

Original Article

Granulocyte colony-stimulating factor suppresses SDF-1 α /CXCR4 and mobilizes regulatory T cells in acute myelogenous leukemia

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Abstract: In acute myelogenous leukemia (AML), the bone marrow microenvironment provides growth and survival signals that may confer resistance to chemotherapy. Granulocyte colony-stimulating factor (G-CSF) potently inhibits lymphopoiesis by targeting stromal cells that comprise the lymphoid niche in the bone marrow; nonetheless, its healing effects in AML are unknown. In this current study, we demonstrated that G-CSF significantly inhibits the CXCR4 expression in a time-dependent manner, and antagonized SDF-1 α -induced migration of AML cells. Furthermore, G-CSF effectively mobilized regulatory T cells into the circulation and inhibits the SDF-1 α in *in vivo* xenograft models. These findings indicate that G-CSF is useful as an effective ingredient in antileukemia activity.

Keywords: Acute monocytic leukemia, G-CSF, regulatory T cells, SDF-1 α , CXCR4

Introduction

Leukemia is a malignant cancer in humans [1]. The characteristics of leukemia include uncontrolled cell growth and disrupted differentiation of hematopoietic cells. The clinical therapies for leukemia include chemotherapy, radiotherapy and bone marrow transplant [2]. However, these strategies have not been shown to be satisfactory for the treatment of leukemia, thus leading to researchers focusing on the discovery of novel therapeutics.

The emergence of cancer results from the disruption of cell growth regulation and failure of the host to provoke sufficient immunological antitumor response. Most cancer patients do not develop a satisfactory immunological antitumor response, implicating the existence of tumor-specific immune evasion strategies [3]. Normal immunocompetent hosts contain tumor-reactive T cells whose activation and expansion is suppressed by the presence of

regulatory T cells (Tregs) [4]. Tregs, characterized by the expression of high levels of CD25 and the forkhead box transcription factor P3 (FOXP3) [5, 6], are a subset of CD4+ T cells and account for 5-10% of the normal CD4+ T cell population. Tregs play a critical role in suppressing the host antitumor immune response and enable cancer cells to evade immune surveillance, and might contribute to the progression of human cancers [7]. Therefore, targeting Tregs in AML represents a novel strategy.

The stromal-derived factor 1 α (SDF-1 α) and its receptor CXCR4 have recently emerged as critical mediators of stromal/leukemic cell interactions [8]. SDF-1 α and CXCR4 primarily regulate the homing, migration, and mobilization of hematopoietic cells [9]. Binding of SDF-1 α to CXCR4 causes CXCR4 to be incorporated into lipid rafts [10] and increases its phosphorylation [11]. The phosphorylation of CXCR4 results in prolonged activation of the extracellular signaling-regulated kinase (ERK)

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and phosphoinositol 3-kinase (PI3K) pathways [12], which are key signaling pathways promoting leukemia cells survival [13]. Both surface and intracellular CXCR4 levels were found to be elevated in a subset of AML cases [14]. Altogether, these findings suggest that disruption of SDF-1 α /CXCR4 signaling represents a novel strategy for targeting leukemia/bone marrow microenvironment interactions.

Granulocyte colony-stimulating factor (G-CSF), a myeloid growth factor, promotes proliferation, survival, and differentiation of myeloid-lineage leukemic cells, as well as normal hematopoietic cells [15]. G-CSF has been used for its priming effect, enhancing the sensitivity of leukemia progenitor cells to cytotoxic agents in acute myeloid leukemia (AML) [16]. The clinical usage of G-CSF mobilized donor lymphocytes administered for therapy of relapse in AML has demonstrated efficacy with a similar graft versus leukemia response when compared with conventional non-mobilized donor lymphocytes [17]. These results demonstrated the importance of using G-CSF-mobilized lymphocytes as a starting material for the manufacturing of antileukemia immunotherapies. Although basic studies of G-CSF in various *in vitro* systems have been performed [18], the efficacy of G-CSF on SDF-1 α /CXCR4 signaling and Tregs are not well understood *in vivo* in the context of AML.

In this study, we examined the effects of G-CSF on AML. Our data suggested G-CSF inhibited SDF-1 α /CXCR4 signaling and thus antagonized SDF-1 α -induced migration of AML cells. Furthermore, we report that in murine *in vivo* xenograft models, G-CSF effectively mobilized regulatory T cells into the circulation. These findings strongly support the notion that blockade of SDF-1 α /CXCR4 interactions by G-CSF may have utility in eliminating leukemic cells that are otherwise protected by Tregs in the bone marrow microenvironment.

Materials and methods

Cell culture

Human AML-M5 U937 cells and mice AML-M4WEHI-3 cells were obtained from ATCC (Rockville, MD, USA). Cells were grown in RPMI-1640 medium supplemented with 10% fetal

bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Cell surface markers examination

For phenotypical analysis, cells were incubated with antibodies in Flow cytometry (FCM) buffer (PBS/0.5% bovine serum albumin (BSA; Sigma)) for 30 minutes at 4°C. After washing, cells were resuspended in FCM buffer and analyzed for the levels of specific cell surface markers by flow cytometry as described elsewhere [19]. The following conjugated monoclonal anti-human or anti-mouse antibodies were used for cell phenotyping: CXCR4-PE, CD4-FITC, CD25-APC, FOXP3-PE. All the antibodies are from Ebioscience.

Cell invasion assay

A cell invasion assay was performed using a BD BioCoatMatrigel invasion chamber (Becton-Dickinson, Franklin Lakes, NJ). AML cells were co-cultured with G-CSF (100 ng/mL) for 18 h. The lower chambers were filled with 600 μ L of RPMI-1640 medium containing 100 ng/ml SDF-1 α or PBS, and the upper chamber of each Transwell was filled with 200 μ L of treated or control cells (1×10^6 cells/mL). Cell invasion into Matrigel was determined after 10 h of culture at 37°C. The migrating cells in the lower chamber (U937) were counted with an inverted phase microscope (Olympus Corporation, Tokyo, Japan). And the invading cells (WEHI-3) on the bottom were stained with 0.1% crystal violet followed by fixation in methyl alcohol for 30 minutes and then were counted.

Animal handling

A total of 40 BALB/c mice of 6 weeks of age and 22-25 g in weight were purchased from the Animal Center of Xi'an Jiaotong University. All animal experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Xi'an Jiaotong University.

Establishment of the leukemic mice model

A total of 40 BALB/c mice were randomly divided into four groups. Group 1 received a subcutaneous injection of the PBS as a control, group 2 received a subcutaneous injection of G-CSF

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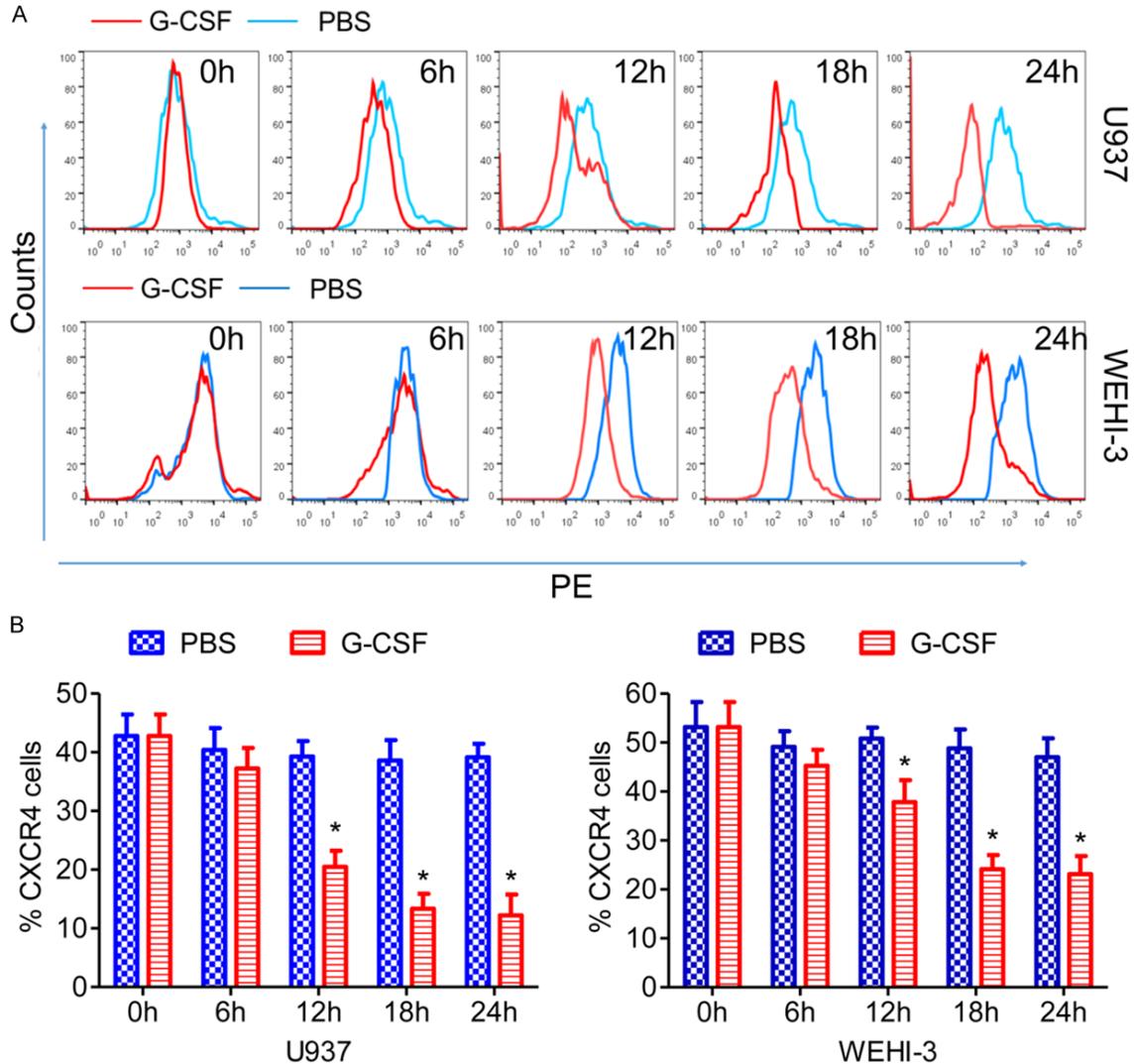


Figure 1. G-CSF inhibits CXCR4 expression in AML cells. A. U937 and WEHI-3 cells were treated with 100 ng/ml G-CSF for the indicated time course, then incubated with CXCR4 antibodies and detected by FCM. B. Quantification of CXCR4 expression. All data represent three independent experiments, mean \pm s.d. *: Compared with 0 h group, * $P < 0.05$.

(100 $\mu\text{g}/\text{kg}/\text{day}$) for 5 days, group 3 received an intravenous injection of 1×10^6 WEHI-3 cells, and group 4 received an intravenous injection of 1×10^6 WEHI-3 cells for 29 days and were then administered an subcutaneous injection of G-CSF (100 $\mu\text{g}/\text{kg}/\text{day}$) for 5 days. At day 34, all animals were sacrificed by euthanasia with CO_2 .

Tissue harvest

To harvest cells from engrafted mice, peripheral blood, spleen and bone marrow were processed as follows. Peripheral blood was col-

lected into EDTA-coated tubes and 500 μl 2% Dextran sulfate in PBS was added to blood and was incubated for 20 minutes at 37°C . Post-incubation, peripheral blood mononuclear cell (PBMC)-enriched supernatant was collected, spun down and washed once with PBS. Cells were red cell depleted by ammonium chloride lysis and washed with PBS. Spleens were collected and crushed through nylon mesh filters (to obtain spleen samples). Harvested cells were red cell depleted as previously described and washed with PBS. After resuspension in PBS, cells were filtered through polystyrene

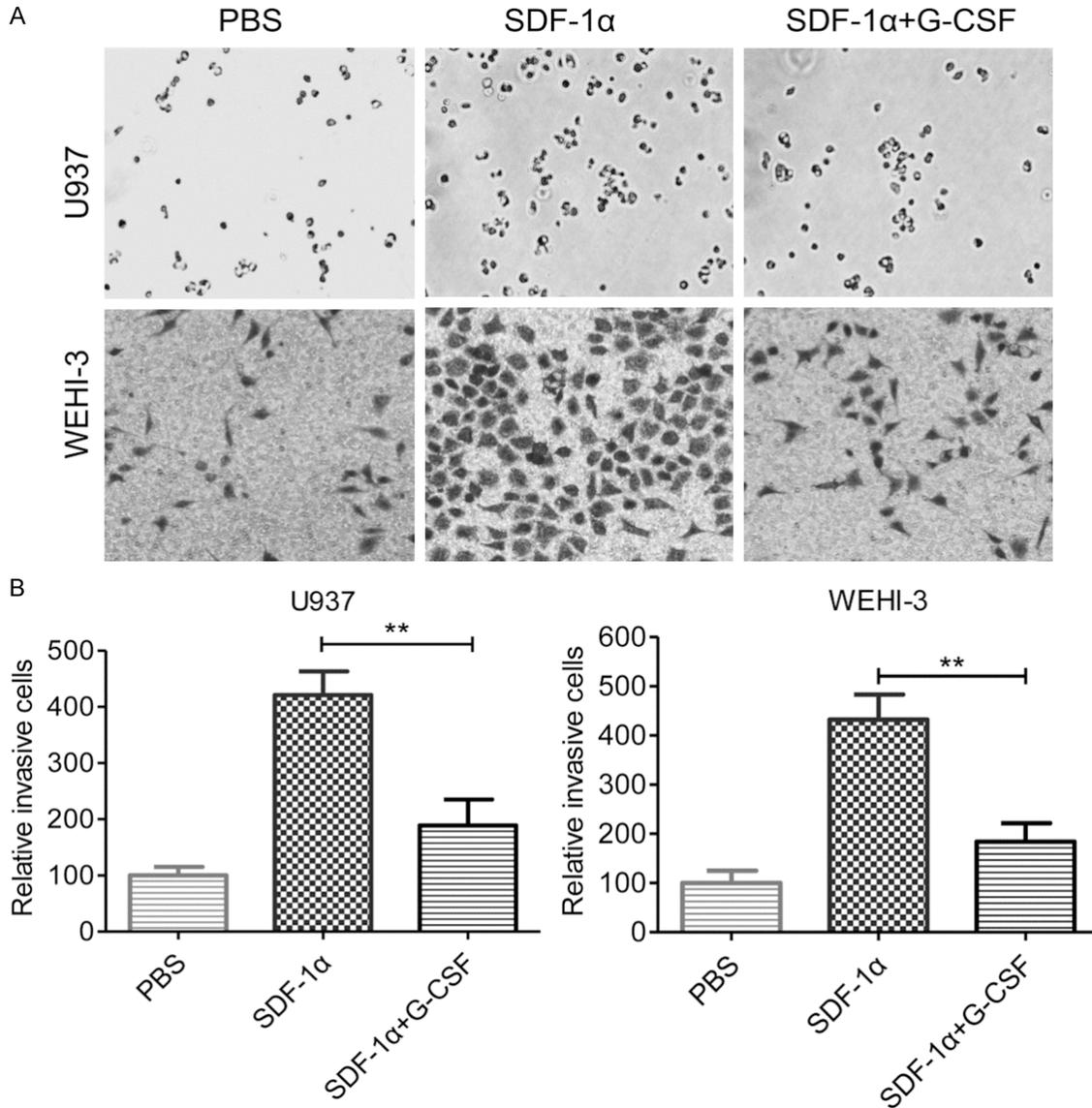


Figure 2. G-CSF inhibits tumor migration and invasion. A. SDF-1 α -induced migration of AML cells whereas the migration was antagonized by additional treatment of G-CSF. B. Quantification of invasive cells. All data represent three independent experiments, mean \pm s.d. **P < 0.01.

round-bottom tube with cell-strainer cap (Falcon, Corning, NY, USA) to create single-cell suspensions. The isolated leukocytes were then examined for cell markers based on being stained with antibodies described above. The positive cells were counted and the percentage of CD25+ and FOXP3+ cells was determined in relation to the total number of CD4+ T lymphocytes. Femurs were collected from mice, then flushed with PBS (to obtain bone marrow samples) and then centrifugation for 10 min at

1500 rpm at 4°C to obtain supernatant for ELISA.

Enzyme-linked immunosorbent assays (ELISA)

The SDF-1 α in serum and bone marrow supernatant were analyzed with ELISA kit (mlbio, ml002079). Briefly, the test samples (10 μ L) were added to the wells and incubated for 30 min at 37°C. Then, the liquid in the wells was discarded, the wells were dried by swinging the plate, and washing buffer was added to every

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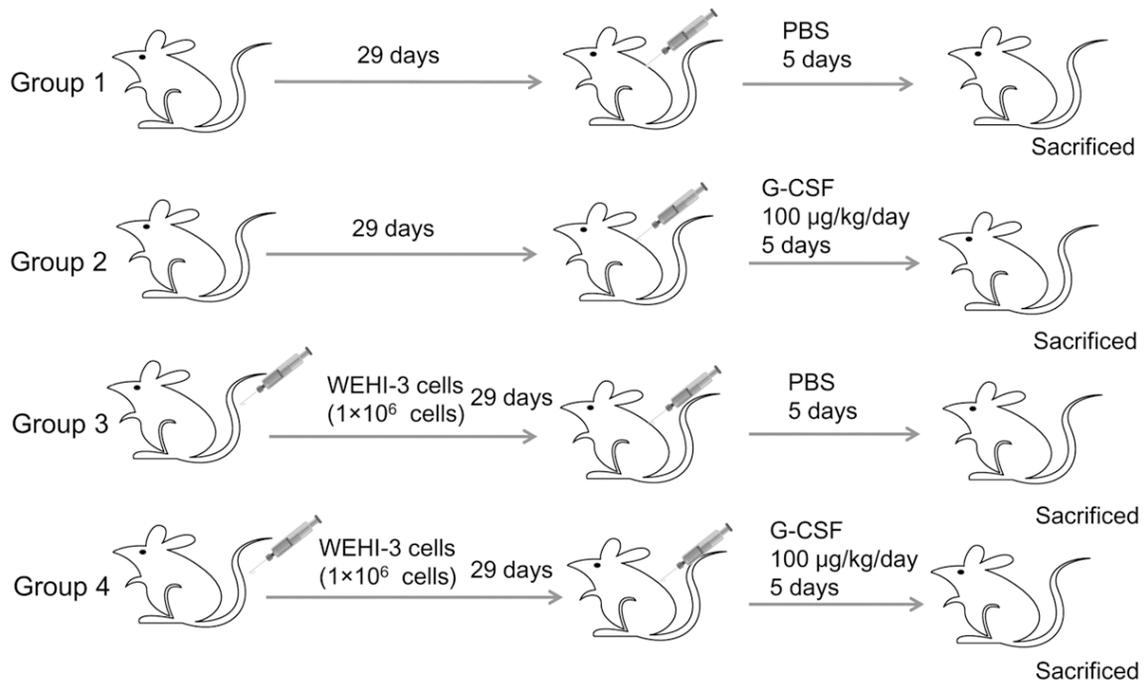


Figure 3. WEHI-3 cell allograft model. Forty BALB/c mice were randomly divided into four groups. Group 1 received an subcutaneous injection of PBS treatment as a control, group 2 received an subcutaneous injection of G-CSF (100 µg/kg/day) for 5 days, group 3 received an intravenous injection of 1×10^6 WEHI-3 cells and PBS for 5 days, group 4 received an intravenous injection of 1×10^6 WEHI-3 cells for 29 days and then received an subcutaneous injection of G-CSF (100 µg/kg/day) for 5 days. At day 34, all animals were sacrificed.

well. The plate was left to stand for 30 s and then drained, and the plate dried by patting. Horseradish peroxidase-(HRP-) conjugated reagent was then added and incubated for 30 min at 37°C. Chromogen solution A (50 µL) and chromogen solution B (50 µL) were added to each well, and the plate was incubated for 10 min at 37°C in the dark. Stop solution (50 µL) was added to each well to stop the reaction. Taking the blank well as zero, the absorbance of each well was read at 450 nm. The concentrations of SDF-1α in the samples were determined by comparing the absorbance of the samples with standard curves. Each sample included three repeated measurements.

Statistical analysis

Results from different experiments are described as mean ± standard deviations. Statistical analysis for the results was done using Student t test when only two groups, or one-way analysis of variance when more than two groups, and the level of statistical significance was set at $P < 0.05$. The calculations were performed using IBM SPSS Statistics software, release 16.0.

Results

G-CSF inhibits CXCR4 expression in AML cells

To evaluate the effect of G-CSF on CXCR4 expression in AML cells, we treated U937 and WEHI-3 cells with G-CSF for 6, 12, 18 and 24 h. The CXCR4 expression was stained by antibody and detected by FCM. G-CSF showed suppressive effect on the CXCR4 expression with a time-dependent manner after the exposure to 100 ng/ml G-CSF (**Figure 1**). G-CSF produced a decrease of nearly 52% (12 h), 69% (18 h), 71% (24 h) in U937 and 30% (12 h), 55% (18 h) and 57% (24 h) in WEHI-3 cells. These results demonstrated that G-CSF was potent in inhibiting CXCR4 expression in AML cells.

SDF-1/G-CSF affects cell invasion in AML cells

In order to investigate whether SDF-1α and G-CSF affects AML cell invasion, we used Transwell migration and invasion assays to compare the cell migration rate and invasive capacity in each group. **Figure 2A** and **2B** showed that the migration rate of the AML cells

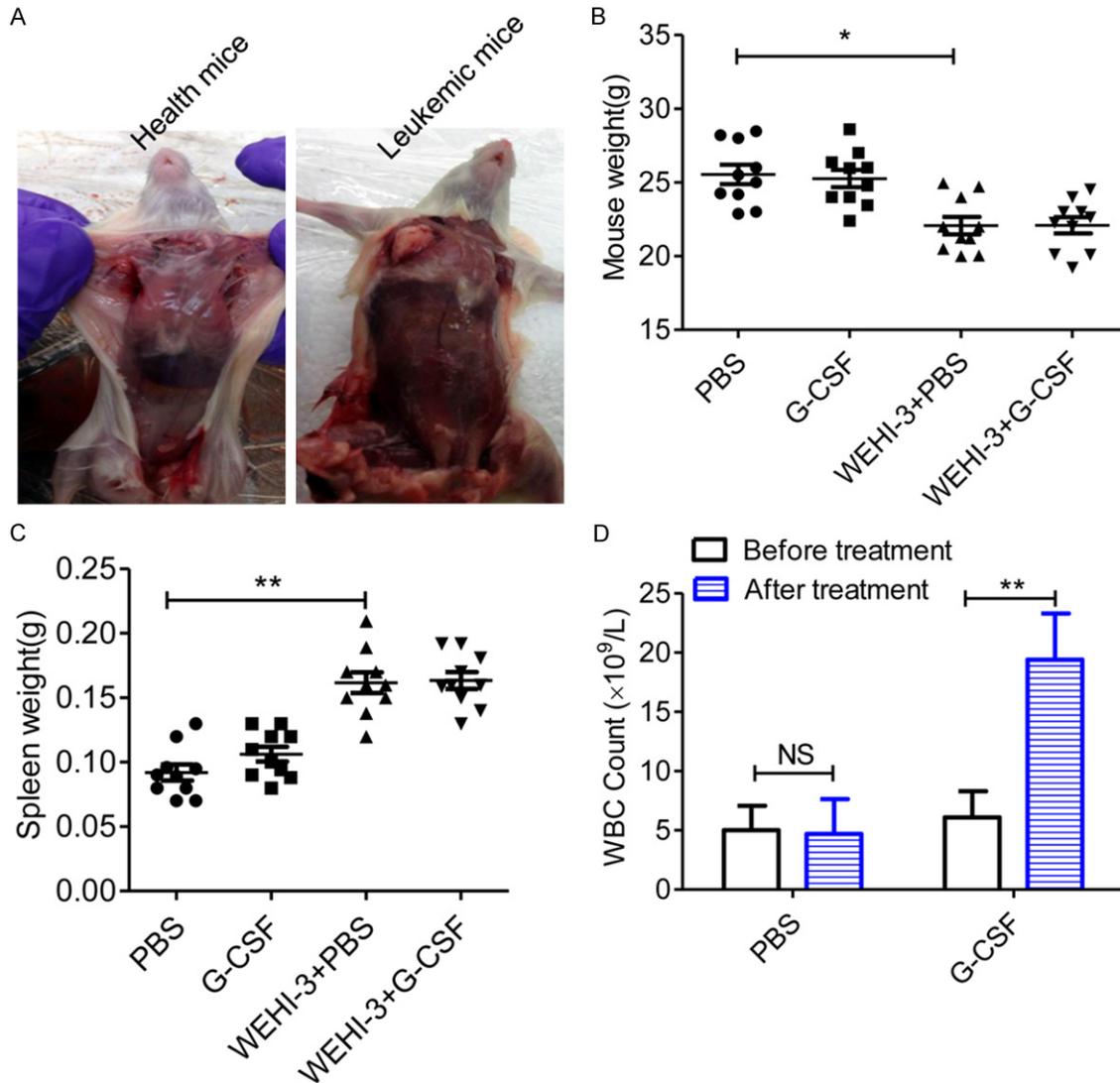


Figure 4. WEHI-3 leukemic mice allograft model. A. Representative animals of health BALB/c mice and WEHI-3 leukemic mice. B. Data of body weight from each group are presented of ten animals at days 34 after tumor implantation. C. Representative data of spleen weight from each group are presented at days 34 after tumor implantation. D. Representative data of WBC count from BALB/c mice are presented before and after G-CSF treatment. *P < 0.05, **P < 0.01.

to the lower chamber containing SDF-1 α was significantly increased compared to the PBS group. However, the invasive capacity of AML cells was decreased by additional treatment with G-CSF, which was reduced by 50% in U937 and 26% in WEHI-3, respectively. These results indicated that G-CSF can impede cell migration and inhibit cell invasion.

G-CSF increases the percentage of regulatory T cells in vivo

To evaluate the effect of G-CSF on the regulatory T cells, we established BALB/c mouse

model and the experimental design and protocol of the leukemic mouse model are shown in **Figure 3**. Representative mice images are shown in **Figure 4A**. The body weight of mouse in the G-CSF group was not changed compared with that of the control mouse; however, the body weight of mice in the leukemia group was significantly decreased compared with that of the control group (**Figure 4B**). Furthermore, the spleen weight of leukemia group increased significantly; while the spleen weights of G-CSF did not differ significantly from PBS group (**Figure 4C**). In addition, the white blood cell

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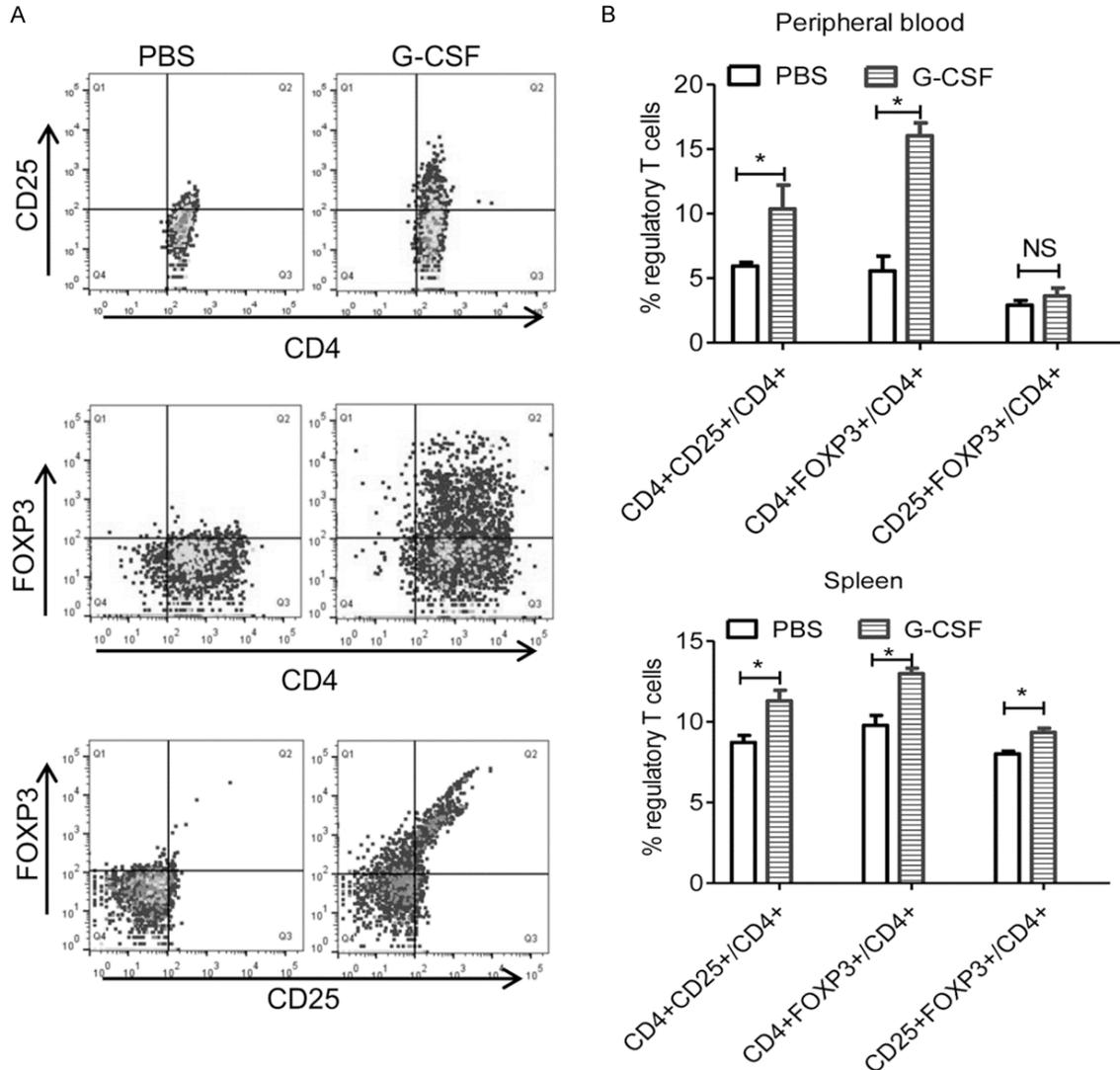


Figure 5. Effects of G-CSF on the levels of regulatory T cells from the peripheral blood and spleen of BALB/c mice. Blood and spleen was collected from each animal and analyzed for cell markers (CD4, CD25 and FOXP3) by flow cytometry as described in Materials and methods. A. Representative percentages of leukocyte subtypes from each group are shown. B. Data are presented as mean \pm s.d. *P < 0.05.

increased significantly after G-CSF treatment in BALB/c mouse (**Figure 4D**).

G-CSF increases the regulatory T cells and SDF-1 α in WEHI-3 leukemic BALB/c mice

The isolated leukocytes from peripheral blood and spleen in BALB/c mice were examined for cell markers. The results shown in **Figure 5** indicate that G-CSF increased the percentage of regulatory T cells derived from both peripheral blood and spleen of BALB/c mice.

To explore the impact of G-CSF on the tumorigenicity, we established the leukemic

mice model by intravenous injection of WEHI-3 cells into the BALB/c mice. All of the 20 mice in two groups were sacrificed at day 34 for regulatory T cells and SDF-1 α detecting. **Figure 6A** and **6B** show that the regulatory T cells isolated from peripheral blood and spleen of WEHI-3 leukemic mice were increased by administration of G-CSF. In addition, the concentration of SDF-1 α was increased in serum but decreased in bone marrow in WEHI-3 leukemic mice (**Figure 6C**). These results were consistent with the SDF-1 α levels identified in the G-CSF treated BALB/c mice (**Figure 6D**), indicating that G-CSF may be an important factor in regulating regulatory T cells and SDF-1 α .

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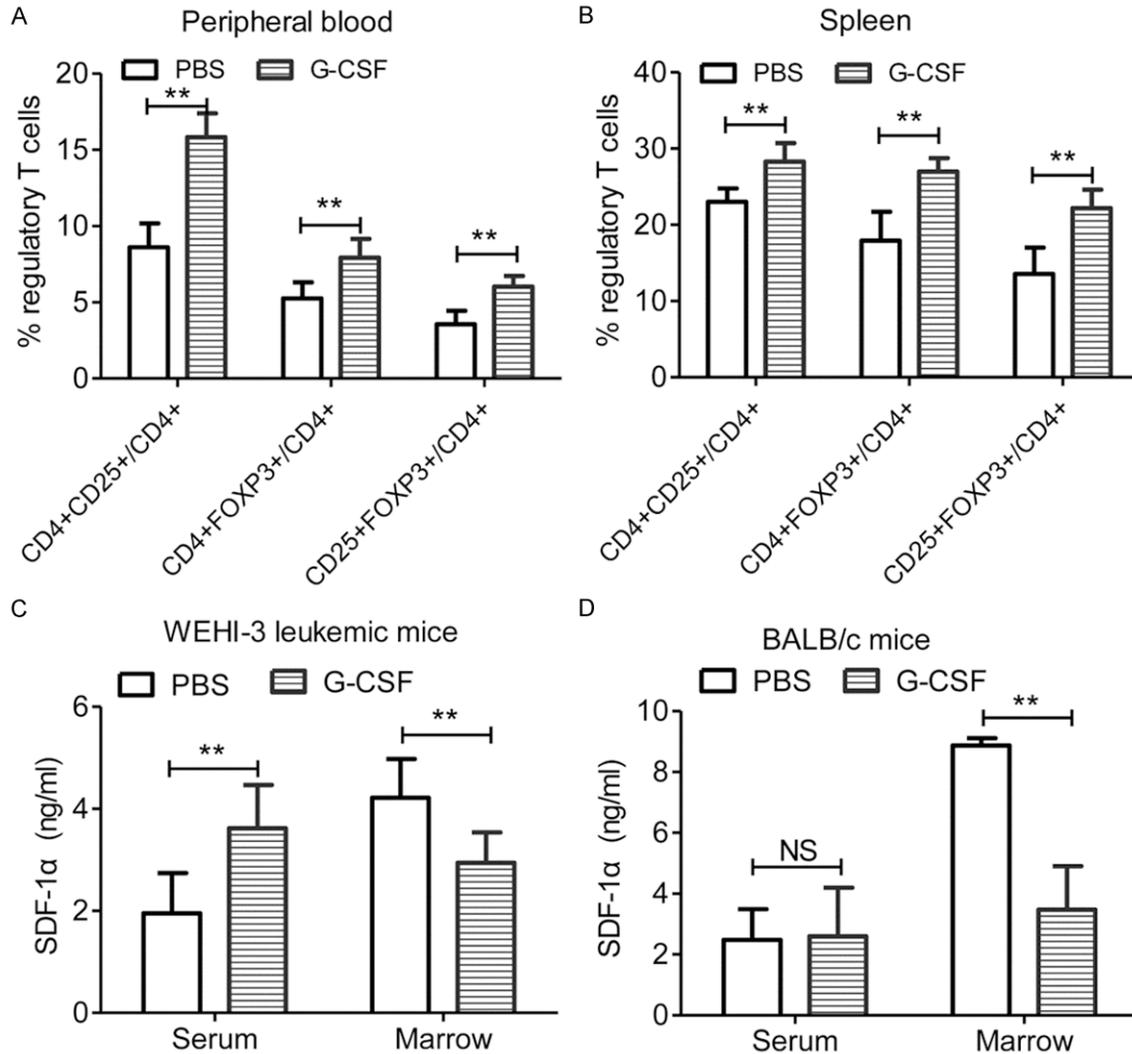


Figure 6. G-CSF mobilize regulatory T cells and suppress SDF-1 α in WEHI-3 cell allograft model. Blood (A) and spleen (B) was collected from each animal and analyzed for cell markers (CD4, CD25 and FOXP3) by flow cytometry as described in Materials and Methods. (C, D) SDF-1 α concentration in the serum and marrow of WEHI-3 leukemic mice or BALB/c mice were detected by ELISA as described in the Materials and Methods. Data are presented as mean \pm s.d. **P < 0.01.

Discussion

G-CSF mobilization is known to elicit broad immunomodulatory effects on both myeloid cells and lymphoid lineages cells. Stem cell mobilization with G-CSF is known to profoundly influence T cell differentiation. These effects include the promotion of Th2 and Th17 differentiation at low and high doses [20] without impairment in cytolytic responses and thus graft versus leukemia effects [21]. However the actual mechanism by which G-CSF alters T cell function remains to be elucidated. The protective effects of G-CSF mediated through

haematopoietic tissue may be via direct effects on T cells or as is the current paradigm, indirect effects, downstream of soluble products, including at least IL-10, generated by expanded myeloid and non-hematopoietic cells.

Similarly, the mobilization of hematopoietic stem cells (HSC) interaction of CXCR4 with SDF1/CXCL12 modulated by G-CSF stimulation generates signals not only regulating the trafficking of HSC from the bone marrow to peripheral blood [22], but also of T cell populations [23]. During G-CSF induced HSC

mobilization, CXCL12 protein expression in the bone marrow decreases, and CXCR4 expressing HSC and Tregs can be consequently released from bone marrow. In the present study, G-CSF application leads to a numerical increase of circulating Tregs yield in both BALB/c mice and the WEHI-3 leukemic mice. The higher circulating Tregs could facilitates further immunotherapy.

Identification of the factors contributing to microenvironment-mediated chemoresistance remains an unresolved challenge important for the eradication of residual chemosensitive leukemic blasts hiding in the bone marrow niches. Our findings indicate that G-CSF inhibits SDF-1 α /CXCR4 and antagonizes the migration of AML cell lines induced by SDF-1 α . Monitoring of circulating Tregs confirmed the ability of G-CSF to induce the mobilization of Tregs. While physical disruption of leukemia/stroma interactions with resulting mobilization is clearly one of the principal effects of G-CSF, our studies also indicated G-CSF mobilized Tregs.

Other mechanisms likely contribute to their chemo-sensitizing effects. For example, researchers have highlighted the potential of stromal cells to activate multiple signaling cascades in leukemic cells, including PI3K/AKT and MAPK pro-survival pathways [24]. Identification of the key downstream targets of these and other signaling pathways activated by stromal cells may be important for combination strategies targeting microenvironment-mediated resistance. *In vitro* and *in vivo* studies indicate that several components of the bone marrow microenvironment including endothelial cells [25], osteoblasts [26], and adipocytes [27] contribute to the enhanced survival of the leukemic cells. These findings indicate that disrupting several key factors involved in the leukemia/stroma interactions may be necessary for complete eradication of bone marrow-resident AML cells from bone marrow niches. Further studies are ongoing to confirm our preliminary observation of the ability of CXCR4 blockade by G-CSF to cause mobilization of AML stem cells in an *in vivo* model of human leukemia.

In summary, these studies for the first time provide evidence for G-CSF affecting SDF-1 α /CXCR4 and induce the mobilization of Tregs. As

SDF-1 α /CXCR4 interactions largely contribute to the protection mediated by the microenvironment, hence arguing for the use of murine models as better predictors of the efficacy of G-CSF *in vivo*. Altogether, these results provide rationale for studies of G-CSF in combination with standard or targeting immunotherapy with the goal to attenuate microenvironment-triggered therapy resistance.

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Disclosure of conflict of interest

None.

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