Etoposide-mediated interleukin-8 secretion from bone marrow stromal cells induces hematopoietic stem cell mobilization

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Ka-Won Kang
Korea University College of Medicine and School of Medicine
ORCiD: 0000-0003-1462-0502

Seung-Jin Lee
Korea University

Ji Hye Kim
Korea University

Byung-Hyun Lee
Korea University College of Medicine and School of Medicine

Seok Jin Kim
Sungkyunkwan University School of Medicine

Yong Park
Korea University College of Medicine and School of Medicine

Byung Soo Kim kbs0309@korea.ac.kr
Corresponding Author
ORCiD: 0000-0003-1462-0502

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Abstract

Background This study assessed the mechanism of hematopoietic stem cell (HSC) mobilization using etoposide with granulocyte-colony stimulating factor (G-CSF) and determined how it differed from that using cyclophosphamide with G-CSF or G-CSF alone. Methods The study analyzed data from 173 non-Hodgkin’s lymphoma patients who underwent autologous peripheral blood stem cell transplantation (auto-PBSCT), in vitro experiments using HSCs and bone marrow stromal cells (BMSCs), and in vivo mouse model studies. Results The etoposide with G-CSF mobilization group showed the highest yield of CD34+ cells and the lowest change in white blood cell counts during mobilization. Etoposide triggered interleukin (IL)-8 secretion from BMSCs and caused long-term BMSC toxicity, which were not observed with cyclophosphamide treatment. The expansion of CD34+ cells cultured in BMSC-conditioned medium containing IL-8 was more remarkable than that without IL-8. The expression of CXCR2, mTOR, and cMYC in HSCs was gradually enhanced at 1, 6, and 24 h after IL-8 stimulation. In animal studies, the etoposide with G-CSF mobilization group presented stronger expression of IL-8-related cytokines and MMP9 and scantier expression of SDF-1 in the bone marrow, compared to the other groups not treated with etoposide. Conclusion Collectively, the unique mechanism of etoposide with G-CSF-mediated mobilization is associated with the secretion of IL-8 from BMSCs, causing the enhanced proliferation and mobilization of HSCs in the bone marrow, which was not observed in the mobilization using cyclophosphamide with G-CSF or G-CSF alone. Moreover, the long-term toxicity of etoposide to BMSC emphasizes the need for further studies to develop more efficient and safe chemo-mobilization strategies.

Background

Successful autologous peripheral blood stem cell transplantation (auto-PBSCT) for
hematologic malignancies requires harvesting a sufficient number of human hematopoietic stem cells (hHSCs) mobilized from the bone marrow (BM) to the peripheral blood (PB). In clinical practice, mobilization protocols generally include chemotherapy and granulocyte colony-stimulating factor (G-CSF) (chemo-mobilization), as reduction of the cancer burden during mobilization is crucial. Since G-CSF mobilization was first applied by Dührsen et al. in 1988 [1-3], cyclophosphamide chemo-mobilization has been commonly used. Cyclophosphamide induces the release of stress signals that cause inflammation, activating the host immune system, which may increase hHSC mobilization [4, 5]. However, this protocol has some disadvantages, including, primarily, the unpredictability of the number of hHSCs that can be collected from the PB and the possibility of mobilization-related toxicities, such as febrile neutropenia [5-7]. Reiser et al. first reported the use of etoposide as an alternative to cyclophosphamide to effectively mobilize PBSCs in patients in whom cyclophosphamide chemo-mobilization failed [8], leading to the continuing study of etoposide chemo-mobilization (Additional file 1: Table S1) [9-13]. However, concerns regarding the use of etoposide include its inhibition of topoisomerase 2, which damages DNA. Cancer patients undergoing chemotherapy, including etoposide, have experienced secondary hematologic malignancies [14, 15]. Moreover, Gibson et al. demonstrated that etoposide could damage human bone marrow stromal cells (hBMSCs) [16]. These findings suggest that etoposide may influence the BM niche by not only enhancing mobilization but also inducing BM damage. Therefore, the mobilization mechanism of etoposide may differ from that of G-CSF or cyclophosphamide, which proceeds through the demargination of HSCs from the BM to PB due to systemic inflammation [17]. However, to date, this topic appears to have received scarce attention. Furthermore, verification of the mobilization mechanisms may be difficult due to the interference of complex physical conditions in patients, which could confound the
interpretation of the associated clinical findings. To overcome these barriers, we designed a three-step study involving: 1) the analysis of clinical data associated with auto-PBSCT in patients with non-Hodgkin’s lymphoma (NHL); 2) performing in vitro experiments to assess changes in hBMSCs, which support HSCs in the BM niche, after exposure to cyclophosphamide or etoposide (we also investigated the effect of IL-8 on CD34 + HSCs as IL-8 is the cytokine predominantly secreted by hBMSCs stimulated by etoposide but not cyclophosphamide); 3) performing in vivo animal studies under standardized conditions to exclude confounding factors and mimic conditions of clinical practice and verify changes in the BM niche caused by etoposide chemo-mobilization or other mobilization protocols.

Methods

Clinical data

A retrospective analysis was performed of clinical data from NHL patients who underwent PB stem cell collection (PBSCC) at the Korea University Anam Hospital and the Samsung Medical Center from 2005 to 2019 and an internal board-approved retrospective chart review was conducted (IRB No. 2019AN0386 and 2019-09-085-001, respectively).

Primary cell culture of hBMSCs

The internal review board of the Korea University Anam Hospital (IRB No. 2015AN0267) approved all the procedures. Written informed consent was obtained from all human subjects. The subjects were healthy individuals who donated BM blood during BM harvesting. A total of 20 mL BM blood was collected from each subject. Mononuclear cells (MNCs) were separated using Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Seoul, South Korea) medium; the remaining cells were cultured in mesenchymal stem cell growth medium (Lonza, Walkersville, MD, USA). This study used isolated hBMSCs within five
passages from the start of the subculture.

**Flow cytometry analysis**

Antibodies against anti-human CD73-PE, CD90-PE, CD105-PE, CD34-FITC, and CD45-PE (Becton Dickinson, San Jose, CA, USA) were used at 1:100 dilution. Cells were sorted on a FACSCalibur™ Flow Cytometer (Becton Dickinson).

**Chemotherapeutic agents and cytotoxic assay**

Commercially available preparations of cyclophosphamide (Endoxan injection, 500 mg; Boxter Inc., Seoul, South Korea) and etoposide (Lastet injection, 100 mg/5 mL; Dong-A Inc., Seoul, South Korea) were used. Cell Counting Kit-8 (CCK-8 assay, Dojindo Laboratories, Japan) was used for cytotoxic assays, according to the manufacturer’s instructions. Absorbance was measured at 450 nm using a SpectraMax Plus 384 spectrophotometer (Molecular Devices Corporation, CA, USA).

**Human and mouse cytokine arrays**

The Human Cytokine Antibody Array C1000 and Mouse Cytokine Antibody Array C1000 (both Ray Biotech, GA, USA) were used, according to the manufacturer’s instructions. Images were acquired using a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA) and quantified using ImageJ software (National Institutes of Health, MD, USA). The signal was normalized to those of the internal positive controls and the background using the RayBio® Antibody Array Analysis Tool (Ray Biotech).

**Apoptosis and cell cycle analysis**
Apoptosis analysis was performed using the EzWay Annexin V-FITC Apoptosis Detection Kit (Koma Biotech Inc., Seoul, South Korea). Cell-cycle distribution analysis was performed using propidium iodide at 50 mg/mL (Sigma-Aldrich, catalog no. P4170). Both assays were performed according to the manufacturers’ instructions.

**Culture of HSCs and IL-8 treatment**

Human BM CD34+ HSCs were purchased from Lonza (catalog no. 2M-101) and cultured in Stemline® II Hematopoietic Stem Cell Expansion Medium (Sigma-Aldrich, catalog no. S0192) containing 100 ng/mL stem cell factor, thrombopoietin, and G-CSF (all obtained from R&D Systems, Inc., Minneapolis, MN, USA). Recombinant human IL-8/CXCL8 protein was acquired from R&D Systems (catalog no. 208-IL).

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA was isolated from cells using the Qiagen RNeasy kit (Qiagen, Hilden, Germany) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized by adding 2 µg total RNA to a 20-µL reaction mixture containing oligos, primers, and Superscript II reverse transcriptase (Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions. Synthesized cDNA was amplified using the iQ SYBR Green qPCR Master Mix (Bio-Rad) in a Bio-Rad iCycler iQ system (Bio-Rad). Comparative threshold cycle values were normalized to those of glyceraldehyde-3-phosphate dehydrogenase. The primers used are described in Additional file 2: Table S2.

**Mice**

All experimental procedures using animals complied with the guidelines of the Laboratory
Animal Research Center of the Korea University College of Medicine (IRB No. KOREA-2017-0176). C57BL/6N mice were purchased from Orient Bio (Seongnam, South Korea). Mice, 8 weeks of age and 20g of body weight, were maintained in polypropylene cages under specific pathogen-free conditions, with light/dark 12-h cycles, at 21 ± 2 °C, and fed ad libitum with maintenance diet. Sample sizes were calculated using a pilot study and the G*Power program (http://www.gpower.hhu.de/). All analyses were conducted blindly to minimize the effects of subjective bias.

Protocol for HSC mobilization in mice

The mouse model of HSC mobilization was designed according to a similar protocol used in human patients (Fig. 4a–b). A previously reported model of cyclophosphamide chemomobilization was used in this study [18]. We developed a new model of etoposide chemomobilization due to the apparent lack of a related animal model. Mice were injected intraperitoneally with 4 mg cyclophosphamide (≈ 200 mg/kg) on day 1 (D1) or with 0.8 mg etoposide (≈ 40 mg/kg) on days 1 and 2 (D1, D2). Then, 5 µg human G-CSF (250 µg/kg per day; Leucostim prefilled syringe INJ, Dong-A Inc.) was administered daily as a single subcutaneous injection, on each successive day from day 3, for a total of 4 days. All mice were euthanized on D7 by cardiac puncture and cervical dislocation under anesthesia. On day 7 (D7), we isolated hematopoietic progenitor cells (HPCs) using an EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit and performed colony-forming unit (CFU) assays using MethoCult™ GF M3434 medium (both from Stem Cell Technologies, Vancouver, BC, Canada), according to the manufacturer’s instructions.

Enzyme-linked immunosorbent assay (ELISA)
Plasma levels of stromal cell-derived factor-1 (SDF-1), matrix metalloproteinase-2 (MMP2), and matrix metalloprotease-9 (MMP9) in mice were measured using the Magnetic Luminex® Screening Assay (R&D Systems), according to the manufacturer’s instructions.

**Immunohistochemistry of BM sections**

Immunohistochemical (IHC) staining was performed on 3-µm formalin-fixed, paraffin-embedded sections from the BM. The following primary antibodies were used: anti-keratinocyte-derived cytokine (KC) (Cloud-Clone Corp., Houston, TX, USA, catalog no. PAA041Mu01, 1:50), anti-macrophage inflammatory protein 2 (MIP-2) (Cloud-Clone Corp., catalog no. PAB603Mu01, 1:100), anti-lipopolysaccharide-inducible CXC chemokine (LIX) (Cloud-Clone Corp., catalog no. PAA860Mu01, 1:100), anti-MMP2 (Abcam, catalogue no. ab37150, 1:200), anti-MMP9 (Abcam, catalog no. ab38898, 1:200), and anti-SDF-1 (Abcam, catalog no. ab9797, 1:500). In the case of anti-KC, anti-MIP-2, anti-LIX, anti-MMP9, and anti-SDF-1, antigen retrieval was performed using a citrate buffer. All slides were scanned using a virtual microscopy scanner (Axio Scan Z1 scanner; Carl Zeiss, Jena, Germany); the positive contributions were calculated by summing the highly positive, positive, and low positive fractions for each staining using the IHC profiler Plugin of ImageJ software [19].

**Statistical analysis**

Patient demographics and baseline characteristics were compared using Kruskal-Wallis H and chi-square tests. Multivariate analysis using the Cox proportional hazards method was performed. Mann-Whitney U and Student’s t-tests and analysis of variance were used to analyze differences in data from the *in vitro* and *in vivo* experiments, according to the variables involved. A post hoc analysis with Bonferroni correction was performed when statistical differences were identified among the three groups. Data analysis was
performed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., NY, USA). p-values < 0.05 denoted significant differences.

Results

**Etoposide chemo-mobilization is highly effective, exhibiting different clinical features, compared to other mobilization methods**

We analyzed data from 173 patients with NHL who underwent PBSCC in the presence of various chemotherapeutic agents: G-CSF only, \( n = 33 \); cyclophosphamide + G-CSF, \( n = 24 \); and etoposide + G-CSF, \( n = 116 \). The baseline characteristics of the patients are summarized in Table 1. The highest yield of CD34+ cells was observed for etoposide + G-CSF (Fig. 1a), a result that remained statistically significant even after adjusting for baseline characteristics (Additional file 3: Table S3). The increase in white blood cell (WBC) count (from at nadir to the time of PBSCC) was modest for etoposide + G-CSF, compared with those for G-CSF only and cyclophosphamide + G-CSF (Fig. 1b). In etoposide + G-CSF, WBC counts at nadir (cyclophosphamide + G-CSF, 41 (9–3,258); etoposide + G-CSF, 262 (1–3,160)) were higher and those at the time of PBSCC (cyclophosphamide + G-CSF, 10,350 (1,000–70,900); etoposide + G-CSF, 4,380 (500–122,150)) were lower than cyclophosphamide + G-CSF (\( p = 0.056 \) and 0.005, respectively). Previous reports have shown a positive correlation between the degree of WBC count increase during mobilization and the increase in CD34+ cell yield [20-22]. Etoposide chemo-mobilization led to the highest CD34+ cell yield, despite the fact that the differences in WBC counts between the nadir and the time of PBSCC were the lowest. Therefore, we suspected that the mobilization mechanism of etoposide might differ from those of G-CSF only and cyclophosphamide. However, our hypothesis must be confirmed because of the heterogeneity of the patients in each group and the presence of other confounding factors.
**Etoposide increases IL-8 secretion from BMSCs and causes long-term MBSC toxicity**

hBMSCs, which constitute the major cell component of the BM niche [23], were isolated from BM blood (Fig. 2a–b) and treated with various concentrations of cyclophosphamide (0–12.5 mg/mL) or etoposide (0–2.0 mg/mL) for 24 h. Drug concentrations sufficient to cause the death of 10 %, 25 %, and 50 % of viable hBMSCs were defined as cytotoxic concentration (CC) 10, CC 25, and CC 50, respectively (Fig. 2c). Then, we compiled data from the literature on the blood concentrations of the two drugs from patients receiving high-dose cyclophosphamide or etoposide treatment. For high-dose cyclophosphamide treatment (1,850–7,000 mg/m²), the maximum reported serum concentration (Cmax) was 2.664 mg/mL [24, 25]. For high-dose etoposide treatment (1,480–1,665 mg/m²), the reported Cmax was 0.1 mg/mL [26, 27]. Based on this information, the CC10 was selected as the drug concentration for further experiments.

hBMSCs were cultured in a medium containing normal saline (control group, n = 4), cyclophosphamide (dose of CC10, n = 5), or etoposide (dose of CC10, n = 5) for 24 h; subsequently, human cytokine analysis was performed using these conditioned media. The level of IL-8, a mobilization-associated cytokine [28, 29], was significantly higher in the etoposide-treated group than in the cyclophosphamide-treated group (p = 0.021 after Bonferroni correction) (Fig. 2d–e). The other mobilization-associated cytokines showed no significant differences among the groups.

The degree of expansion of etoposide-treated hBMSCs was significantly lower than that of cyclophosphamide-treated hBMSCs for all passages (p < 0.001 after Bonferroni correction for both) (Fig. 2f). No significant differences in apoptosis were observed among the groups.
(Fig. 2g). However, cell-cycle analysis revealed a significantly higher proportion of etoposide-treated hBMSCs arrested in the G0/G1 phase than cyclophosphamide-treated and untreated hBMSCs ($p = 0.03$ and $p = 0.01$ after Bonferroni correction, respectively; Fig. 2h).

**IL-8 enhances HSC expansion and is associated with CXCR2, mTOR, and cMYC activation**

We observed significantly increased IL-8 secretion in hBMSCs treated with etoposide, compared to hBMSCs treated with cyclophosphamide. To investigate the manner in which the hBMSC-released IL-8 affects hHSCs in the BM niche, we cultured $2.5 \times 10^6$ hHSCs with 100 ng/mL IL-8 ($n = 12$) or without IL-8 ($n = 12$) for 24 h in a conditioned medium collected from 24-h cultures of healthy hBMSCs grown in mesenchymal stem-cell growth medium. Previous experiments confirmed the distribution of cytokines in this conditioned medium in human cytokine analysis (Fig. 2d, control group) and the relatively low IL-8 expression (Fig. 2e, control group). The numbers of total, CD34+, and CD34+/CD45- cells determined using a hemocytometer and flow cytometric analysis of CD34+ cells cultured with IL-8 were significantly higher than those of cells cultured without IL-8 ($p = 0.014$, 0.020, and 0.039, respectively) (Fig. 3a). To identify the mechanism of IL-8 action on hHSCs, the expression levels of CXCR2 (a representative IL-8 receptor) and mTOR and cMYC (components of an IL-8-related signaling pathway) were measured by qRT-PCR. The relative gene expression levels of CXCR2, mTOR, and cMYC gradually increased 1, 6, and 24 h after IL-8 treatment (Fig. 3b).

**Etoposide chemo-mobilization increases IL-8-associated cytokine levels, especially in the BM**
We developed mouse models for PB HSC mobilization based on the actual mobilization protocol used in human patients (G-CSF only, \( n = 8 \); cyclophosphamide + G-CSF, \( n = 8 \); etoposide + G-CSF, \( n = 8 \); Fig. 4a-b). The changes in WBC counts at nadir and at the time of collection (D7) showed patterns similar to those observed in clinical settings (Fig. 1b and Fig. 4c). On D7, HPCs were isolated from the PB, and CFUs (CFU-granulocytes, erythrocytes, monocytes, and megakaryocytes; CFU-granulocytes, macrophages; and burst forming unit-erythroid) were counted (Fig. 4d). No significant differences in the total number of CFUs were observed between the cyclophosphamide-treated (total 200 mg/kg) and etoposide-treated (total 80 mg/kg) groups (G-CSF only, \( n = 5 \); cyclophosphamide + G-CSF, \( n = 5 \); etoposide + G-CSF, \( n = 5 \); Fig. 4e). Thus, this condition might be appropriate to investigate the differences in mobilization mechanisms between etoposide-mediated chemo-mobilization and others.

Plasma cytokine levels in whole blood collected from mice on D7 were analyzed. The levels of KC, MIP-2, and LIX, which are IL-8 homologs in mice [30-32], were measured (G-CSF only, \( n = 9 \); cyclophosphamide + G-CSF, \( n = 9 \); etoposide + G-CSF, \( n = 9 \)). The level of KC was significantly increased in the etoposide-treated group, compared with that in the cyclophosphamide-treated group (\( p = 0.001 \) after Bonferroni correction). The levels of the other IL-8 homologs, MIP-2 and LIX, were also increased in the etoposide-treated group, compared with those in the cyclophosphamide-treated group; however, the differences were not statistically significant. None of the three homologs showed statistically significant differences between the etoposide-treated and G-CSF-only groups (Fig. 5a-b). To confirm that the changes in the plasma levels of KC, MIP-2, and LIX reflected similar changes in the BM, we quantified IHC images of BM sections using the IHC profiler Plugin of ImageJ (G-CSF only, \( n = 7 \); cyclophosphamide + G-CSF, \( n = 7 \); etoposide + G-CSF, \( n = 7 \)). The levels of KC, MIP-2, and LIX were all significantly increased.
in the BM sections from the etoposide-treated group, compared with those from the G-CSF-only and cyclophosphamide-treated groups ($p < 0.001$ and $p < 0.001$; $p = 0.004$ and $p < 0.001$; $p < 0.001$ and $p < 0.001$ after Bonferroni correction, respectively; Fig. 5c–e).

**Etoposide chemo-mobilization is associated with increased MMP9 and decreased SDF-1 levels in the BM**

The total cytokine network was analyzed to identify the potential mechanisms underlying HSC mobilization stimulated by etoposide. Cytokines exhibiting a significant ($p < 0.05$) increase via etoposide chemo-mobilization, compared to G-CSF-only or cyclophosphamide chemo-mobilization, in mouse cytokine assays were analyzed by Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA; Additional file 4 and 5: Fig. S1 and S2). Network analysis showed that cytokines exhibiting increased levels via etoposide chemo-mobilization were associated with the activation of matrix metalloproteinases (MMPs), which affect the CXCR4/SDF-1 axis and are involved in HSC mobilization [33, 34]. Therefore, the expressed levels of representative MMPs related to HSC mobilization, MMP2 and MMP9, were assessed. In the PB, the expression levels of MMP2, MMP9, and SDF-1 did not differ significantly among the groups (G-CSF-only, $n = 4$; cyclophosphamide chemo-mobilization, $n = 4$; etoposide chemo-mobilization, $n = 4$). In the BM, however, MMP9 expression was significantly increased and SDF-1 expression was significantly decreased in the etoposide chemo-mobilization group, compared to the other groups (G-CSF-only, $n = 7$; cyclophosphamide + G-CSF, $n = 7$; etoposide + G-CSF, $n = 7$; Fig. 6).

**Discussion**

Our retrospective analysis of clinical data showed that etoposide chemo-mobilization results in the highest yield of CD34+ cells, compared with G-CSF or cyclophosphamide chemo-mobilization, despite relatively modest changes in PB WBC counts. To our
knowledge, this is the first analysis of clinical data regarding etoposide chemomobilization and suggests the possibility of a different mechanism underlying etoposide chemomobilization. Our in vitro experiments showed that etoposide treatment significantly increased hBMSC secretion of IL-8, whereas cyclophosphamide treatment did not. IL-8 is a senescence-associated secretory phenotype; therefore, this finding might be associated with the influence of etoposide on hBMSC subcultures, which was not observed for cyclophosphamide. This finding might be a clue to explain the more efficient chemomobilization mediated by etoposide than by cyclophosphamide. Studies by Pelus et al. and Fukuda et al. support this hypothesis by showing that the CXCR2 ligand GRO-β rapidly mobilizes HSCs and enhances engraftment, although the underlying mechanism has not yet been elucidated [33, 34]. Moreover, we previously reported that CXCR2 stimulation is crucial to maintain human pluripotent stem cell (hPSC) proliferation [33, 34]. Therefore, we hypothesized that IL-8 activates CXCR2 as well as proliferation of hHSCs suppressed by chemotherapeutic agents in a manner similar to that of hPSCs, resulting in more efficient mobilization. To confirm this hypothesis, we performed an in-vitro experiment to determine the effect of IL-8 on hHSCs in a simulated BM environment using conditioned medium from healthy hBMSCs and observed the expansion of CD34+ and CD34+/CD45- cells. We also observed the concomitant significant enhancement of CXCR2, mTOR, and cMYC expression levels in CD34+ cells following IL-8 stimulation. Our findings showing that IL-8 stimulated CXCR2 and mTOR expression were consistent with the results of our studies with hPSCs [35] and with the observation that mTOR activated cMYC [36]. Regarding the role of cMYC in hematopoiesis, Wilson et al. reported that cMYC controls the balance between stem cell self-renewal and differentiation, presumably by regulating the interaction between HSCs and their niche [37]. Laurenti et al. demonstrated that the loss of cMYC alone resulted in the inability of HSCs to
differentiate into progenitors; furthermore, the majority of early and late progenitors stopped proliferating, resulting in HSC accumulation in the BM niche [38]. Ehninger et al. showed that, although HSCs express low levels of the cMYC protein, its expression increases steadily during progenitor differentiation [39]. A recent study showed that IL-8 activates mTOR and increases endogenous cMYC production, inducing PDL1 expression in gastric cancer [40]. In this study, IL-8 significantly increased not only the number of CD34+ cells but also that of CD34+/CD45- cells. Considering the results of our previous studies showing the role of CXCR2 to support hPSCs proliferation [33, 34], CXCR2 activation by IL-8 may have enhanced hHSC proliferation; however, further studies are necessary to confirm this hypothesis. Therefore, etoposide may induce IL-8 secretion from hBMSCs, which stimulates CXCR2 in HSCs, activating mTOR and cMYC and leading to HSCs proliferation and progenitor cell differentiation. This is the first HSC mobilization study to report such a mechanism. Furthermore, this mechanism may also explain the excellent yield at PBSCC during etoposide chemo-mobilization associated with a modest change in WBC count in the PB.

In clinical practice, it is difficult to observe changes in the BM niche in patients undergoing PBSCC. Moreover, cytokine measurements in the PB do not always accurately reflect levels in the BM niche due to systemic confounding factors. To overcome these obstacles, we established in vivo standardized animal mobilization models that excluded such confounding factors. We were able to simulate the cyclophosphamide and etoposide mobilization patterns observed in clinical practice in two distinct mouse models. Using these models, we confirmed the significantly increased expression of IL-8 homologs and MMP9 and decreased expression of SDF-1 in the BM during etoposide chemo-mobilization, compared to G-CSF only and cyclophosphamide chemo-mobilization. The levels of IL-8 homologs in the PB during G-CSF mobilization were comparable to those during etoposide mobilization.
chemo-mobilization. Watanabe et al. previously reported that G-CSF increased WBC counts and IL-8 levels during mobilization. Increased IL-8 levels were correlated with a higher number of CD34+ cells in the PB [41]. G-CSF was associated with polymorphonuclear neutrophils, which leads to increased IL-8 levels and, potentially, mobilization [42-44]. Moschella et al. reported that cyclophosphamide induced the transcriptional modulation of PB MNCs and IFN-1-related sterile inflammatory responses. In that study, the levels of IL-8, an IFN-1-induced proinflammatory mediator in the PB [45], also increased significantly. Thus, the reason for the IL-8 increment in the PB after etoposide treatment could be an inflammatory response; however, few studies have addressed this issue [46, 47]. Previous studies have reported that IL-8 is produced by phagocytes and mesenchymal cells exposed to inflammatory stimuli [48] and that etoposide affects BMSCs [16, 49]. Therefore, increased IL-8 levels in the PB may be due to inflammatory responses as well as hBMSCs. Additionally, IL-8 may enhance MMP9 production [50], leading to SDF-1 degradation and subsequent mobilization [33, 51, 52]. The results of our animal study revealed that etoposide increases IL-8 homologs (KC, MIP-2, and LIX) and MMP9 expression and decreases SDF-1 expression in the BM, although the levels of these molecules in the PB were similar in all the groups. These findings suggested that the origin of IL-8 in the PB during etoposide chemo-mobilization was predominantly the BM niche rather than systemic inflammation. Synthetically, etoposide stimulated IL-8 secretion from hBMSCs, which activated CXCR2, mTOR, and cMYC in HSCs, resulting in their proliferation. Moreover, MMP9 levels increased and SDF-1 decreased in the BM niche, resulting in mobilization.

The results of this study showed that etoposide causes long-term hBMSC toxicity associated with cell-cycle arrest at the G0/G1 phase. Hare et al. reported that exposure of hBMSCs to sub-lethal doses of etoposide resulted in an increased proportion of cells
arrested in the G0/G1 phase [53]. Moreover, BMSCs could not activate non-homologous end-joining repair following etoposide-induced stress after successive passages [53]. Clinical data also suggest that the toxicity of etoposide in the BM niche is higher and lasts longer than that of cyclophosphamide [9, 10]. However, currently no definitive data are available regarding the adverse effects of etoposide on engraftment or survival. Studies on this topic need to be conducted in future.

This study focused predominantly on cytokine or enzyme changes in the BM niche rather than systemic inflammation because previous studies have generally assessed the role of systemic inflammation on mobilization; moreover, we suspected that the effect of etoposide on the BM niche might be the main mechanism of mobilization. To this end, we used healthy hBMSC-conditioned medium, reflecting the environment of the normal BM niche, for the culture of CD34+ hHSCs. A study design investigating both aspects of mobilization would be very complex. Nevertheless, to our knowledge, this is the first study on the mechanism of etoposide chemo-mobilization to focus on the BM niche. Additionally, this study describes the establishment of the first mouse model of etoposide chemo-mobilization, reflecting the conditions encountered in clinical practice.

Conclusion

In conclusion, etoposide chemo-mobilization is highly effective for harvesting HSCs in PB. Its mechanism of action is associated with IL-8 secretion by hBMSCs, which induces the expansion of HSCs associated with CXCR2, mTOR, and c-MYC activation as well as MMP9 increase and SDF-1 decrease in the BM niche. Finally, our results suggest that etoposide exposure should be minimized before and after PBSCT because of its long-term toxicity to hBMSCs. These findings emphasize the need for further studies to develop more efficient and safe chemo-mobilization strategies.
List Of Abbreviations

Auto-PBSCT: Autologous peripheral blood stem cell transplantation; BM: Bone marrow; CFU: Colony-forming unit; Cmax: Maximum reported serum concentration; G-CSF: granulocyte colony-stimulating factor; hBMSCs: Human bone marrow stromal cells; hHSCs: Human hematopoietic stem cells; HPC: Hematopoietic progenitor cells; hPSC: Human pluripotent stem cell; IHC: Immunohistochemical; KC: Keratinocyte-derived cytokine; LIX: Lipopolysaccharide-inducible CXC chemokine; MIP-2: Macrophage inflammatory protein 2; MMP: Matrix metalloproteinase; MMP2: Matrix metalloproteinase-2; MMP9: Matrix metalloprotease-9; MNC: Mononuclear cells; NHL: Non-Hodgkin’s lymphoma; PB: Peripheral blood; PBSCC: Peripheral blood stem cell collection; SDF-1: Stromal cell-derived factor-1; WBC: White blood cell

Declarations

Ethical approval and consent to participate

The internal review board approved the project protocol.

Consent for publication

All authors agree to submit for consideration for publication in the journal.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information file.

Competing interest

The authors declare that they have no competing interests.

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Author Contributions

B.S.K. designed the study. K.W.K., S.J.L., and J.H.K. performed the experiments. B.H.L., S.J.K., Y.P., and B.S.K. critically reviewed the data analysis. K.W.K. analyzed the data. K.W.K. and B.S.K. wrote the manuscript. All authors approved the final version of the manuscript.

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References

1. Duhrsen U, Villeval JL, Boyd J, Kannourakis G, Morstyn G, Metcalf D. Effects of recombinant human granulocyte colony-stimulating factor on hematopoietic progenitor cells in cancer patients. Blood. 1988;72:2074-81.

2. Pavone V, Gaudio F, Guarini A, Perrone T, Zonno A, Curci P, et al. Mobilization of peripheral blood stem cells with high-dose cyclophosphamide or the DHAP regimen plus G-CSF in non-Hodgkin's lymphoma. Bone Marrow Transplant. 2002;29:285-90.

3. Haverkos BM, Huang Y, Elder P, O'Donnell L, Scholl D, Whittaker B, et al. A single center's experience using four different front line mobilization strategies in lymphoma patients planned to undergo autologous hematopoietic cell transplantation. Bone Marrow Transplant. 2017;52:561-6.

4. Cottler-Fox MH, Lapidot T, Petit I, Kollet O, DiPersio JF, Link D, et al. Stem Cell Mobilization. ASH Education Program Book. 2003;2003:419-37.

5. Levesque JP, Hendy J, Takamatsu Y, Simmons PJ, Bendall LJ. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization
induced by GCSF or cyclophosphamide. J Clin Invest. 2003;111:187-96.

6. de Mel S, Chen Y, Lin A, Soh TG, Ooi M, Yap ES, et al. Vinorelbine-Cyclophosphamide compared to cyclophosphamide in peripheral blood stem cell mobilization for multiple myeloma. Hematol Oncol Stem Cell Ther. 2018;11:225-32.

7. Jantunen E, Putkonen M, Nousiainen T, Pelliniemi TT, Mahlamaki E, Remes K. Low-dose or intermediate-dose cyclophosphamide plus granulocyte colony-stimulating factor for progenitor cell mobilisation in patients with multiple myeloma. Bone Marrow Transplant. 2003;31:347-51.

8. Reiser M, Josting A, Draube A, Mapara MY, Scheid C, Chemnitz J, et al. Successful peripheral blood stem cell mobilization with etoposide (VP-16) in patients with relapsed or resistant lymphoma who failed cyclophosphamide mobilization. Bone Marrow Transplant. 1999;23:1223-8.

9. Milone G, Leotta S, Battiato K, Murgano P, Mercurio S, Strano A, et al. Intermediate dose etoposide plus G-CSF 16 g/kg is more effective than cyclophosphamide 4 g/m(2) plus G-CSF 10 g/kg in PBSC mobilization of lymphoma patients. Leuk Lymphoma. 2007;48:1950-60.

10. Hyun SY, Cheong JW, Kim SJ, Min YH, Yang DH, Ahn JS, et al. High-dose etoposide plus granulocyte colony-stimulating factor as an effective chemomobilization regimen for autologous stem cell transplantation in patients with non-Hodgkin Lymphoma previously treated with CHOP-based chemotherapy: a study from the Consortium for Improving Survival of Lymphoma. Biol Blood Marrow Transplant. 2014;20:73-9.

11. Wood WA, Whitley J, Goyal R, Brown PM, Sharf A, Irons R, et al. Effectiveness of etoposide chemomobilization in lymphoma patients undergoing auto-SCT. Bone Marrow Transplant. 2013;48:771-6.

12. Wood WA, Whitley J, Moore D, Sharf A, Irons R, Rao K, et al. Chemomobilization with
Etoposide is Highly Effective in Patients with Multiple Myeloma and Overcomes the Effects of Age and Prior Therapy. Biol Blood Marrow Transplant. 2011;17:141-6.

13. Park Y, Kim DS, Jeon MJ, Lee BH, Yu ES, Kang KW, et al. Single-dose etoposide is an effective and safe protocol for stem cell mobilization in patients with multiple myeloma. J Clin Apher. 2019. https://doi.org/10.1002/jca.21734.

14. Kollmannsberger C, Beyer J, Droz JP, Harstrick A, Hartmann JT, Biron P, et al. Secondary leukemia following high cumulative doses of etoposide in patients treated for advanced germ cell tumors. J Clin Oncol. 1998;16:3386-91.

15. Relling MV, Boyett JM, Blanco JG, Raimondi S, Behm FG, Sandlund JT, et al. Granulocyte colony-stimulating factor and the risk of secondary myeloid malignancy after etoposide treatment. Blood. 2003;101:3862-7.

16. Gibson LF, Fortney J, Landreth KS, Piktel D, Ericson SG, Lynch JP. Disruption of bone marrow stromal cell function by etoposide. Biol Blood Marrow Transplant. 1997;3:122-32.

17. Tay J, Levesque JP, Winkler IG. Cellular players of hematopoietic stem cell mobilization in the bone marrow niche. Int J Hematol. 2017;105:129-40.

18. Morrison SJ, Wright DE, Weissman IL. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci U S A. 1997;94:1908-13.

19. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. PLoS One. 2014;9:e96801.

20. Grigg AP, Roberts AW, Raunow H, Houghton S, Layton JE, Boyd AW, et al. Optimizing dose and scheduling of filgrastim (granulocyte colony-stimulating factor) for mobilization and collection of peripheral blood progenitor cells in normal volunteers.
21. Lack NA, Green B, Dale DC, Calandra GB, Lee H, MacFarland RT, et al. A pharmacokinetic-pharmacodynamic model for the mobilization of CD34+ hematopoietic progenitor cells by AMD3100. Clin Pharmacol Ther. 2005;77:427-36.

22. Abraham M, Biyder K, Begin M, Wald H, Weiss ID, Galun E, et al. Enhanced unique pattern of hematopoietic cell mobilization induced by the CXCR4 antagonist 4F-benzoyl-TN14003. Stem Cells. 2007;25:2158-66.

23. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature. 2014;505:327-34.

24. Balasubramanian P, Desire S, Panetta JC, Lakshmi KM, Mathews V, George B, et al. Population pharmacokinetics of cyclophosphamide in patients with thalassemia major undergoing HSCT. Bone Marrow Transplant. 2012;47:1178-85.

25. Hassan M, Ljungman P, Ringden O, Hassan Z, Oberg G, Nilsson C, et al. The effect of busulphan on the pharmacokinetics of cyclophosphamide and its 4-hydroxy metabolite: time interval influence on therapeutic efficacy and therapy-related toxicity. Bone Marrow Transplant. 2000;25:915-24.

26. Wurthwein G, Klingebiel T, Krumpelmann S, Metz M, Schwenker K, Kranz K, et al. Population pharmacokinetics of high-dose etoposide in children receiving different conditioning regimens. Anticancer Drugs. 2002;13:101-10.

27. Mross K, Bewermeier P, Kruger W, Stocksclader M, Zander A, Hossfeld DK. Pharmacokinetics of undiluted or diluted high-dose etoposide with or without busulfan administered to patients with hematologic malignancies. J Clin Oncol. 1994;12:1468-74.

28. Laterveer L, Lindley IJ, Hamilton MS, Willemze R, Fibbe WE. Interleukin-8 induces rapid mobilization of hematopoietic stem cells with radioprotective capacity and
long-term myelolymphoid repopulating ability. Blood. 1995;85:2269-75.

29. Fibbe WE, Pruij JF, Velders GA, Opdenakker G, van Kooyk Y, Figdor CG, et al. Biology of IL-8-induced stem cell mobilization. Ann N Y Acad Sci. 1999;872:71-82.

30. Hol J, Wilhelmsen L, Haraldsen G. The murine IL-8 homologues KC, MIP-2, and LIx are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies. J Leukoc Biol. 2010;87:501-8.

31. Singer M, Sansonetti PJ. IL-8 is a Key Chemokine Regulating Neutrophil Recruitment in a New Mouse Model of Shigella-Induced Colitis. The Journal of Immunology. 2004;173:4197-206.

32. Hang L, Frendeus B, Godaly G, Svanborg C. Interleukin-8 receptor knockout mice have subepithelial neutrophil entrapment and renal scarring following acute pyelonephritis. J Infect Dis. 2000;182:1738-48.

33. Fibbe WE, Pruij JF, van Kooyk Y, Figdor CG, Opdenakker G, Willemze R. The role of metalloproteinases and adhesion molecules in interleukin-8-induced stem-cell mobilization. Semin Hematol. 2000;37:19-24.

34. Shirvaikar N, Marquez-Curtis LA, Janowska-Wieczorek A. Hematopoietic Stem Cell Mobilization and Homing after Transplantation: The Role of MMP-2, MMP-9, and MT1-MMP. J Biochemistry Research International. 2012;2012:11.

35. Jung JH, Kang KW, Kim J, Hong SC, Park Y, Kim BS. CXCR2 Inhibition in Human Pluripotent Stem Cells Induces Predominant Differentiation to Mesoderm and Endoderm Through Repression of mTOR, beta-Catenin, and hTERT Activities. Stem Cells Dev. 2016;25:1006-19.

36. Luo G, Li B, Duan C, Cheng Y, Xiao B, Yao F, et al. cMyc promotes cholangiocarcinoma cells to overcome contact inhibition via the mTOR pathway. Oncol Rep. 2017;38:2498-506.
37. Wilson A, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM, et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev. 2004;18:2747-63.

38. Laurenti E, Varnum-Finney B, Wilson A, Ferrero I, Blanco-Bose WE, Ehninger A, et al. Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity. Cell Stem Cell. 2008;3:611-24.

39. Ehninger A, Boch T, Uckelmann H, Essers MA, Mudder K, Sleckman BP, et al. Posttranscriptional regulation of c-Myc expression in adult murine HSCs during homeostasis and interferon-alpha-induced stress response. Blood. 2014;123:3909-13.

40. Sun L, Wang Q, Chen B, Zhao Y, Shen B, Wang H, et al. Gastric cancer mesenchymal stem cells derived IL-8 induces PD-L1 expression in gastric cancer cells via STAT3/mTOR-c-Myc signal axis. Cell death & disease. 2018;9:928-.

41. Watanabe T, Kawano Y, Kanamaru S, Onishi T, Kaneko S, Wakata Y, et al. Endogenous interleukin-8 (IL-8) surge in granulocyte colony-stimulating factor-induced peripheral blood stem cell mobilization. Blood. 1999;93:1157-63.

42. Cassatella MA, Bazzoni F, Ceska M, Ferro I, Baggiolini M, Berton G. IL-8 production by human polymorphonuclear leukocytes. The chemoattractant formyl-methionyl-leucyl-phenylalanine induces the gene expression and release of IL-8 through a pertussis toxin-sensitive pathway. The Journal of Immunology. 1992;148:3216-20.

43. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. Immunol Today. 1995;16:21-6.

44. Pruijt JF, Verzaal P, van Os R, de Kruijf EJ, van Schie ML, Mantovani A, et al. Neutrophils are indispensable for hematopoietic stem cell mobilization induced by interleukin-8 in mice. Proc Natl Acad Sci U S A. 2002;99:6228-33.

45. Moschella F, Torelli GF, Valentini M, Urbani F, Buccione C, Petrucci MT, et al.
Cyclophosphamide Induces a Type I Interferon-Associated Sterile Inflammatory Response Signature in Cancer Patients' Blood Cells: Implications for Cancer Chemoimmunotherapy. Clinical Cancer Research. 2013;19:4249-61.

46. Kawagishi C, Kurosaka K, Watanabe N, Kobayashi Y. Cytokine production by macrophages in association with phagocytosis of etoposide-treated P388 cells in vitro and in vivo. Biochim Biophys Acta. 2001;1541:221-30.

47. Tokarz P, Błasiak J. [Role of DNA methylation in colorectal cancer]. Postepy biochemii. 2013;59:267-79.

48. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 1992;307:97-101.

49. Li J, Law HK, Lau YL, Chan GC. Differential damage and recovery of human mesenchymal stem cells after exposure to chemotherapeutic agents. Br J Haematol. 2004;127:326-34.

50. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 Directly Enhanced Endothelial Cell Survival, Proliferation, and Matrix Metalloproteinases Production and Regulated Angiogenesis. The Journal of Immunology. 2003;170:3369-76.

51. Pruijt JFM, Fibbe WE, Laterveer L, Pieters RA, Lindley IJD, Paemen L, et al. Prevention of interleukin-8-induced mobilization of hematopoietic progenitor cells in rhesus monkeys by inhibitory antibodies against the Metalloproteinase gelatinase B (MMP-9). 1999;96:10863-8.

52. Jin F, Zhai Q, Qiu L, Meng H, Zou D, Wang Y, et al. Degradation of BM SDF-1 by MMP-9: the role in G-CSF-induced hematopoietic stem/progenitor cell mobilization. Bone Marrow Transplantation. 2008;42:581.

53. Hare I, Gencheva M, Evans R, Fortney J, Piktel D, Vos JA, et al. In Vitro Expansion of Bone Marrow Derived Mesenchymal Stem Cells Alters DNA Double Strand Break
Yield of CD34+ cells and changes in white blood cell counts according to mobilization method. a. Data for 173 patients diagnosed with lymphoma who underwent peripheral blood stem cell collection (G-CSF only, n = 33; cyclophosphamide + G-CSF, n = 24; etoposide + G-CSF, n = 116) were analyzed. The highest yield of CD34+ cells was observed for etoposide + G-CSF (1st day: G-CSF only, 1.36 (0.01–14.60); cyclophosphamide + G-CSF, 0.81 (0.05–18.70); etoposide + G-CSF, 4.32 (0.03–32.77), 2nd day: G-CSF-only, 0.96 (0.09–7.25); cyclophosphamide + G-CSF, 0.70 (0.06–13.20); etoposide + G-CSF, 3.37 (0.14–32.60), Total: G-CSF only, 3.13 (0.01–14.60); cyclophosphamide + G-CSF, 2.05 (0.12–31.9); etoposide + G-CSF, 7.22 (0.18–59.20)). b. The change in white blood cell counts.
cell (WBC) counts at nadir and at the time of collection during mobilization was the lowest for the etoposide + G-CSF group among the three groups (ΔWBC: G-CSF only, 15,305 (-1,412-574,000); cyclophosphamide + G-CSF, 10,320 (916-70,884); etoposide + G-CSF, 3,770 (254-120,780)). Note: ‘At nadir’ refers to the lowest WBC value during peripheral blood stem cell collection. ‘ΔWBC’ refers to the increase in WBC counts from the nadir to the time of peripheral blood stem cell collection. Note: *** p < 0.001 after Bonferroni correction; ** p < 0.01 after Bonferroni correction. Note: Values are reported as the median with range.

Abbreviations: G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; ETO, etoposide
Figure 2

Primary culture of human bone marrow stromal cells and results of the cytotoxicity assays, cytokine arrays, and apoptosis and cell cycle analyses a.

Mononuclear cells were collected from a healthy donor during bone marrow harvest. After 1–2 weeks of primary culture, adherent cells showed spindle-shaped morphology and reached 65–70 % confluence. b. Flow cytometry analysis indicated that these cells were positive for the human bone marrow stromal cells (hBMSC) markers CD73, CD90, and CD105 and negative for the hematopoietic stem cell markers CD34 and CD45. These results indicate that hBMSCs were properly isolated. c. Cytotoxic concentration (CC) 10, CC 25, and CC 50, defined as the concentration sufficient to cause the death of 10 %, 25 %, and 50 % of viable hBMSCs, were calculated for various concentrations of both cyclophosphamide and etoposide. d, e. Next, hBMSCs were cultured with normal saline (control group, n = 4), cyclophosphamide (dose of CC10, n = 5), or etoposide (dose of CC10, n = 5) for 24 h. Human cytokine analysis was performed with the conditioned media. The level of IL-8, a mobilization-associated cytokine, was significantly higher in the etoposide-treated group than in the cyclophosphamide-treated group (p = 0.021 after Bonferroni correction). f. Expansion of etoposide-treated hBMSCs was significantly lower than that of cyclophosphamide-treated hBMSCs in both P1 and P2 (control, n = 7; cyclophosphamide, n = 7; etoposide, n = 7; both, p < 0.001 after Bonferroni correction). g. No differences in the numbers of early apoptotic and necrotic cells or late apoptotic cells were observed among the groups (control, n = 3; cyclophosphamide, n = 6; etoposide, n = 6). h. Etoposide-treated hBMSCs showed a higher proportion of cells arrested in the G0/G1 phase of the cell-cycle than the
cyclophosphamide-treated and untreated hBMSCs (control, n = 3; cyclophosphamide, n = 3; etoposide, n = 3; p = 0.03 and p = 0.01 after Bonferroni correction, respectively). Note: *p < 0.05 after Bonferroni correction.

Note: Values are reported as the mean ± standard error of the mean (SEM).

Abbreviations: P1, passage 1; P2, passage 2; CC, cytotoxic concentration

Figure 3

Effects of increased IL-8 levels on hematopoietic stem cells a. Conditioned media was collected from 24-h cultures of healthy hBMSCs grown in mesenchymal stem
cell growth medium. Then, 2.5 × 10^6 hHSCs were cultured for 24 h in conditioned media with 100 ng/mL IL-8 (n = 12) and without IL-8 (n = 12). The numbers of total, CD34+, and CD34+/CD45- cells were significantly higher in the hHSCs cultured in the presence of IL-8, compared to hHSCs cultured without IL-8 (p = 0.014, p = 0.020, and p = 0.039, respectively). b. The relative gene expression levels of CXCR2, mTOR, and cMYC gradually increased with increasing time after IL-8 treatment (1 h, 6 h, and 24 h). Each experiment was repeated thrice. Note: **p < 0.01; *p < 0.05. Note: Values are reported as the median with range (A) and the mean ± SEM (B). Abbreviations: hBMSCs, human bone marrow stromal cells; hHSC, human hematopoietic stem cell
Mouse model of peripheral blood hematopoietic stem cell mobilization a, b, c. The mouse model of hematopoietic stem cell (HSC) mobilization was designed based on a protocol used in human patients (G-CSF only, n = 8; cyclophosphamide + G-CSF, n = 8; etoposide + G-CSF, n = 8). d. On day 7 (D7) of the protocol, HPCs were isolated from the peripheral blood and CFUs (CFU-granulocytes, erythrocytes, monocytes, and megakaryocytes; CFU-granulocytes, macrophages; and burst-forming unit-erythroid) were counted. e. No significant difference was
observed in the total number of CFUs between the cyclophosphamide-treated (200 mg/kg) and etoposide-treated (80 mg/kg) groups (G-CSF only, n = 5; cyclophosphamide + G-CSF, n = 5; etoposide + G-CSF, n = 5). Note: ** p < 0.01 after Bonferroni correction; * p < 0.05 after Bonferroni correction. Note: Values are reported as the mean ± SEM. Abbreviations: S.C., subcutaneous injection; I.P., intraperitoneal injection; NS, normal saline; G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; ETO, etoposide; CFU, colony-forming unit; GEMM, granulocytes, erythrocytes, monocytes, and megakaryocytes; GM, granulocytes, macrophages; BFU-E, burst forming unit-erythroid; n.s., not significant
Figure 5
Keratinocyte-derived cytokine (KC), macrophage inflammatory protein 2 (MIP-2), and lipopolysaccharide-inducible CXC (LIX) expression in the mouse model of peripheral blood hematopoietic stem cell mobilization. a, b. Plasma cytokine analysis was performed in the mouse model on day 7. Levels of KC, MIP-2, and LIX (IL-8 homologs in mice) were measured (G-CSF only, n = 9; cyclophosphamide + G-CSF, n = 9; etoposide + G-CSF, n = 9). KC levels significantly increased in the etoposide-treated group, compared with those in the cyclophosphamide-treated group (p = 0.001 after Bonferroni correction). Levels of the other IL-8 homologs, MIP-2 and LIX, were also increased in the etoposide-treated group but did not show statistically significant differences compared to the cyclophosphamide-treated group. c, d, e. To confirm local changes in KC, MIP-2, and LIX in the bone marrow, we quantified IHC images using the IHC profiler plugin of the ImageJ software. KC increased significantly in the etoposide-treated group, compared to the G-CSF-only and cyclophosphamide-treated groups (p < 0.001 and p < 0.001 after Bonferroni correction, respectively). Levels of the other IL-8 homologs, MIP-2 and LIX, increased significantly in the etoposide-treated group, compared to the G-CSF-only group and cyclophosphamide-treated group (MIP-2, p = 0.004 and p < 0.001 after Bonferroni correction, respectively; LIX, p < 0.001 and p < 0.001 after Bonferroni correction, respectively). Note: *** p < 0.001 after Bonferroni correction; ** p < 0.01 after Bonferroni correction. Note: Values are reported as the mean ± SEM. Abbreviations: G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; ETO, etoposide; n.s., not significant; IHC, immunohistochemistry.
Matrix metalloprotease (MMP) 2, MMP9, and stromal cell-derived factor-1 (SDF-1) expression in the mouse model of peripheral blood hematopoietic stem cell mobilization. a. MMP2, MMP9, and SDF-1 expression in the peripheral blood did not differ significantly among the groups (G-CSF only, n = 4; cyclophosphamide + G-CSF, n = 4; etoposide + G-CSF, n = 4).

b, c, d. In the bone marrow, however, the etoposide + G-CSF group showed a significant increase in MMP9 and decrease in SDF-1 expression, compared to the G-CSF only and cyclophosphamide + G-CSF groups (G-CSF only, n = 7; cyclophosphamide + G-CSF, n = 7; etoposide + G-CSF, n = 7).

Note: ** p <0.01 after Bonferroni correction; * p <0.05 after Bonferroni correction. Note: Values are reported as the mean ± SEM. Abbreviations: G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; ETO, etoposide; n.s., not significant

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