it was shown that SDF-1 can act together with thrombopoietin to stimulate the development of megakaryocytic progenitor cells (CFU-MK).[34] In addition, it was also shown that SDF-1 can induce a selective inhibition of human erythroid development through the functional upregulation of Fas/CD95 ligand.[35] In contrast to the studies that suggested a role for SDF-1 in promoting the survival, proliferation and differentiation of hematopoietic stem cells and progenitors, other reports suggested a role for SDF-1 in inhibiting the cycling of very primitive human and mouse stem cells *in vitro* and *in vivo*.[19,36] Our results suggest that during the recovery phase, strong retention signals provided by the BM stroma prevent the proliferation and maturation of hematopoietic progenitors, as well as the release of mature cells from the BM to the periphery and that CXCR4 is predominantly controlling this phenomenon.

G-CSF has been shown to be relatively safe and effective in accelerating the recovery of neutrophil counts after a variety of chemotherapy regimens; however, its effect is limited and far from being satisfactory.[37,38] Moreover, though G-CSF affects hematopoiesis, its influence is exerted on cell migration and mobilization only at late stages of neutrophil production, when its effect on the number of neutrophils in the blood is not as crucial as in the first stages, not to mention that it has no effect whatsoever on the recovery of red blood cells.

AMD3100, a selective antagonist of CXCR4, was recently shown to rapidly mobilize CD34+ HPCs from the human marrow into the peripheral blood. Furthermore, AMD3100

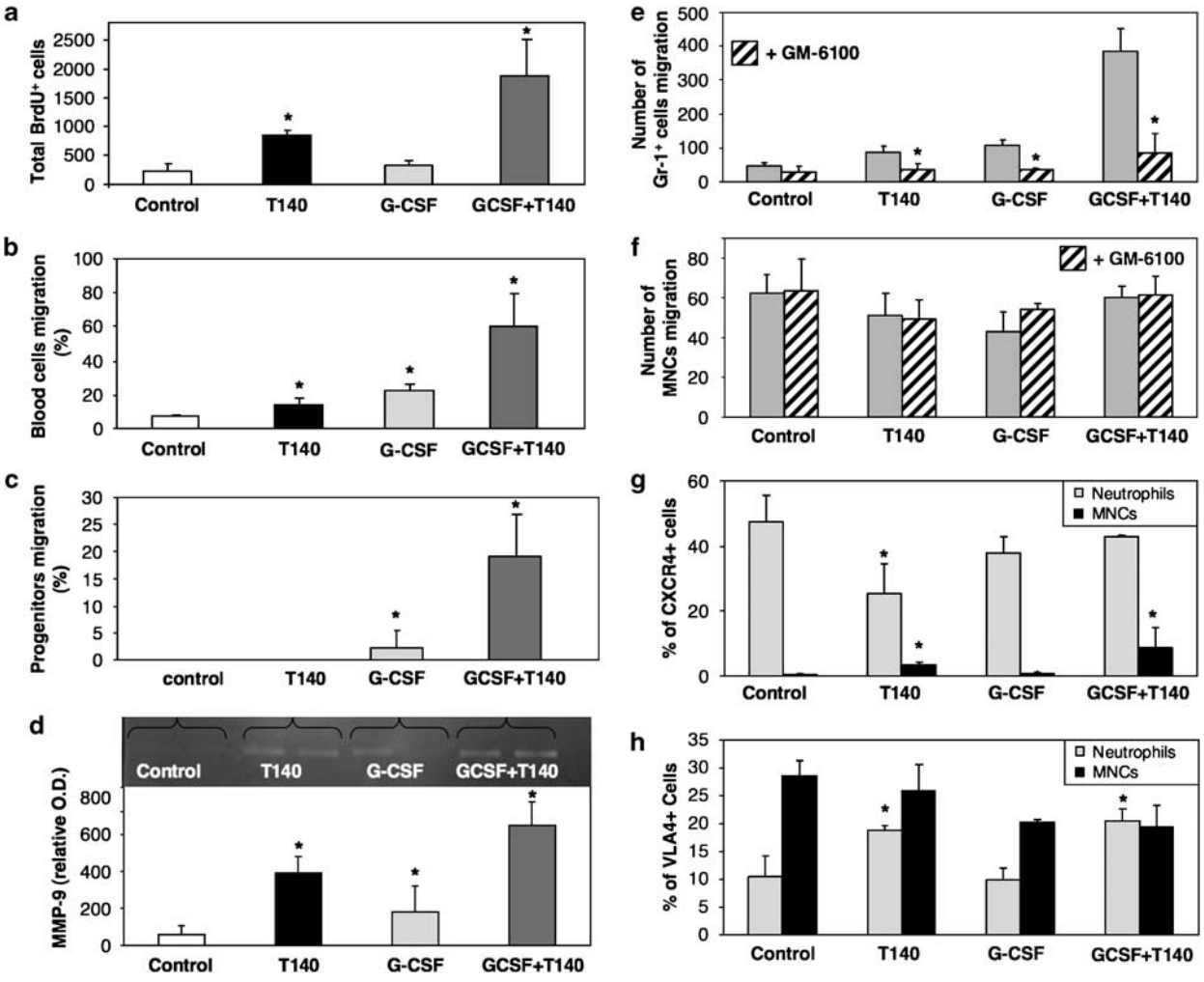


**Figure 6** Characterization of CXCR4- and granulocyte colony-stimulating factor (G-CSF)-dependent mobilized cells. Mice were subcutaneously injected with 0.2 ml of T140 (5 mg/kg), G-CSF (2.5 μg/mouse) or their combination, twice a day for 2 days. Another injection was performed on day 3, and 1 h later blood was collected and isolated. Control mice were injected with phosphate-buffered solution (PBS) at the appropriate volume. (**a**) Proliferation of mobilized cells was measured by the BrdU flow kit. (**b**) Motility of mobilized cells was measured using transendothelial migration assay. A total of $3 \times 10^5$ bone marrow (BM) cells were placed in the upper chamber and the number of migrating cells was counted by FACS after 4 h. (**c**) Motility of progenitor cells was evaluated after 6 h of migration. Migrating cells were cultured for colony assay and the percentage of migrating progenitors was calculated. (**d**) Secretion and activity of matrix metalloproteinase-9 (MMP-9) from mobilized cells were determined after culture of equal numbers of mobilized cells ($0.25 \times 10^6$/well) in serum-free medium. Supernatants were collected after 24 h and run on gelatin zymography gel. Histograms show the mean densitometric intensity (± s.d.), as assessed in three separate experiments. (**e** and **f**) GM-6100 (pan-MMPs inhibitor) was added to the transendothelial migration assay culture at a final concentration of 10 μM/ml and the numbers of migrated granulocytes (**e**) or MNC cells (**f**) were counted by FACS after 4 h. Data show the number of cells migrating with or without the MMP inhibitor. Expression of CXCR4 (**g**) and VLA4 (**h**) on mobilized neutrophils and MNCs was detected by flow cytometry after staining with FITC-anti-CXCR4 and FITC-anti-VLA4. The data shown are the average ± s.d. of four separate experiments performed (*$P<0.05$).

significantly increased both G-CSF stimulated mobilization of CD34+ cells and leukapheresis yield of CD34+ cells. In addition, AMD3100 was reported to be a safe and effective agent for the mobilization of CD34+ cells in patients with multiple myeloma or lymphoma receiving before chemotherapy.[39,40] On the other hand, a clinical trial with AMD3100 in HIV-infected individuals produced premature ventricular contraction side effects, resulting in the discontinuation of this trial.[41]

In our earlier work, we have found that T140 can induce mobilization of hematopoietic stem cells and progenitors within a few hours after treatment in a dose-dependent manner. Furthermore, T-140 can also increase the number of WBC in blood. T-140 was found to efficiently synergize with G-CSF in its ability to mobilize WBC and progenitors. Comparison between the CXCR4 antagonists, T-140 and AMD3100, showed that T-140 with or without G-CSF was significantly more potent in its ability to mobilize hematopoietic stem cells and progenitors into blood. These results show that different CXCR4 antagonists may have different therapeutic potential.[14]

Comparative studies between T-140 and AMD3100 found that each of these agents inhibited CXCR4 through different mechanisms.[11,12,14] Analysis of antagonists revealed that exposure to AMD3100 induced G protein activation by CXCR4, whereas T-140 decreased autonomous signaling.[12] Therefore, AMD3100 was defined to have a weak partial agonist activity, whereas T-140 functions as an inverse agonist. In our current work, we found that SDF-1α at high levels, as well as T140, but not AMD3100, similarly affect the proliferation and differentiation of

progenitors, granulocytes and erythroblasts when co-cultured with stromal cells. It was already reported that high plasma levels of SDF-1α can stimulate the mobilization of hematopoietic cells from the BM probably by desensitization and internalization of the CXCR4 receptor.[22] Furthermore, it was also reported that low levels of SDF-1α inhibit the production of erythroid colonies *in vitro* in semisolid agar.[35] These results may therefore suggest that either T140 or high levels of SDF-1α block the CXCR4 axis and release cells from the stromal suppression effect leading to an increased mobilization and production of cells from and in the BM.

The CXCR4 antagonist, T140, acts through a mechanism different from that of G-CSF. This is shown by the compromised ability of G-CSF to release neutrophils into the periphery in the early days after recovery from chemotherapy, as compared with T140 (Figure 3b). In addition, when we measured the effect of T140 ± G-CSF on the proliferation of BM cells in the presence of stromal cells, we found that only T140, but not AMD3100 or G-CSF, stimulated Ter $119^+$ erythroblasts production (Figure 5c). This specific effect of T140 on the erythroblast proliferation is compatible with our earlier work,[14] in which indeed, we found that the most dramatic effect of T140 on the induction of progenitor's mobilization was observed with the mobilization of the erythroid progenitors, BFU-E. This effect could not be observed when we used either G-CSF or the CXCR4 antagonist AMD3100.

In semisolid assays, relatively low levels of SDF-1α (20–200 ng/ml) significantly reduced the number of plurifocal erythroid colonies (erythroid blast-forming units; BFU-E), whereas these levels did not affect CFU-GM.[35] We suggest that both SDF-1α at high levels (5 μg/ml), as well as T140 may block the CXCR4 axis and allow more erythroid progenitors to develop. Future molecular studies should address the questions about the specific mechanism(s) by which T-140 induces and high levels SDF-1α allow for the production of erythroblasts. G-CSF was found to induce stem cell mobilization by stimulating neutrophils to secrete proteases that cleave SDF-1 and decrease its BM concentration.[16] Indeed, we did not observe any alteration in the levels of SDF-1 during the decline and the beginning of the recovery phase (day 3–6); however, on day 7, when the number of neutrophils increased, the amount of SDF-1 dropped significantly (Figure 3c). Whereas the CXCR4 antagonist, AMD3100, predominantly synergized with G-CSF to induce mobilization of hematopoietic cells,[13] T140 strongly synergized with G-CSF to induce the exit from the BM of mature cells and progenitors.[14] These results may be explained by the exclusive ability of T140 to release cells from the suppressive effect of the stroma and expose them to either G-CSF or other stromal-derived cytokines.

Characterization of the mobile cells after CXCR4 blockage revealed that T140 alone or in combination with G-CSF stimulated the mobilization of recently proliferating cells characterized by increased transendothelial migration, MMP-9 secretion and CXCR4 expression. Increased motility coupled with a recent proliferation event and high MMP-9 may define a transient population of maturing cells a step before they are released from the suppression of the stroma and exit the BM. Indeed, it was already shown that in MMP-9−/− mice, the release of soluble Kit-ligand (stem cell factor, SCF) and motility of hematopoietic stem cells were impaired, resulting in the failure of hematopoietic recovery and increased mortality, although exogenous SCF restored hematopoiesis and survival after BM ablation.[42] G-CSF stimulates neutrophils to secrete a variety of proteolytic enzymes, including elastase, cathepsin G, MMP-2 and MMP-9.[9,16] Interestingly, we found that T140 alone, or with G-CSF, stimulated the secretion of MMP-9 from mobile cells. Most of the mobilized cells are neutrophils, and blocking MMP9 with GM-6100 blocks specifically neutrophils, but not MNCs, suggesting that MMPs are functionally secreted mainly from mobilized neutrophils. The specific downregulation of CXCR4 on neutrophils mobilized by T140 may be a result of internalization of the receptor CXCR4 by T140. The β 1 integrin, very late antigen 4 (VLA-4), plays a central role in SDF-1α-induced mobilization and homing of CD34 + cells.[43–45] When we looked at the expression of VLA-4 on mobilized cells, we found that T140 exclusively upregulates its expression on mobilized neutrophils. Changes in the expression of VLA-4 may be the outcome of increased stimulation of mobilized cells with T140 and the ability of G-CSF to antagonize for such stimulation when it is injected with T140 or alone. The differential expression of CXCR4 and VLA4 on mobilized MNCs can show the specific effect of T140 on mobilization of different subpopulations or may be the outcome of low numbers of MNCs expressing CXCR4. Recent work published by Nie *et al*.[19] reports that CXCR4 is required for the quiescent state of primitive hematopoietic cells. In this report, SDF-1 produced by BM stromal cells is not only the major chemoattractant for HSCs, but also a regulatory factor that controls the quiescence of primitive hematopoietic cells.

Our results suggest that during the recovery phase from irradiation or chemotherapy, CXCR4 plays a critical role in keeping the progenitor pool in close contact with the stroma and allows these cells to suppress both differentiation as well as progenitor cell proliferation. Releasing the cells from the suppressive effect of the stroma allows a faster response of these cells to outside signals such as G-CSF and accelerates the recovery of the BM after transplantation or chemotherapy. Furthermore, our results suggest a unique effect to the CXCR4 antagonist, T140 as compared with AMD3100, and show that different CXCR4 antagonists may have different therapeutic potential.

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