

Cimigenol depresses acute myeloid leukemia cells protected by breaking bone marrow stromal cells via CXCR4/SDF-1 α

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Abstract. The purpose of the present study was to evaluate cimigenol (Cim) treatment effects to cell proliferation by breaking bone marrow stromal cells (BMSCs) through C-X-C chemokine receptor type 4 (CXCR4)/stromal cell-derived factor-1 α (SDF-1 α) pathway. MV-4-11 and U937 cell lines were used. The present study was divided into two parts. First, the cell lines were divided into normal control (NC), BMSC (cells co-cultured with BMSCs), BMSC + DMSO, BMSC + Low (treated with 5 mg/ml Cim), BMSC + Middle (treated with 10 mg/ml Cim), BMSC + High (treated with 20 mg/ml Cim). In the second step, the cell lines were divided into NC, BMSC, BMSC + BL8040 (treated with BL8040 which inhibits CXCR4), BMSC + Cim and BMSC + Cim + BL8040. EdU positive cell numbers were measured by EdU assay and apoptosis rate by flow cytometry and TUNEL assay. Relative gene and protein expression was measured by reverse transcription-quantitative PCR and western blotting assay. BMSCs were able to protect proliferation of cancer cells and decreased cell apoptosis compared with the NC group ($P < 0.001$, respectively). With Cim supplement, the cell proliferation was decreased with cell apoptosis increasing compared with NC group ($P < 0.001$ respectively). However, the anti-tumor effects of Cim were not significantly different from the BL8040 treated groups ($P < 0.001$, respectively). In conclusion Cim decreased acute myeloid leukemia cells protected by BMSCs through the CXCR4/SDF-1 α pathway.

Introduction

Acute myeloid leukemia (AML), characterized by blocked differentiation and clonal proliferation of hematopoietic

stem/progenitor cells, is the commonest hematological malignancy and seriously endangers human health. So far, multiple methods including combined chemotherapy, targeted therapy and hematopoietic stem cell transplantation have not been effective in the treatment of AML (1). Therefore, exploring new therapies for AML is a focus of research. Bone marrow microenvironment (BMME) is a vital research field. Abnormal BMME has long been considered as one of the important factors affecting the onset of AML. Relevant studies have also confirmed that some components of BMME can promote the survival of AML cells and induce chemotherapy resistance by secreting cytokines and interacting with AML cells (2,3).

Natural drug extracts play an important role in the prevention and treatment of tumors in various systems, and have their own distinctiveness in the clinical treatment of leukemia. The regulation of the tumor microenvironment through multi-target effect on tumor cells is one of the important characteristics of anti-tumor therapy of traditional Chinese medicine. Cimigenol (Cim) is one of the main effective components of natural *Cimicifugae Rhizoma* (4). Research results have shown that Cim has a clear inhibitory effect on the biological activity of tumor cells (5-7). However, the role of Cim in AML and relevant mechanisms remains to be elucidated. In clinical treatment, it was found that Cim did not directly affect AML cells promoted by BMSCs (*in vitro* research data not shown as it is a part of another study), and due to this result, the present study did not use Cim to treat MV-4-11 or U937 cells. Therefore, in present study, the role of Cim in inducing AML cell apoptosis and inhibiting AML-related angiogenesis was explored. Cim might affect the occurrence and progression of AML by regulating BMME, but the specific regulatory mechanism is unclear and requires further exploration.

Materials and methods

Reagents and instruments. Cim was purchased from MilliporeSigma, and RPMI 1640 culture medium and fetal bovine serum from Gibco; Thermo Fisher Scientific, Inc. EdU kit, TUNEL kit and Annexin V-APC/7-AAD apoptosis kit were purchased from Jiangsu KeyGEN Biotech Co., Ltd. RNA extraction kit, reverse transcription kit, One Step TB Green PrimeScript RT-PCR kit II (SYBR Green) were purchased from Takara Bio, Inc. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Rabbit anti-human GAPDH was

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purchased from Jiangsu KeyGEN Biotech Co., Ltd., and rabbit anti-human C-X-C chemokine receptor type 4 (CXCR4), stromal cell-derived factor-1 α (SDF-1 α), vascular cell adhesion molecule 1 (VCAM1), leukocyte function-associated antigen-1 (LFA-1), Fms like tyrosine kinase receptor 3 (FLT3), nucleophosmin 1 (NPM-1), CCAAT/enhancer-binding protein alpha (C/EBPA), AKT, phosphorylated (p-)AKT, mTOR and p-mTOR were purchased from Abcam; very-late-antigen-4 (VLA-4) was purchased from ProteinTech Group, Inc. Flow cytometer FC500 was from Beckman Coulter, Inc., ND2000 ultra-microspectrophotometer was from Thermo Fisher Scientific, Inc., and gel image analysis system was produced by GeneGenius. BL8040 (CXCR4 antagonist) was from Roche Diagnostics.

Cell culture. Human AML cell lines MV-4-11 and U937 were purchased from ATCC and primary BMSCs cell were purchased from Procell Life Science & Technology Co., Ltd. (cat. no. CP-H166) and cultured in RPMI 1640 medium containing 10% fetal bovine serum in a 5% CO₂ incubator at 37°C. According to cell proliferation, the medium was changed from time to time for subculture. Cells in the logarithmic growth phase were collected for subsequent experiment. MV-4-11 and U937 cell lines were not contaminated and the STR profiles were positive. The present study was approved by ethics Committee of Affiliated Hospital of Nanjing University of Chinese Medicine (approval no. 2021010606).

Cell treatment. MV-4-11 or U937 cells were inoculated onto 96-well plate (2x10⁵/well), and subsequent experiments were performed when confluence reached >80%. The cells in the NC group were routinely cultured. In the BMSC group, MV-4-11 or U937 cells were co-cultured with BMSCs. In the BMSC + DMSO group, MV-4-11 or U937 cells were co-cultured with BMSCs, to which was added 250 μ l DMSO. In the BMSC + Low group, MV-4-11 or U937 cells were co-cultured with BMSCs and to 250 μ l of the mixed solution was added 5 mg/l Cim (final concentration). In the BMSC + Middle group, MV-4-11 or U937 cells were co-cultured with BMSCs and to 250 μ l of the mixed solution was added 10 mg/l Cim (final concentration). In the BMSC + High group, MV-4-11 or U937 cells were co-cultured with BMSCs and to 250 μ l of the mixed solution was added 20 mg/l Cim (final concentration). In the BMSC + BL8040 group, MV-4-11 or U937 cells were co-cultured with BMSCs, to which was added 10 nM BL8040. In the BMSC + Cim group, MV-4-11 or U937 cells were co-cultured with BMSCs and to 250 μ l of the mixed solution was added 20 mg/l Cim (final concentration). In the BMSC + Cim + BL8040 group, MV-4-11 or U937 cells were co-cultured with BMSCs and to 250 μ l of the mixed solution was added 20 mg/l Cim (final concentration) and 10 nM BL8040. After the cells in each group were treated for 48 h at room temperature, the subsequent experiment was conducted.

The co-culture method for the BMSCs cells was to inoculate on a treated glass slide and when the cells adhered, place the slide into the dish of AML cells and co-culture them.

Detection of cell proliferation by EdU staining. After corresponding treatment in each group for 48 h, 50 μ mol/l EdU staining solution was added to each well for incubation for

2 h at room temperature, followed by washing with PBS for 3 times. Then, 4% paraformaldehyde was added for fixation for 30 min, and 50 μ l 2 mg/ml glycine was added for incubation on a shaking table for 5 min. Additionally, 100 μ l 0.5% TritonX-100 was added for penetration enhancement at room temperature, followed by PBS washing for 3 times. Afterwards, each well had 100 μ l Hoechst33342 staining solution added for reaction at room temperature in the dark for 30 min, followed by washing with PBS for 3 times. Finally, observation and capturing of images were conducted under a fluorescence microscope, with three duplicated wells in each group.

TUNEL assay. After corresponding treatment for 48 h, MV-4-11 or U937 cells were collected from each group, fixed with 4% paraformaldehyde for 30 min at room temperature, and then washed twice with PBS. After adding 100 μ l TUNEL balanced buffer and incubation at room temperature for 5 min, 50 μ l reaction buffer was finally added for incubation in the dark for 60 min at room temperature. Following centrifugation (8,000 x g, 4°C, 2 min), the supernatant was discarded, followed by washing with 5x10⁻³ mg/l BSA. The morphological changes of cells were observation and capturing of images were conducted under a fluorescence microscope.

Flow cytometry. After corresponding treatment for 48 h, MV-4-11 or U937 cells were collected from each group. In 1 h after Annexin V/PI staining at room temperature, 10,000 cells were collected and fixed in each group, and the apoptotic rate of hepatoma cells was detected by flow cytometry. Samples were repeated three times in each group. The quantification was analyzed by FlowJo 7.6.5 software (FlowJo LLC). The apoptosis rate=early + late apoptotic cells/all cells x100%

Reverse transcription-quantitative (RT-q) PCR. After corresponding treatment for 48 h, MV-4-11 or U937 cells (10,000 cells) were collected from each group. Total RNA was extracted from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Then, 0.5 μ g RNA was converted into cDNA at 37°C for 1 h using PrimeScript RT MasterMix (Takara Bio, Inc.). qPCR was performed using ChamQ SYBR® qPCR MasterMix (Vazyme Biotech, Co., Ltd.). Primer sequences are listed in Table I. The reaction system (20 μ l) included: 10 μ l SYBR FAST qPCR Mix (2X), 1 μ l upstream primer (10 μ mol/l), 1 μ l downstream primer (10 μ mol/l), 2 μ l cDNA template and 6 μ l ddH₂O. The reaction conditions were as follows: 95°C for 5 min, 95°C for 15 sec, 60°C for 1 min for 40 cycles. Finally, the relative expression of each gene was analyzed using the 2^{- $\Delta\Delta C_q$} method (8). The experiment was repeated three times.

Western blotting. After corresponding treatment for 48 h, MV-4-11 or U937 cells were collected from each group. Total protein was extracted from cells using RIPA buffer (Changsha Auragene Biological Technology Co., Ltd.) and quantified using a BCA Protein Assay kit (Beijing Dingguo Changsheng Biotechnology, Co., Ltd.). The lysates were incubated at 95°C for 5 min, an equal amount of total protein (30 μ g/lane), separated using 10% SDS-PAGE (Bio-Rad Laboratories) and transferred onto PVDF membranes (MilliporeSigma).

Table I. Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')	Size
AKT	CAGGATGTGGACCAACGTGA	AAGGTGCGTTCGATGACAGT	137 bp
c/EBP α	GACAAGAACAGCAACGAGTACC	GTCATTGTCACTGGTCAGCTC	132 bp
CXCR4	TTCCAGTTTCAGCACATCATGG	GTCGATGCTGATCCCAATGTAG	192 bp
FLT3	GTGAATCCTTACCCCTGGCATTC	GTCAAATTAGGGAAGGATGGCC	164 bp
LFA-1	GGTTGACGTGGTGTATGAGAAG	GAAACCAACCTTGTACAGCACT	109 bp
mTOR	AACCTCCTCCCCTCCAATGA	TCAGCGGTAAAAGTGTCCCC	92 bp
NPM-1	CACCAAAAGGACCTAGTTCTGT	TGCCAGAGATCTTGAATAGCCT	157 bp
SDF-1 α	GATTCTTCGAAAGCCATGTTGC	TCAATGCACACTTGTCTGTTGT	121 bp
VCAM-1	AGTTCTTGTGGCCGAGCTAAA	AAATCTCTGGAGCTGGTAGACC	197 bp
VLA-4	AACATGAGCAGATTGGTAAGGC	CAGACAGAAGCTCCAAAGTACG	112 bp
GAPDH	CAAATTCCATGGCACCCTCA	AGCATCGCCCCACTTGATTT	109 bp

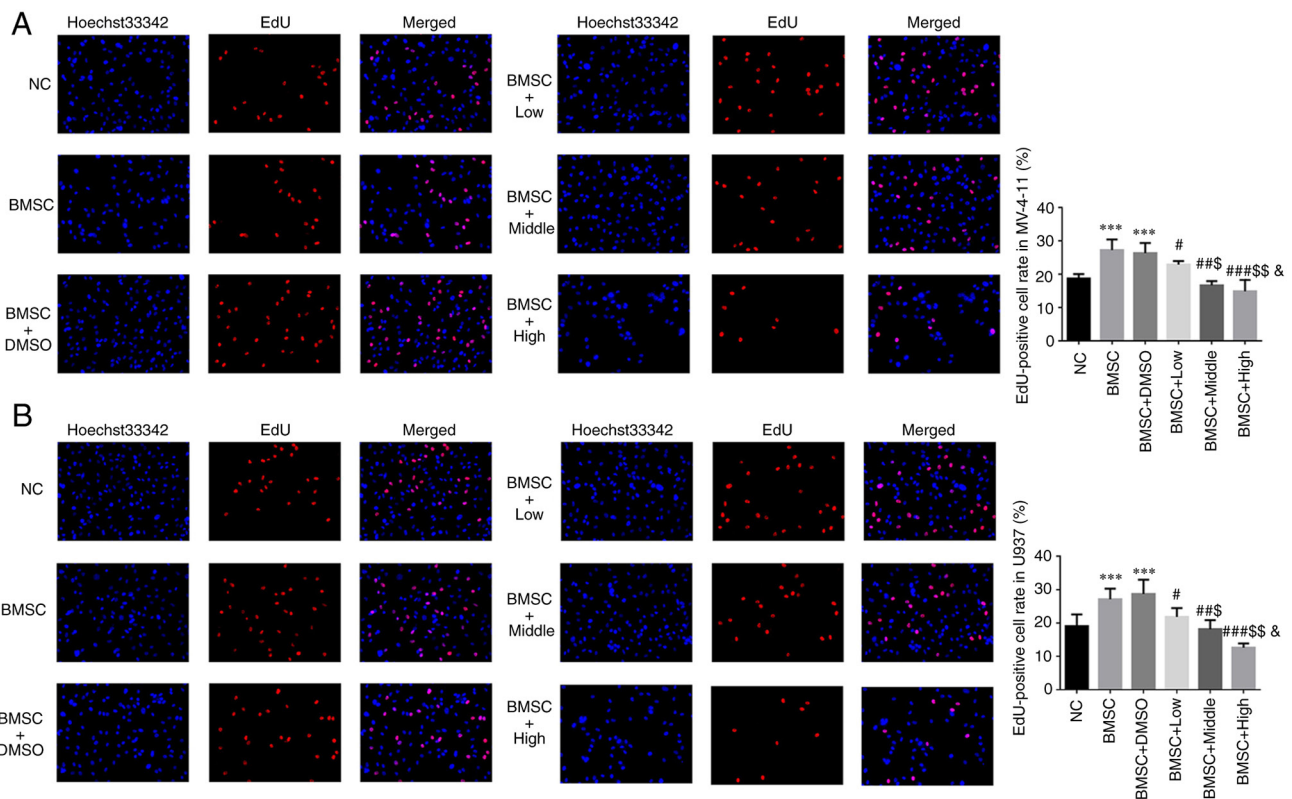


Figure 1. Different concentrations of Cim suppress AML cell proliferation protected by BMSC. EdU-positive cell rate in (A) MV-4-11 and (B) U937 cell line (%; magnification, x200). *** $P < 0.001$ vs. NC; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. BMSC; \$ $P < 0.05$, \$\$ $P < 0.01$, vs. BMSC + Low; & $P < 0.05$, vs. BMSC + Middle. Cim, cimigenol; AML, acute myeloid leukemia; BMSC, bone marrow stromal cells; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim.

After being blocked with 5% skimmed milk at room temperature for 2 h, the membrane was incubated with primary antibodies CXCR4 (1:1,000; cat. no. ab16502), SDF-1 α (1:1,000; cat. no. ab25117), VLA-4 (1:1,000; ProteinTech Group, Inc., cat. no. 19676-1-AP), VCAM1 (1:1,000; cat. no. ab134047), LFA-1 (1:1,000; cat. no. ab235456), FLT3 (1:1,000; cat. no. ab52648), NPM-1 (1:1,000; cat. no. ab10530), C/EBP α (1:1,000; cat. no. ab40761), AKT (1:1,000; cat. no. ab179463), mTOR (1:1,000; cat. no. ab2732), p-AKT (1:1,000; cat.

no. ab81283), p-mTOR (1:1,000; cat. no. ab109268) or GAPDH (1:500; cat. no. ab8245) at 4°C overnight. The membrane was washed with TBST three times for 10 min. Subsequently, HRP-labeled goat anti-rabbit IgGII antibody (1:5,000) was added for incubation at room temperature for 1 h, and the membrane was washed with TBST three times for 10 min. Finally, Proteins were visualized using an ECL reagent kit (Shanghai Yeasen Biotech Co., Ltd.) and were semi-quantified using ImageJ software (1.46r; National Institutes of Health).

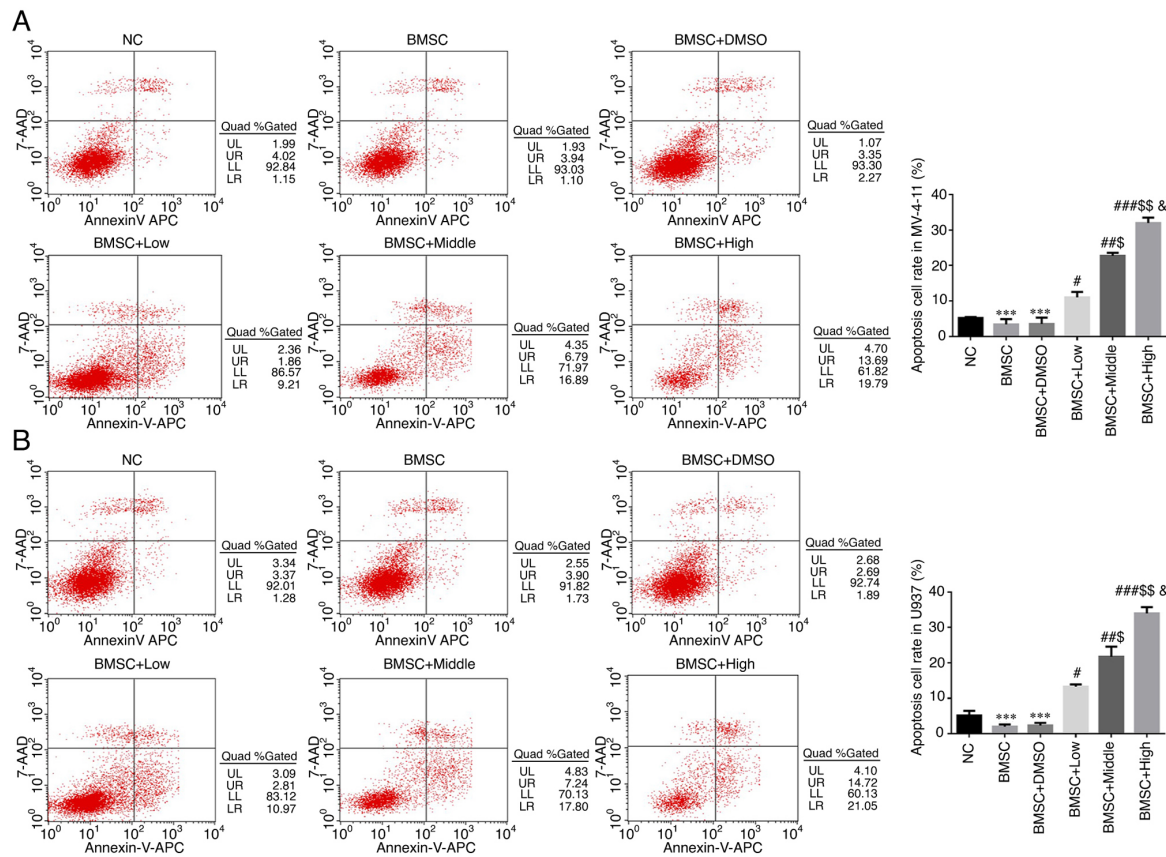


Figure 2. Different concentrations of Cim suppress increased AML cell apoptosis protected by BMSC by flow cytometry. Apoptosis cell rates in (A) MV-4-11 and (B) U937 (%). *** $P < 0.001$, vs. NC; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. BMSC; $P < 0.05$, $P < 0.01$, vs. BMSC + Low; $P < 0.05$, vs. BMSC + Middle. Cim, cimigenol; AML, acute myeloid leukemia; BMSC, bone marrow stromal cells; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim.

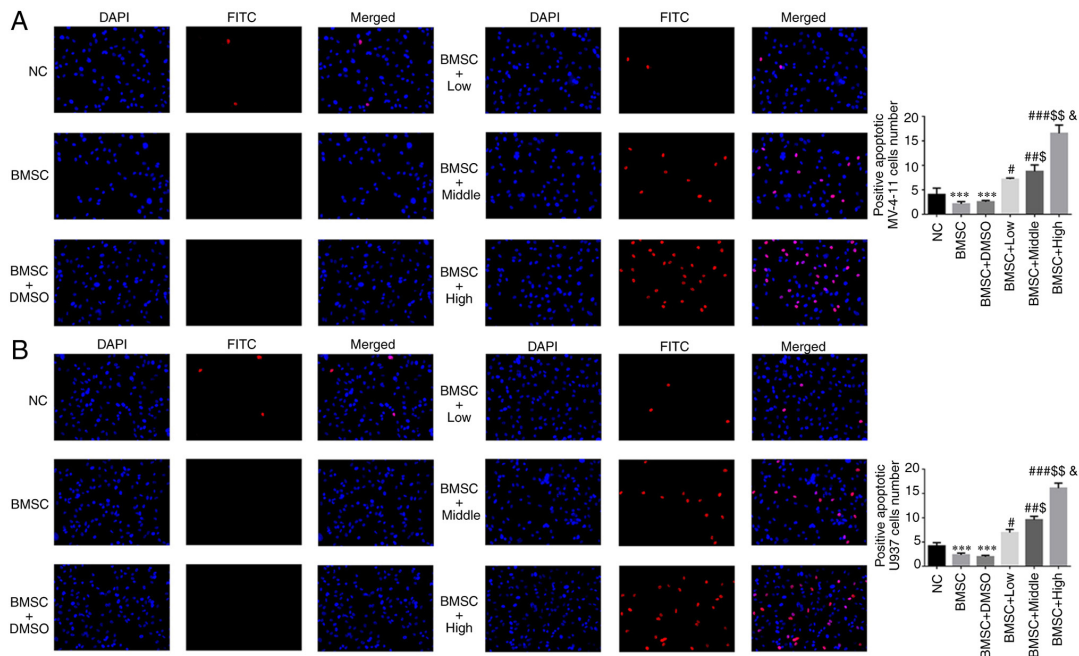


Figure 3. Different concentrations of Cim suppress increased apoptosis AML cell number protected by BMSC by TUNEL assay. Positive apoptotic cell number in (A) MV-4-11 and (B) U937 (magnification, x200). *** $P < 0.001$, vs. NC; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. BMSC; $P < 0.05$, $P < 0.01$, vs. BMSC + Low; $P < 0.05$, vs. BMSC + Middle. Cim, cimigenol; AML, acute myeloid leukemia; BMSC, bone marrow stromal cells; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

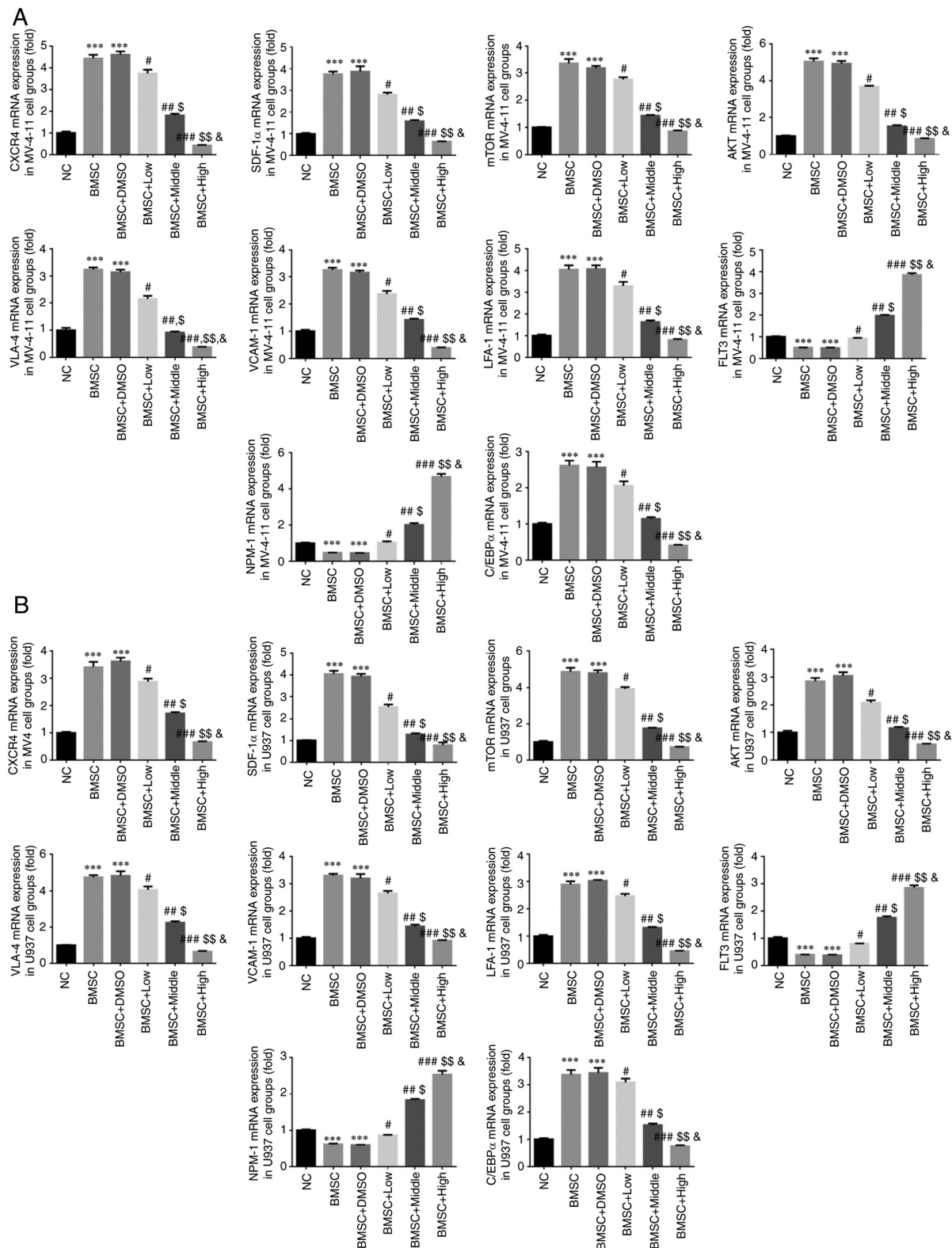


Figure 4. Different concentrations of Cim suppress affect relative gene expression by reverse transcription-quantitative PCR. Relative gene expression in (A) MV-4-11 and (B) U937. *** $P < 0.001$, vs. NC; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, vs. BMSC; $^{\$}P < 0.05$, $^{ss}P < 0.01$, vs. BMSC + Low; $^{\&}P < 0.05$, vs. BMSC + Middle. Cim, cimigenol; AML, acute myeloid leukemia; BMSC, bone marrow stromal cells; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

Statistical analysis. Experiments were performed in triplicate at minimum. Data are presented as the mean \pm standard deviation and were analyzed using GraphPad Prism 8.0 (GraphPad

Software, Inc.). For statistical analysis, pairwise comparisons between two groups were analyzed using the unpaired Student's t-test. One-way ANOVA followed by Tukey's post

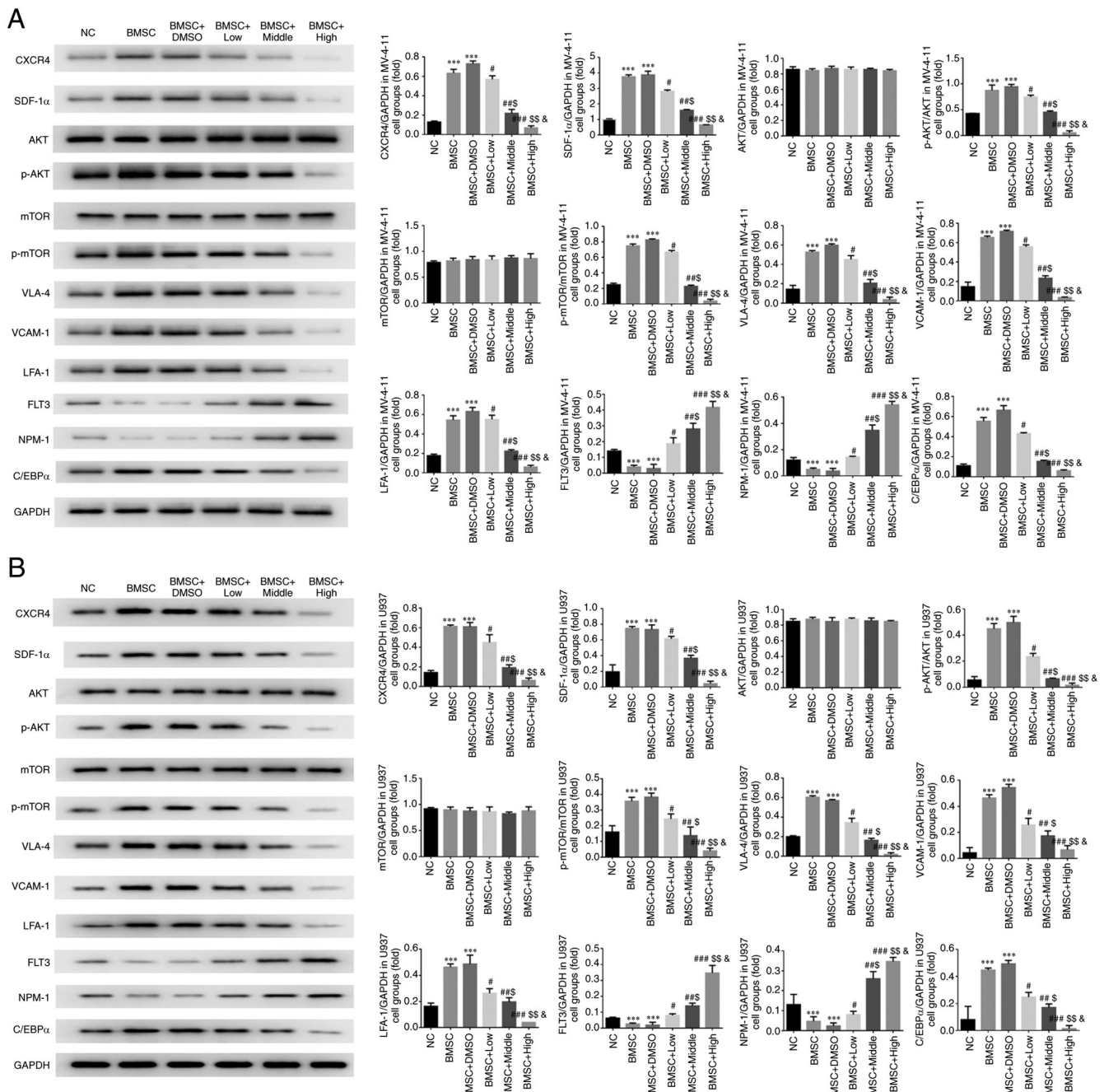


Figure 5. Different concentrations of Cim suppress affected relative protein expression by western blotting. Relative protein expression in (A) MV-4-11 and (B) U937. *** $P < 0.001$, vs. NC; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, vs. BMSC; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, vs. BMSC + Low; $^{\&}P < 0.05$, vs. BMSC + Middle. Cim, cimigenol; CXCR4, C-X-C chemokine receptor type 4; SDF-1 α , stromal cell-derived factor-1 α ; p-, phosphorylated; VLA-4, very-late-antigen-4; VCAM1, vascular cell adhesion molecule 1; LFA-1, leukocyte function-associated antigen-1; FLT3, Fms like tyrosine kinase receptor 3; NPM-1, nucleophosmin 1; C/EBP α , CCAAT/enhancer-binding protein alpha; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

hoc test was used for comparisons between >2 groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Different Cim concentrations suppresses AML cell proliferation protected by BMSC. Compared with NC group, EdU positive cell rates of BMSC and BMSC + DMSO groups were significantly upregulated in MV-4-11 and U937 cell lines

($P < 0.001$, respectively, Fig. 1A and B). With Cim supplement, compared with BMSC group, EdU positive cell rates of Cim treated groups were significantly depressed in MV-4-11 and U937 cell lines ($P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively, Fig. 1A and B) and was dose-dependent ($P < 0.05$, respectively, Fig. 1A and B).

Different Cim concentrations increase AML cell apoptosis protected by BMSC by flow cytometry. Compared with NC

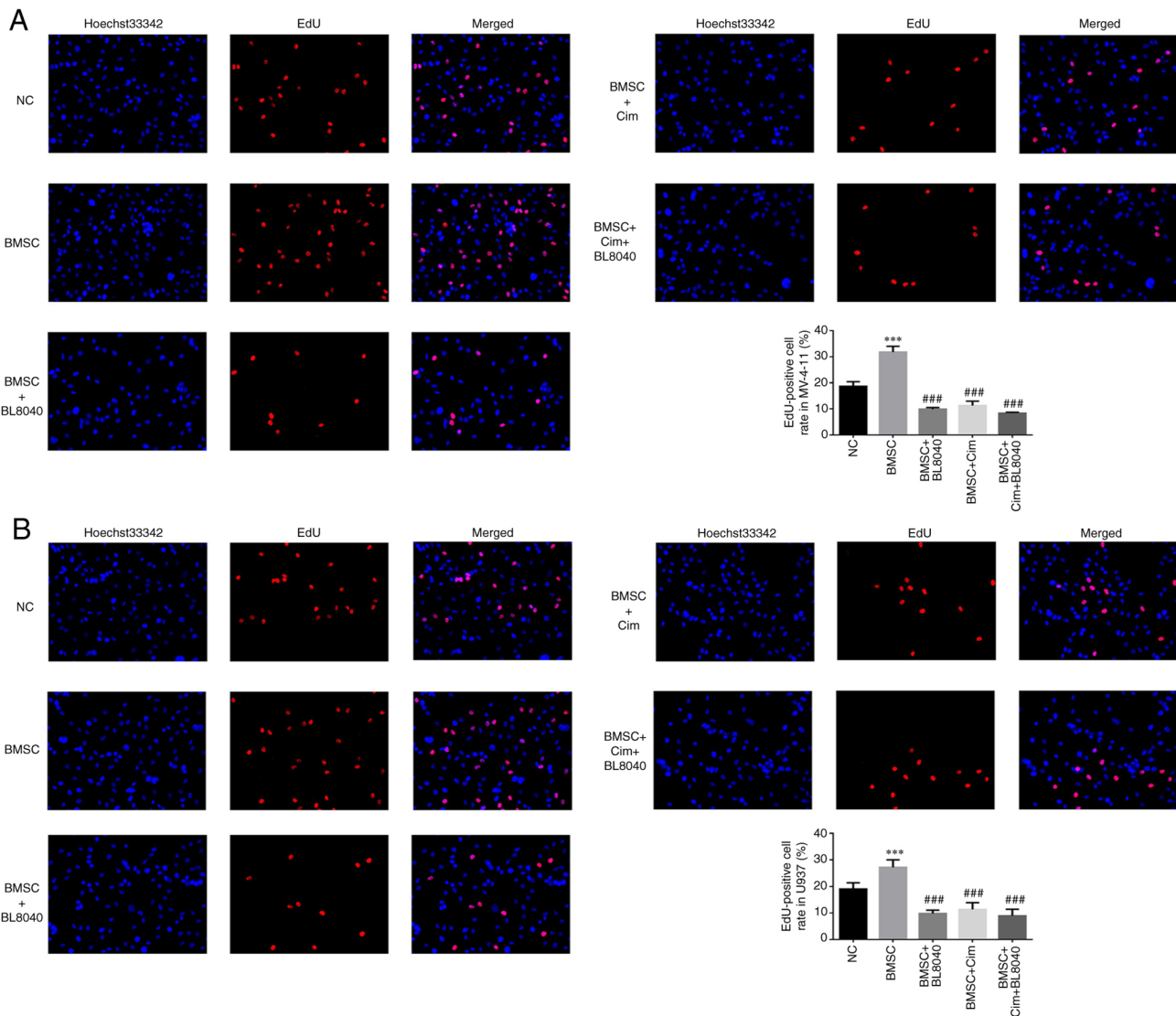


Figure 6. Effect of CXCR4 on the anti-tumor effects of Cim in cell proliferation. EdU-positive cell rate in (A) MV-4-11 and (B) U937 (%). *** $P < 0.001$, vs. NC; ### $P < 0.001$, vs. BMSC. CXCR4, C-X-C chemokine receptor type 4; Cim, cimigenol; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

group, apoptosis rates of BMSC and BMSC + DMSO groups were significantly downregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 2A and B). With Cim supplement, compared with BMSC group, apoptosis rates of Cim treated groups were significantly increased in MV-4-11 and U937 cell lines ($P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively, Fig. 2A and B) with dose-dependent ($P < 0.05$, respectively, Fig. 2A and B).

Different Cim concentrations increase number of apoptotic AML cells protected by BMSC by TUNEL assay. Compared with the NC group, the number of positive apoptotic cells in BMSC and BMSC + DMSO groups was significantly downregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 3A and B). With Cim supplement, compared with BMSC group, the number of positive apoptotic cells in Cim treated groups was significantly increased in MV-4-11 and U937 cell lines ($P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively; Fig. 3A and B) dose-dependently ($P < 0.05$, respectively; Fig. 3A and B).

Different Cim concentrations affect relative gene expression by RT-qPCR assay. Compared with the NC group, CXCR4, SDF-1 α , mTOR, AKT, VLA-4, VCAM-1, LFA-1 and C/EBP α mRNA expression in BMSC and BMSC + DMSO groups was significantly upregulated and FLT3 and NPM-1 mRNA expression in BMSC and BMSC + DMSO groups was significantly downregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 4A and B). With Cim, compared with the BMSC group, CXCR4, SDF-1 α , mTOR, AKT, VLA-4, VCAM-1, LFA-1 and C/EBP α mRNA expression was significantly downregulated and FLT3 and NPM-1 mRNA expression was significantly upregulated in MV-4-11 and U937 cell lines ($P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively, Fig. 4A and B) dose-dependently ($P < 0.05$, respectively, Fig. 4A and B).

Different Cim concentrations affect relative protein expression by western blotting. Compared with the NC group,

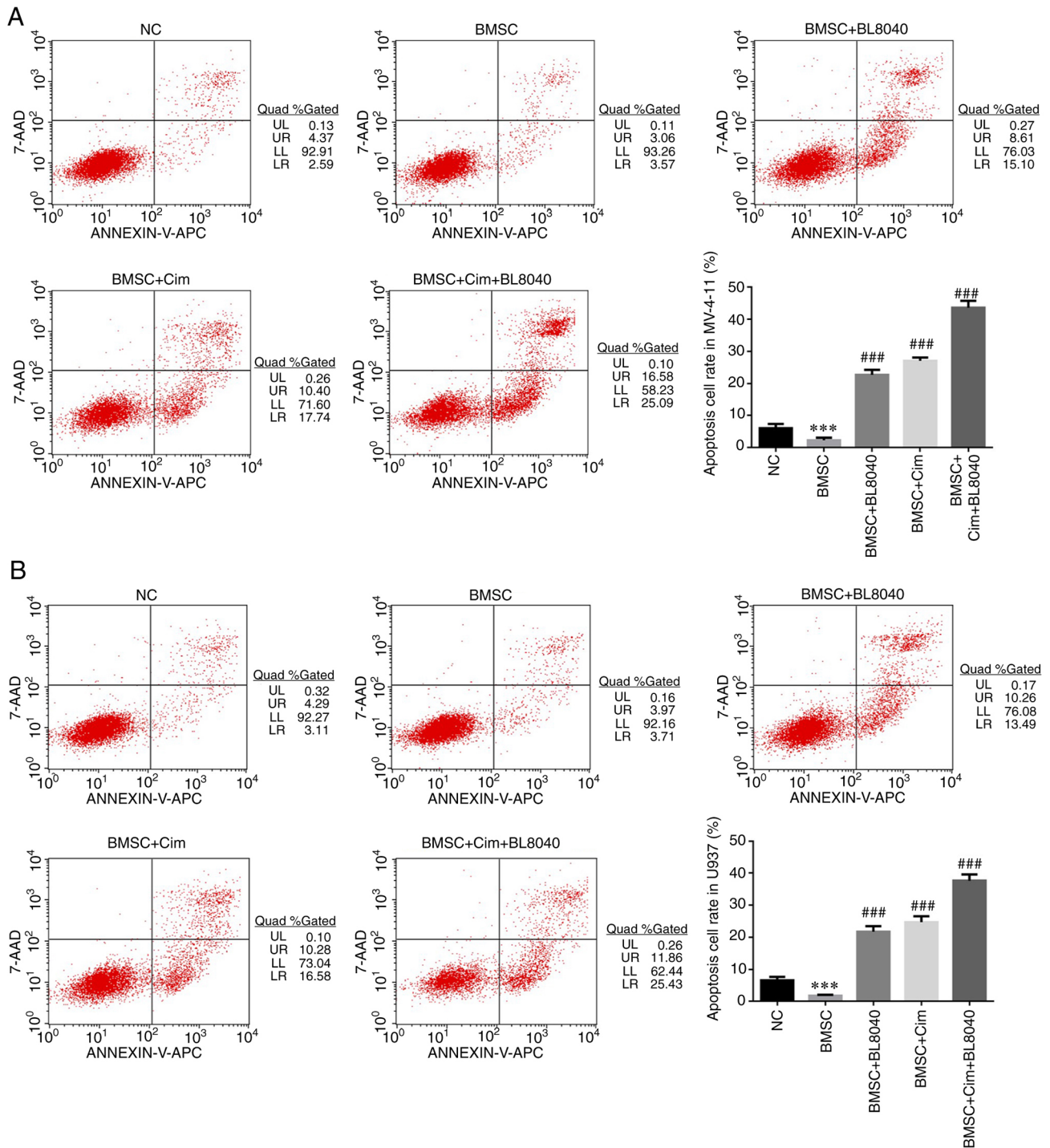


Figure 7. Effect of CXCR4 on the anti-tumor effects of Cim in cell apoptosis by flow cytometry. Apoptosis cell rate in (A) MV-4-11 and (B) U937 (%). *** $P < 0.001$, vs. NC; ### $P < 0.001$, vs. BMSC. C-X-C chemokine receptor type 4; Cim, cimigenol; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

CXCR4, SDF-1 α , p-mTOR, p-AKT, VLA-4, VCAM-1, LFA-1 and C/EBP α protein expression of BMSC and BMSC + DMSO groups was significantly upregulated, and FLT3 and NPM-1 protein expression of BMSC and BMSC + DMSO groups was significantly downregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 5A and B). With Cim, compared with the BMSC group, CXCR4, SDF-1 α , p-mTOR, p-AKT, VLA-4, VCAM-1, LFA-1 and C/EBP α protein expression was

significantly downregulated and FLT3 and NPM-1 protein expression was significantly upregulated in MV-4-11 and U937 cell lines ($P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively, Fig. 5A and B) dose-dependently ($P < 0.05$, respectively, Fig. 5A and B).

Effect of CXCR4 on the anti-tumor effects of Cim in cell proliferation. Compared with the NC group, EdU positive cell rates

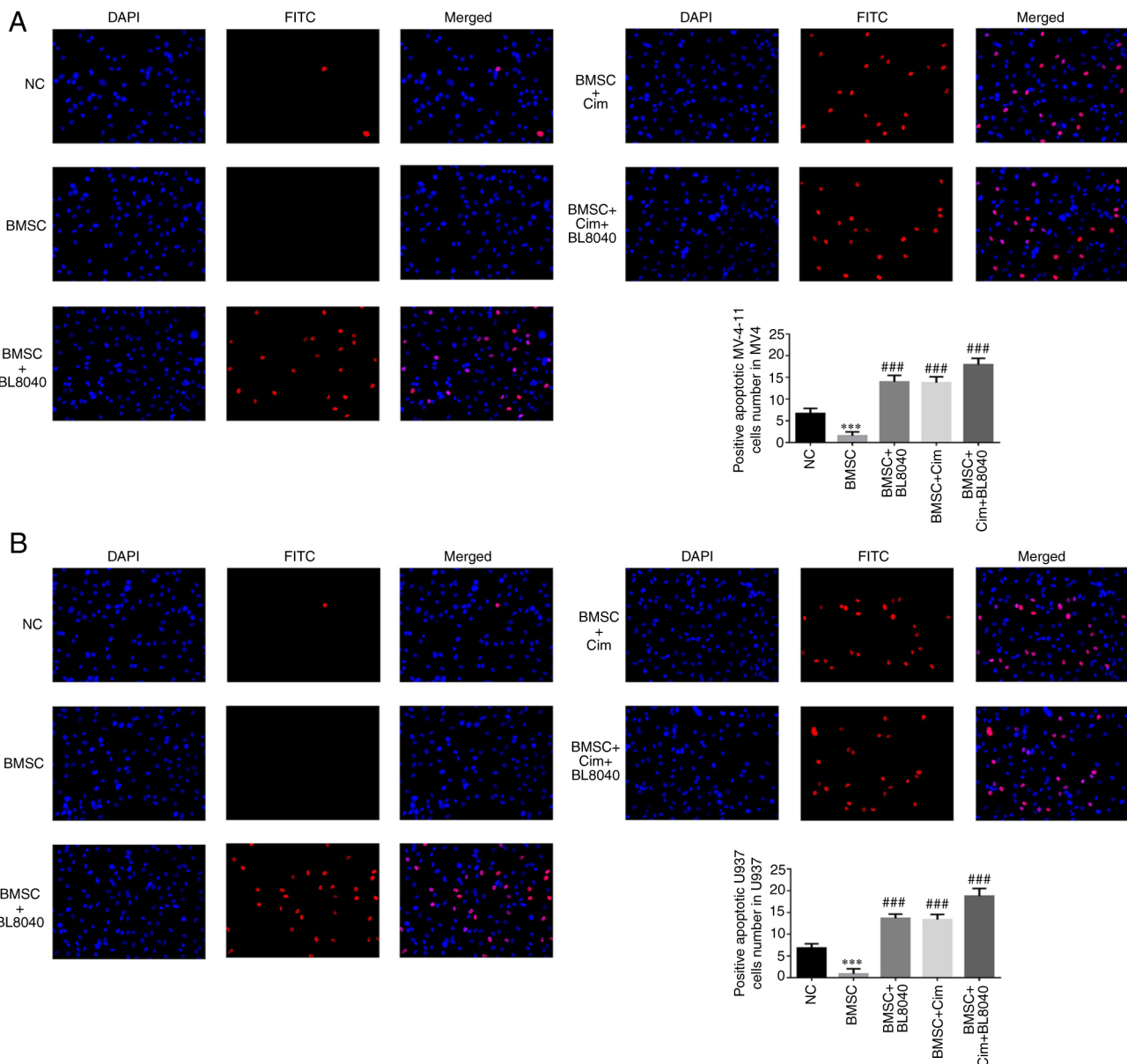


Figure 8. Effect of CXCR4 on the anti-tumor effects of Cim in cell apoptosis by TUNEL assay. Number of positive apoptotic cells in (A) MV-4-11 and (B) U937 (magnification, x200). ***P<0.001, vs. NC; ###P<0.001, vs. BMSC. C-X-C chemokine receptor type 4; Cim, cimigenol; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

in BMSC groups were significantly upregulated in MV-4-11 and U937 cell lines ($P<0.001$, respectively, Fig. 6A and B). With BL8040 (CXCR4 inhibitor) and/or Cim treatment, compared with BMSC group, EdU positive cell rates of BMC + BL8040, BMSC + Cim and BMSC + Cim + BL8040 groups were significantly suppressed ($P<0.001$, respectively, Fig. 6A and B).

Effect of CXCR4 on the anti-tumor effects of Cim in cell apoptosis by flow cytometry. Compared with the NC group, apoptosis rates in BMSC groups were significantly downregulated in MV-4-11 and U937 cell lines ($P<0.001$, respectively, Fig. 7A and B). With BL8040 (CXCR4 inhibitor) and/or Cim treatment, compared with BMSC group, apoptosis rates of BMC + BL8040, BMSC + Cim and BMSC + Cim + BL8040

groups were significantly increased ($P<0.001$, respectively, Fig. 7A and B).

Effect of CXCR4 on the anti-tumor effects of Cim in cell apoptosis by TUNEL assay. Compared with the NC group, number of positive apoptotic cells in the BMSC groups was significantly downregulated in MV-4-11 and U937 cell lines ($P<0.001$, respectively, Fig. 8A and B). With BL8040 (CXCR4 inhibitor) and/or Cim treatment, compared with BMSC group, the number of positive apoptotic cells in the BMC + BL8040, BMSC + Cim and BMSC + Cim + BL8040 groups was significantly increased ($P<0.001$, respectively, Fig. 8A and B).

CXCR4 inhibitor affects relative gene expression. Compared with the NC group, CXCR4, SDF-1 α , mTOR, AKT, VLA-4,

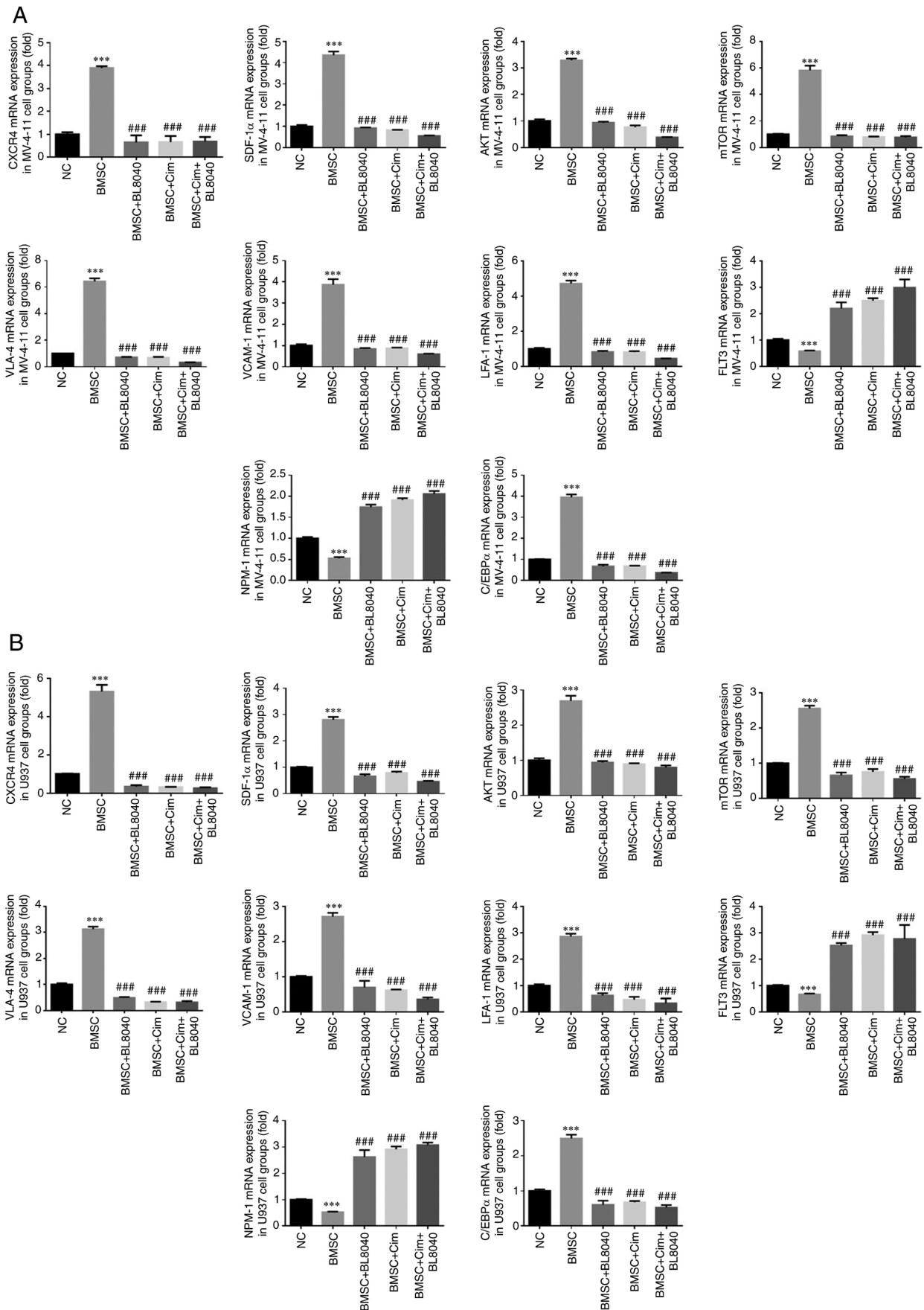


Figure 9. CXCR4 inhibitor affected relative gene expression. Relative gene expression in (A) MV-4-11 and (B) U937. *** $P < 0.001$, vs. NC; ### $P < 0.001$, vs. BMSC. C-X-C chemokine receptor type 4; Cim, cimigenol; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

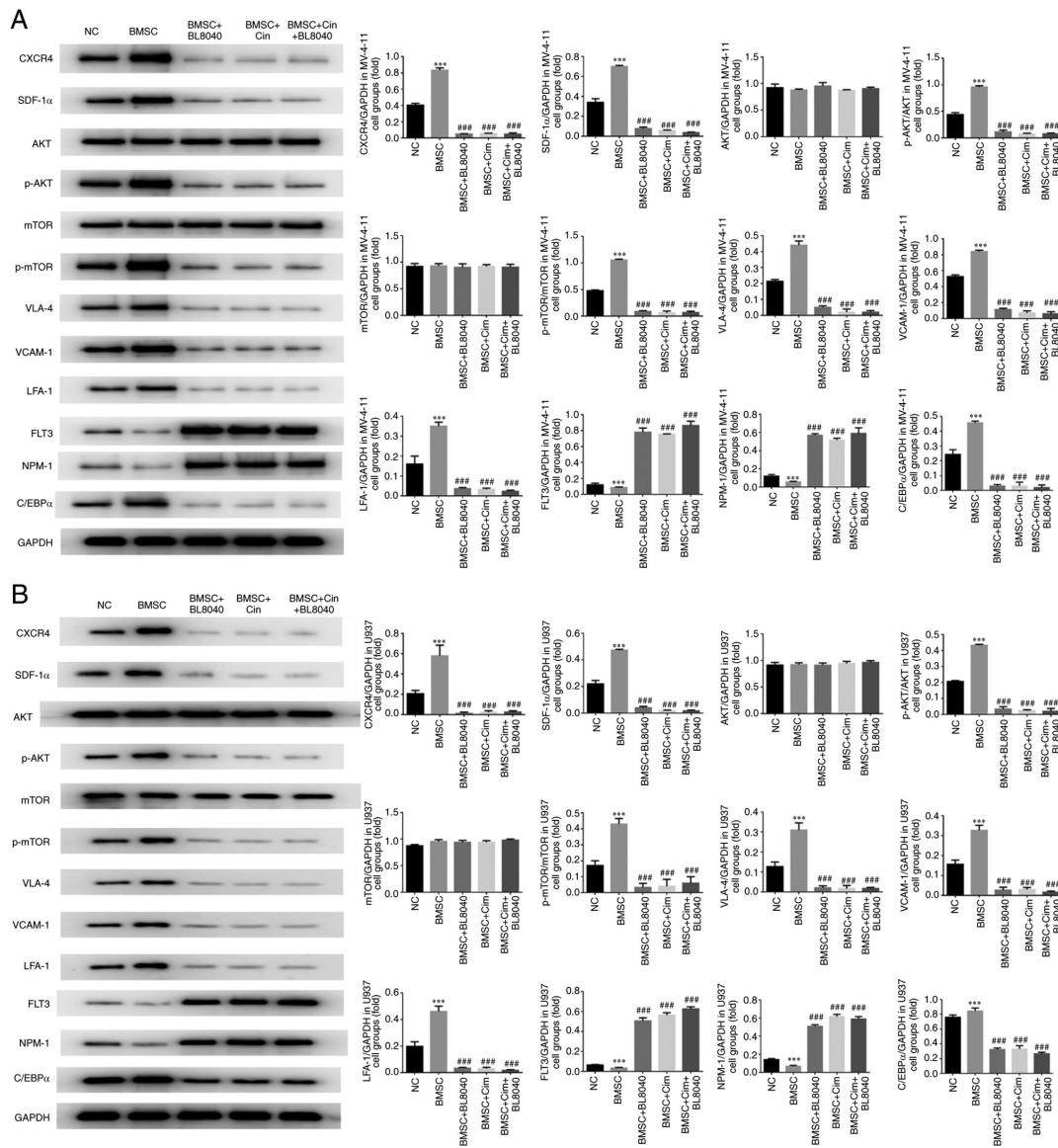


Figure 10. CXCR4 inhibitor affects relative protein expression. Relative protein expression in (A) MV-4-11 and (B) U937. *** $P < 0.001$, vs. NC; ### $P < 0.001$, vs. BMSC. Cim, cimigenol; CXCR4, C-X-C chemokine receptor type 4; SDF-1 α , stromal cell-derived factor-1 α ; p-, phosphorylated; VLA-4, very-late-antigen-4; VCAM1, vascular cell adhesion molecule 1; LFA-1, leukocyte function-associated antigen-1; FLT3, Fms like tyrosine kinase receptor 3; NPM-1, nucleophosmin 1; C/EBP α , CCAAT/enhancer-binding protein alpha; NC, cells in normal culture medium; BMSC, cells co-cultured with BMSC; BMSC + DMSO, cells co-cultured with BMSCs and treated with 250 μ l DMSO; BMSC + Low, cells co-cultured with BMSCs and treated with 5 mg Cim; BMSC + Middle, cells co-cultured with BMSCs and treated with 10 mg Cim; BMSC + High, cells co-cultured with BMSCs and treated with 20 mg Cim; BMSCs, breaking bone marrow stromal cells.

VCAM-1, LFA-1 and C/EBP α mRNA expression in BMSC groups was significantly upregulated, and FLT3 and NPM-1 mRNA expression in BMSC groups was significantly downregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 9A and B). With BL8040 (CXCR4 inhibitor) and/or Cim treatment, compared with the BMSC group, CXCR4, SDF-1 α , mTOR, AKT, VLA-4, VCAM-1, LFA-1 and C/EBP α mRNA expression was significantly downregulated and FLT3 and NPM-1 mRNA expression was significantly upregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 9A and B).

CXCR4 inhibitor affects relative protein expression. Compared with the NC group, CXCR4, SDF-1 α , mTOR, AKT, VLA-4, VCAM-1, LFA-1 and C/EBP α protein expression of

BMSC groups was significantly upregulated, and FLT3 and NPM-1 protein expression of BMSC groups was significantly downregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 10A and B). With BL8040 (CXCR4 inhibitor) and/or Cim treatment, compared with BMSC group, CXCR4, SDF-1 α , mTOR, AKT, VLA-4, VCAM-1, LFA-1 and C/EBP α protein expression was significantly downregulated and FLT3 and NPM-1 protein expression was significantly upregulated in MV-4-11 and U937 cell lines ($P < 0.001$, respectively, Fig. 10A and B).

Discussion

Under the physiological state, BMSCs in BMME can produce a variety of adhesion molecules and chemokines,

thus mediating multiple signal cascades to ensure and maintain the normal localization and homeostasis of HSCs in BM. In the pathological state of AML, these products can be hijacked and shared by AML cells, so that AML cells can obtain environmental conditions conducive to their own survival, expansion and progression, finally leading to the weakening of apoptosis in AML cells (9,10). The bindings and interactions of the chemokine receptor family represented by CXCR4 and its relevant ligands play a representative role in this process. CXCR4 directly or indirectly activates a variety of signal cascades by binding with its ligands to ensure the correct localization, homeostasis maintenance and normal survival of HSCs in BMME (11). CXCR4 may eventually cause the progression, difficulties in treatment and recurrence of AML by affecting the proliferation, migration, chemotaxis and angiogenesis as well as increasing the chemotherapy resistance of leukemia cells (12).

SDF-1 α is one of the members of the chemokine family in BMME and also the only ligand of CXCR4. In BMME, many components such as BMSCs, immature osteoblasts and bone marrow endothelial cells can secrete SDF-1 α (13). Among these cell components, BMSCs are the main source (14). SDF-1 α is often expressed in bone marrow microvascular hot spots that attract the aggregation of AML cells in BMME (15). It can promote the survival and expansion of AML cells by guiding AML cells to a favorable environment in BMME (16). In addition, the adhesion between AML cells and bone marrow stroma in BMME is vital for the survival and proliferation of AML cells and SDF-1 α participates in the regulation of this mechanism. This regulation can be briefly summarized as: SDF-1 α regulates the adhesion of AML cells to matrix components in BMME by activating adhesion molecules, such as integrins CD44 and VLA-4, so that AML cells can obtain 'concealment' and produce chemotherapy resistance (17). The binding of CXCR4 and its ligand SDF-1 α can initiate multiple Ca²⁺-dependent or independent signal events, leading to actin cytoskeleton reorganization and activating integrins, which results in its appropriate interaction with the endothelium of BM sinus and stromal cells, finally affecting the survival, chemotaxis, homing and proliferation of cells (18). A relevant study (19) demonstrated that CXCR4 inhibitor AMD3465 can promote the peripheral mobilization of AML cells and enhance the anti-leukemia effect of chemotherapeutic drugs. A further study (3) found that blocked CXCR4/SDF-1 α interaction affects the activity of related downstream signaling pathways such as PI3K/AKT and MAPK and increases the mobilization rate of AML cells, finally leading to the increase in chemotherapy sensitivity. Experiments have confirmed that other CXCR4 antagonists and monoclonal antibodies, including LY2510924, CX-01, POL6326 and NOX-A12, can also effectively inhibit the growth of AML cells and produce sustained pharmacodynamic effects on peripheral mobilization of cells (20-22). Some related studies showed that the CXCR4/SDF-1 α signaling pathway stimulation could improve cancer cell biological activating *in vitro* and *in vivo* studies (23,24). The results of the present study showed that after AML cells were co-cultured with BMSCs, the apoptosis of AML

cells MV-4-11 and U937 was protected, the proliferation was increased and the CXCR4/SDF-1 α signaling pathway was activated. Therefore, it was hypothesized that BMSCs possess a protective effect on AML cells.

Cim has an inhibitory effect on the activity of tumor cells *in vivo* and *in vitro* (5-7). The present study showed that Cim also had a certain killing effect on AML cells under the protection of BMSCs and that its mechanism might be through the inhibition of the CXCR4/SDF-1 α signaling pathway, which further inhibited downstream AKT and mTOR activities and acted on the terminal VLA-4 (25), VCAM-1 (26), LFA-1 (27), FLT3 (28), NPM-1 (29) and c/EBP α (30). Therefore, it is hypothesized that Cim can promote the apoptosis and inhibit the proliferation of AML cells by inhibiting the CXCR4/SDF-1 α signaling pathway.

However, there were some limitations to the present study. It only studied the effect of cimigenol on AML cell lines via CXCR4/SDF-1 α pathway; the anti-tumor effects of cimigenol might be regulated by other pathways, meanwhile there were some differences between *in vitro* in AML. Future *in vivo* studies will address the bioavailability of cimigenol.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BM, HD and XMS contributed to the conceptualization and the design of the present study. BM, HD and XD performed the experiments and analyzed the data. XD, SQ, XCS and XMS were responsible for the acquisition, analysis and interpretation of the data. HD and XCS contributed to the drafting of the manuscript. XD and SQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the Affiliated Hospital of Nanjing University of Chinese Medicine (approval no. 2021010606).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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