

Effects of triptolide on bone marrow-derived mesenchymal stem cells from patients with multiple myeloma

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Received November 25, 2017; Accepted August 17, 2018

DOI: 10.3892/etm.2019.7373

Abstract. Triptolide (TPL), an extract of the Chinese herb *Tripterygium wilfordii* Hook F, is a potent anti-inflammatory agent that further possesses anticancer activity. Its antiproliferative effects are well established. Only few studies have focused on TPL as a potential treatment in multiple myeloma (MM). In the current study, bone marrow-derived mesenchymal stem cells (BMMSCs) from patients with MM were isolated and treated with TPL at varying concentrations. Thalidomide is currently used as a positive control drug in the treatment of MM. Cell Counting kit-8 assays were performed to assess proliferation activity and flow cytometry with Annexin V-fluorescein/propidium iodide was used to detect cell apoptosis of TPL-treated BMMSCs. Reverse transcription-quantitative polymerase chain reaction assays were applied to measure interleukin (IL)-6, IL-1 β and stem cell factor (SCF or Kit ligand) mRNA expression and western blot assays were performed to analyze transcription factor p65 (P65) expression in TPL-treated BMMSCs. ELISA was applied to measure vascular endothelial growth factor (VEGF) levels in the supernatant of the cultured and treated BMMSCs. TPL treatment significantly inhibited BMMSC proliferation compared with the untreated control ($P<0.05$). At 48 h following TPL treatment, a Cell Counting kit-8 study was performed and the IC₅₀ value was determined at 101.55 \pm 2.45 ng/ml. Apoptotic rates were observed to increase with increasing concentrations of TPL ($P<0.001$), and IL-6, IL-1 β and SCF mRNA expression was significantly decreased with increasing TPL ($P<0.001$).

P65 expression following TPL treatment was significantly decreased compared with the untreated control ($P<0.05$). VEGF levels were significantly reduced in the presence of increasing amounts of TPL ($P<0.05$). These findings suggest that TPL inhibited BMMSC growth and improved the bone marrow hematopoietic microenvironment by decreasing IL-6, IL-1 β and SCF mRNA expression, subsequently inhibiting the proliferation of MM cells. Therefore, TPL may be used in the future to treat patients with MM.

Introduction

Multiple myeloma (MM) is a malignant plasma hematologic tumor and a potent radical treatment is currently not available (1). Thus, it is considered as an incurable tumor. The incidence rate is estimated to be 2-3/100,000, with a male to female ratio of 1.6:1 and a median age of 65 years. Novel drugs for the treatment of MM include second generation of proteasome inhibitors, such as carfilzomib, third generation of immunomodulators, such as pomalidomide and monoclonal antibodies, such as daratumumab. The *Tripterygium wilfordii* extract triptolide (TPL), a Traditional Chinese Medicine, possesses significant anti-inflammatory, antitumor and immunoregulatory effects, and regulates fertility (2,3). According to previous studies (4-8), TPL has been reported to decrease the activity of nucleation factor (NF)- κ B in lymphocytes, endothelial cells and other cells, and further to decrease the levels of inflammatory mediators, including interleukin (IL)-6 and IL-1 β , which are released in the activation of NF- κ B. In previous studies, TPL was observed to inhibit cyclin D1 expression through blocking the signal transducer and activator of transcription 3 signaling pathway and mRNA expression of the positive cell cycle regulatory genes, including c-myc and cyclin A, B, C and D, in various colon cancer cell lines (9,10). Constitutive activation of the NF- κ B pathway is mediated by mutations in certain tumors during progression, such as hepatocellular carcinoma (11). In addition to these oncogenic events, tumor cells are strongly dependent on the bone marrow (BM) microenvironment (12). Substantial advances have been made in understanding the biology of MM by studying the BM microenvironment (13,14). The BM niche serves an important role in differentiation, migration, proliferation, survival and drug resistance of malignant plasma cells (15). It has further

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Abbreviations: TPL, triptolide; BMMSC, bone marrow-derived mesenchymal stem cell; MM, multiple myeloma; VEGF, vascular endothelial growth factor

Key words: triptolide, multiple myeloma, bone marrow-derived mesenchymal stem cells, cytokines, nucleation factor- κ B

been reported that the BM is closely associated with MM, its cell growth, survival and drug resistance (16). These findings provide preclinical evidence for the potential application of BM-derived mesenchymal stem cells (MSCs) as antitumor treatment in MM. The cellular compartment is composed of hematopoietic and non-hematopoietic cells, including fibroblasts, BMMSCs, endothelial cells, osteoclasts and osteoblasts (17). The non-cellular compartment is composed of the extra cellular matrix (ECM) and the liquid milieu, containing cytokines (IL-6, IL-1 β), growth factors and chemokines (17). MM cells can form masses around the BM and adhere to ECM proteins and BM stromal cells (BMSCs) (18). This relocation allows the progression and metastasis of MM to further BM locations (15).

Due to the increasing prices of imported drugs, patients in China cannot afford treatment, including bortezomib and lenalidomide. For example bortezomib costs 6,116 renminbi (RMB)/dose; every patient requires 4-6 courses and each course consisted of 4 doses. The total cost of bortezomib is >100,000 RMB. A total of 21 doses of lenalidomide cost 23,141 RMB for 25 mg doses and 18,186 RMB for 10 mg. Patients on lenalidomide need to administer the drug for 1-2 years. TPL, a traditional Chinese medicine that can be administered orally and intravenously, is inexpensive. Hence, the current study aimed to elucidate the potential application and effectiveness of TPL in the treatment of MM and to provide an experimental basis for clinical applications.

Materials and methods

BMMSCs. BMMSCs were obtained from 10 patients with MM (age range, 40-80 years; mean age, 63 \pm 9.7 years; 4 females and 6 males) recruited at the Tongde Hospital of Zhejiang Province (Hangzhou, China) between October 2014 and September 2015. The study was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province (Hangzhou, China) and patients provided written informed consent.

The following diagnostic criteria applied: i) Symptomatic MM: Presence of M protein in serum or urine; cloned plasma cell or plasma cell tumor in the BM; relevant organ or tissue damage, including anemia, hypercalcemia, renal damage and bone lesions. ii) Asymptomatic myeloma (smoldering myeloma); serum M protein reach myeloma level (>30 g/l); \geq 10% clonal plasma cells in the BM; no associated organ or tissue damage or MM-associated symptoms.

Inclusion criteria were defined as follows: i) Meet diagnostic criteria of MM; ii) onset of disease without remission or relapse; iii) aged 18-80 years; iv) Eastern Cooperative Oncology Group score <2 (19). Exclusion criteria were as follows: i) Perinatal women or mentally ill patients; ii) history of underlying liver or kidney disease; iii) other hematological malignancies.

The majority of patients with MM had M protein levels >30 g/l, IgA >25 g/l and 24-h urine light chains >1 g, some patients exhibited lower M protein levels. A previous study reported that the number of plasmacytoid plasma cells in the BM were >10% in 95% of patients (20). Clinical manifestations of associated target organ damage, including anemia, osteolytic lesions, hypercalcemia, renal insufficiency,

hyperviscosity syndrome, amyloidosis and recurrent infections, were further important diagnostic criteria in patients with MM.

Reagents and equipment. TLP and thalidomide were purchased from Sigma-Aldrich (purity, >98%; Merck KGaA, Darmstadt, Germany). Low-glucose Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Boehringer Ingelheim GmbH (Ingelheim, Germany) and the Cell Counting kit-8 (CCK-8) was from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Dimethyl sulfoxide (DMSO) was from Sangon Biotech Co., Ltd. (Shanghai, China), the Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit was supplied by Hangzhou MultiSciences (Lianke) Biotech Co., Ltd. (Hangzhou, China). Human BM mononuclear lymphocytes separating medium was obtained from Tianjin Haoyang Biological Products Technology Co., Ltd. (TBD2013LHU; Tianjin, China) and antibodies for CD34 phycoerythrin (PE; cat. no. A07776), CD45 PE (cat. no. IM3548; both Beckman Coulter, Inc., Brea, CA, USA) and CD105 PE (MHCD10504; Invitrogen; Thermo Fisher Scientific, Inc.) were. The RNA Extraction kit (antibody total RNA fast extraction kit) was from Generay Biotech Co., Ltd. (Shanghai, China), the reverse transcription (RT) PrimeScript™ RT Master mix was from Takara Biotechnology Co., Ltd. (lot no. RR036A; Dalian, China) and the quantitative polymerase chain reaction (qPCR) SuperRealPreMix color was from Tiangen Biotech Co., Ltd. (SYBR Green; lot no. FP215; Beijing, China). Polyvinylidene difluoride (PVDF) membranes were from Merck KGaA (0.45 μ m) and Bio-Rad Laboratories, Inc. (0.2 μ m; Hercules, CA, USA). Color pre-dyed molecular weight standard were provided by Fermentas (Thermo Fisher Scientific, Inc.), and the electrochemiluminescence (ECL) Plus luminescent kit, the SDS-PAGE loading buffer (5X), Western and IP cell lysis buffer, phenylmethylsulfonyl fluoride and the bicinchoninic acid (BCA) kit were obtained from Beyotime Institute of Biotechnology (Haimen, China). Primer sequences were as follows: IL-6, forward 5'-TAGTGAGGAACAAGCCAGAG-3' and reverse 3'-ATGCTACATTTGCCGAAGAG-5'; IL-1 β , forward 5'-TTACAGTGGCAATGAGGATG-3' and reverse 3'-TGTAGTGGTGGTCGGAGATT-5'; stem cell factor (SCF or Kit ligand), forward 5'-ATGGCTCCAGAAATGTCTAA-3' and reverse 3'-AATCCAATCACAGTTCCCAC-5'; and GAPDH forward, 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse, 3'-AGGGGCCATCCACAGTCTTC-5'. The following ELISA kits were used: VEGF [cat. no. EK1831; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.].

Separation, culturing and in vitro amplification of BMMSCs. Under aseptic conditions, BM from patients with MM (3-4 ml) was collected and placed into EDTA anticoagulant tubes or directly into chromosome culture bottles. To prepare single cell suspensions of the BM, BM cells were collected and mixed with 4-5 ml F solution (Tianjin Hao Yang Biological Products Technology Co., Ltd.), centrifuged for 20 min at 4°C and 500 x g. The supernatant was discarded, then the precipitate was washed twice with F solution and suspended with whole blood and tissue diluent containing 20% FBS, which

as also a part of the F solution kit. Separating medium (≥ 3 ml) was added to a 15-ml centrifuge tube, followed by the addition of an equal volume BM single cell suspension using a straw. The solution was centrifuged at 500 x g and 4°C for 30 min, and the annular milk and white lymphocytes, which formed the second layer from the top, were extracted and placed into a fresh 15-ml centrifuge tube. A total of 10 ml cleaning solution (cat. no. 2010X1118; Tianjin Hao Yang Biological Products Technology Co., Ltd.) was added and mixed with the cells. Following centrifugation at 250 x g and 4°C for 10 min, the supernatant was discarded. Cells were resuspended in 0.5 ml DMEM. Above steps were repeated 3 times. Following the final centrifugation step, cells were suspended with 0.5 ml DMEM. Resuspended cells were counted and seeded at 10^5 cells/cm in a counting plate, containing 10% FBS and low-glucose DMEM. Cells were incubated at 37°C in 5% CO₂ with saturated humidity. Following 24 h, ~25% of cells adhered onto the walls and 75-80% of cells adhered to the walls over the second and third days. Medium was replaced following 4 days. Following 1 week, ~80% cell densities were achieved and cell passage was performed using trypsin.

Morphological observation and flow cytometric analysis of BMMSCs. Second generation BMMSCs were digested using trypsin, collected by centrifugation at 800 x g and 4°C for 5 min and the cellular morphology was observed under an optical microscope at a magnification of x40 and x100. CD34 PE, CD45 PE and CD105 PE antibodies were added to the cells in the experimental group, and ISO IgG1 antibodies (cat. no. IM0670U; Beckman Coulter) were added to the control group at a density of at 1 μ g antibody/ 10^6 for 30 min at 4°C. Cells were incubated for 30 min at 4°C, detected by flow cytometry and analysed by BD Accuri™ C6 software (version 1.0.264.21; BD Biosciences, Franklin Lakes, NJ, USA).

Proliferation by CCK-8 assay. Second generation BMMSCs were digested with 0.25% trypsin, centrifuged at 800 x g and 4°C for 5 min, counted and seeded in 96-well plates at 5×10^3 cells/well. Cells were incubated standing still for 24 h at 37°C. Equal volumes of 10% FBS in low-glucose DMEM (control group) or TPL at 6.25, 12.5, 25, 50 and 100 ng/ml in 10% FBS in low-glucose DMEM were added to the cells. Six replicates were performed in each group. Cells were cultured for 12, 24 and 48 h, and harvested by centrifugation at 800 x g and 4°C for 5 min. The supernatant was discarded and 10 μ l CCK-8 in low-glucose DMEM (100 μ l) was added to each well; cells in DMEM and 10% FBS were prepared as blanks. Reactions were incubated for 2 h at 37°C in the dark and optical density (OD) values was recorded at 450 nm. Half maximal inhibitory concentration (IC₅₀) was calculated by SPSS software (version 17; SPSS, Inc., Chicago, IL, USA).

Apoptosis by flow cytometry. Second generation BMMSCs were digested with 0.25% trypsin, centrifuged at 800 x g and 4°C for 5 min, counted and seeded in 6-well plates at 1×10^5 cells/well. Cells were further incubated for 24 h at 37°C. Cells were incubated in 10% FBS in low-glucose DMEM with TPL at 25, 50 and 100 ng/ml or without TPL (the control group). Following 48 h, cells were collected by centrifugation

at 800 x g and 4°C for 5 min. Cells were washed twice with PBS and resuspended in binding buffer [cat. no. AP101-100-BB; 500 μ l; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.]. A total of 5 μ l Annexin V-FITC and 10 μ l PI were added prior to incubation for 5 min at 4°C in the dark. Samples were detected using flow cytometry. All the experiments were repeated 3 times. Samples were analysed by BD Accuri™ C6 software.

IL-6, IL-1 β and SCF mRNA expressions by RT-qPCR. TRIzol (cat. no. GK3016; Shanghai Generay Biotech Co., Ltd.) was used to extract total RNA from BMMSCs in the TPL group. RT of the total RNA was performed according to the manufacturer's instructions provided by the cDNA RT kit (42°C). The total volume of the qPCR mixture was 30 μ l, including RT product (1 μ l), MgCl₂ (1.5 mM), 1X PCR buffer, dNTPs (0.5 mM), up- and downstream primers (0.25 μ M) and Taq DNA synthetase (1 U). IL-6, IL-1 β and SCF reaction parameters were as follows: 94°C for 35 sec, followed by 40 cycles of 59°C for 45 sec and 72°C for 30 sec, with a final extension at 72°C for 10 min. The internal reference GAPDH. Products were directly used in electrophoresis or stored at 4°C. All the experiments were repeated thrice. Relative gene expression data were analysed using Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Inc.) and the 2^{- $\Delta\Delta$ Ct} method (21).

P65 protein expression by western blotting. BMMSCs were treated with TPL and thalidomide (both 0, 25, 50 and 100 ng/ml) for 48 h at 37°C. Cells were harvested by centrifugation at 800 x g and 4°C for 5 min and the supernatant of the control group, TPL treatment group or thalidomide treatment group was discarded. Cells were washed twice with PBS and lysis buffer and protease inhibitor were added. The resulting solution was mixed by shaking for 1 min and then placed on ice for 10 min. The mixing and incubation on ice were repeated thrice. Following centrifugation at 1,000 x g and 4°C for 5 min, the protein concentration in the supernatant was detected using a BCA assay. Proteins (40 μ g) were separated by SDS-PAGE on a 12% separation gel and 5% concentrated gel, and then transferred to the 0.45 μ m PVDF membranes. Membranes were incubated with 5% skimmed milk in TBST at room temperature for 2 h. Anti-P65 (cat. no. ab7970; Abcam, Cambridge, UK) and anti-GAPDH (AP0063; Bioworld Technology, Inc., St. Louis Park, MN, USA) primary antibodies were added and incubated overnight at 4°C. Subsequently, membranes were immersed in TBST and incubated with shaking 3 times for 15 min at 25°C. Horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (cat. no. bs12478; 1:5,000; Bioworld Technology, Inc.) were added and membranes were incubated with shaking for 2 h at room temperature. Membranes were immersed in TBST and incubated with shaking 3 times for 15 min at 25°C. ECL detection reagent was added according to the manufacturer's instructions. The grey value of each band was analyzed using ImageJ (version 1.4; National Institutes of Health, Bethesda, MD, USA), following exporting the image in TIFF format. All the experiments were repeated thrice.

ELISA analysis of the vascular endothelial growth factor (VEGF) content in the culture supernatant. Cells from the



Figure 1. Identification of BMMSCs from patients with multiple myeloma. (A) Morphology of BMMSCs determined using light microscopy (magnification, x40 and x100). BMMSCs grew with increased adherence and exhibited a long spindle shape. (B) Immunophenotyping of BMMSCs using flow cytometry. Cells were CD34-negative, weak CD45-positive and CD105-positive. BMMSC, bone marrow-derived mesenchymal stem cell.

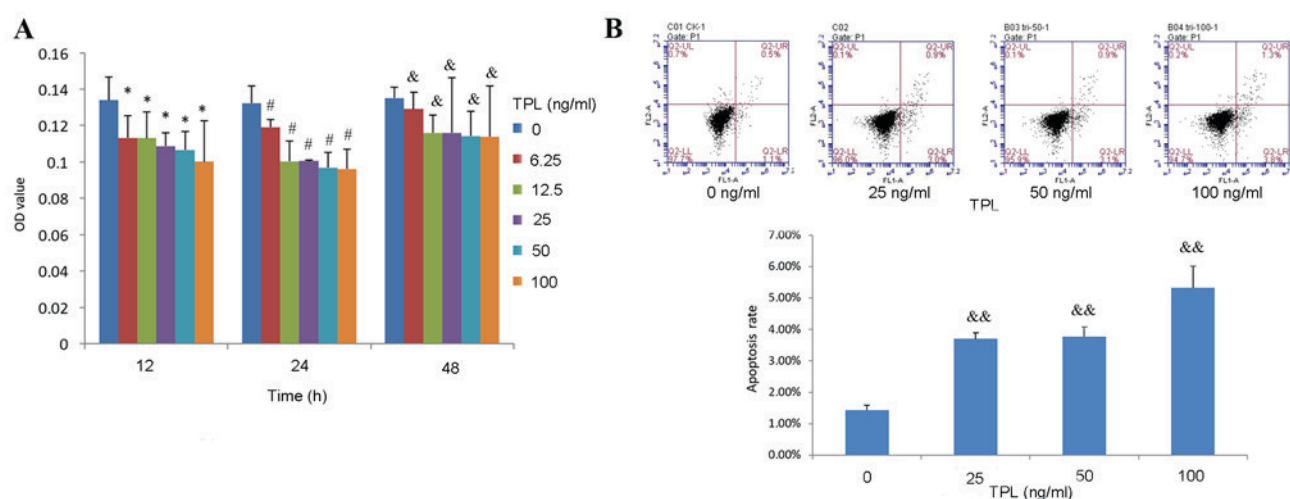


Figure 2. Effects of TPL on proliferation and apoptosis of BMMSCs from patients with multiple myeloma. (A) Effects of TPL (6.25, 12.5, 25, 50 and 100 ng/ml) on BMMSC proliferation determined by Cell Counting Kit-8 assay. (B) Effects of TPL (0, 25, 50 and 100 ng/ml) on apoptosis measured by flow cytometry. * $P < 0.05$ vs. untreated control at 12 h; # $P < 0.05$ untreated control at 24 h; & $P < 0.05$, && $P < 0.001$ vs. untreated control at 48 h. TPL, triptolide; BMMSC, bone marrow-derived mesenchymal stem cell; OD, optical density.

control, and 25, 50 and 100 ng/ml TPL groups were analysed. According to the manufacturer's instructions, cell culture supernatant was removed from -80°C and left to thaw at room temperature for 30 min. The samples of the cells and standards were added into each well in a 96-well plate, except the blank well (with only contained DMEM). Plates were sealed and incubated at 37°C for 120 min to react to the antibodies prior to washing (6 times). Except for the blank, TBST working solution (100 μl) was added, plates were sealed and incubated at 37°C for 45 min prior to washing (6 times). Then, except for the blank, enzyme binding working solution (100 μl) was added, plates were sealed and incubated at 37°C for 30 min prior to washing (6 times). Substrate color developing agent (100 μl) was added and plates were incubated at 37°C for 20 min. Termination reaction solution (100 μl) was added at 25°C for 30 min and OD values were determined at 490, 570 or 630 nm. All experiments were repeated thrice.

Statistical analysis. SPSS software was used to analyze the data. Data are expressed as mean \pm standard deviation. Univariate data was analyzed by one-way analysis of variance followed by the Least Significant Difference post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Separation and identification of BMMSCs in culture. BMMSCs are adherent cells and in the current study they were separated from the BM following the addition of lymphocyte separating medium and screening with low-glucose DMEM. Cells exhibited a long-spindle shaped morphology and flow cytometry results indicated a CD34-negative, CD45 weak positive and CD105-positive expression (Fig. 1). All results complied with the characteristics of BMMSCs, namely a uniform long shuttle shape (22).

Effects of TPL on BMMSC proliferation and apoptosis. In order to investigate proliferation and apoptosis in TPL-treated BMMSCs, CCK-8 assays and flow cytometry were performed at varying TPL concentrations (0, 25, 50 and 100 ng/ml) over time (12, 24 and 48 h). At 12, 24 and 48 h, the OD in TPL-treated BMMSCs (all concentrations) was significantly reduced compared with the untreated control sample ($F = 3.085$, $P = 0.023$; $F = 8.896$, $P < 0.001$; $F = 7.873$, $P < 0.001$, respectively; Fig. 2A). These results indicated that TPL inhibited the proliferation of BMMSCs compared with the untreated control. The IC_{50} of TPL at 48 h was 101.55 ± 2.45 ng/ml. Apoptosis in BMMSCs significantly increased in a concentration-dependent

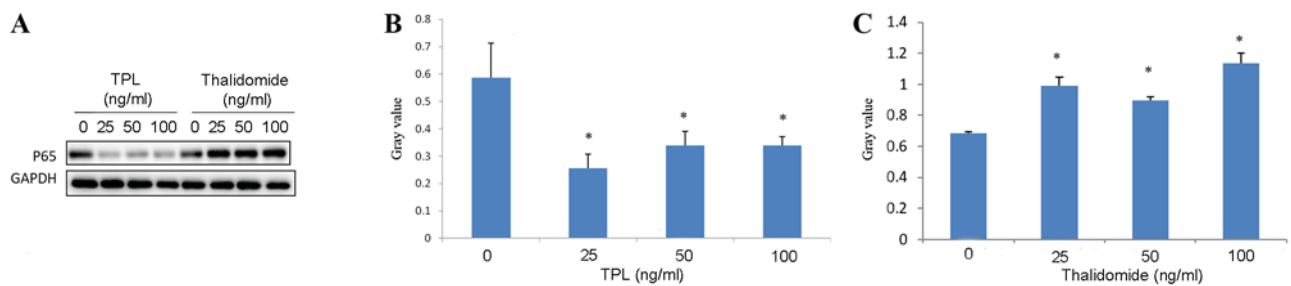


Figure 3. P65 protein expression in TPL- and thalidomide-treated BMMSCs. Western blotting (A) images and gray value analysis of P65 levels in (B) TPL- (0, 25, 50 and 100 ng/ml) and (C) thalidomide-treated (0, 25, 50 and 100 ng/ml) BMMSCs. *P<0.05 vs. untreated control. TPL, triptolide; BMMSC, bone marrow-derived mesenchymal stem cell; P65, transcription factor p65.

Table I. Effects of TPL on IL-6, IL-1 β and SCF mRNA expressions in bone marrow-derived mesenchymal stem cells.

Cytokine	TPL (ng/ml)			
	0	25	50	100
IL-6	1.00 \pm 0.02	0.11 \pm 0.02 ^a	0.06 \pm 0.01 ^a	0.03 \pm 0.00 ^a
IL-1 β	1.00 \pm 0.12	0.69 \pm 0.09 ^b	0.13 \pm 0.05 ^a	0.05 \pm 0.03 ^a
SCF	1.00 \pm 0.01	0.10 \pm 0.00 ^a	0.03 \pm 0.00 ^a	0.03 \pm 0.00 ^a

Data are presented as mean \pm standard deviation. ^aP<0.001 and ^bP<0.05 vs. untreated control. TPL, triptolide; IL, interleukin; SCF, stem cell factor or Kit ligand.

manner at 48 h, with rates of 1.43 \pm 0.15, 3.70 \pm 0.20, 3.77 \pm 0.32 and 5.33 \pm 0.68% for 0, 25, 50 and 100 ng/ml TPL, respectively (F=49.078, P<0.001; Fig. 2B). These results suggested that TPL may induce apoptosis in BMMSCs.

IL-6, IL-1 β and SCF mRNA expression by RT-qPCR. To investigate the effect of TPL on cytokines, RT-qPCR was performed to detect mRNA expression levels of IL-6, IL-1 β and SCF. BMMSCs were treated with TPL (0, 25, 50 and 100 ng/ml) for 48 h. Analysis revealed that IL-6, IL-1 β and SCF expression were significantly decreased with TPL treatment compared with the untreated controls in what appeared to be a dose-dependent manner (F=1433.475, P<0.001; F=90.057, P<0.001; F=11117.534, P<0.001; Table I). These findings suggested that TPL may decrease cytokine mRNA expression.

P65 protein expression analyzed by western blotting. To detect the effect of TPL and thalidomide on P65 protein levels, BMMSCs were treated with different TPL and thalidomide concentrations (0, 25, 50 and 100 ng/ml) for 48 h and western blots were generated. Results indicated that with increasing TPL, P65 expression decreased (F=7.489, P=0.010; Fig. 3). With increasing thalidomide, P65 expression markedly increased compared with the blank control (F=3.916, P=0.054; Fig. 3C). These results suggested that TPL downregulated P65 expression.

VEGF levels in the culture supernatant. To detect effects of TPL on VEGF levels, BMMSCs were treated with TPL

Table II. Effect of TPL on VEGF levels in the culture supernatant of bone marrow-derived mesenchymal stem cells.

TPL (ng/ml)	Vascular endothelial growth factor (pg/ml)
0	2,642 \pm 34
25	1,479 \pm 733 ^a
50	1,052 \pm 244 ^a
100	561 \pm 59 ^a

Data are presented as mean \pm standard deviation. ^aP<0.05 vs. untreated control. TPL, triptolide.

(0, 25, 50 and 100 ng/ml) for 48 h. VEGF levels significantly decreased with TPL treatment compared with the untreated controls in what appeared to be a dose-dependent manner (F=10.500, P=0.023; Table II). The lowest VEGF level (561 \pm 59 pg/ml) was measured at 100 ng/ml TPL.

Discussion

Recently, attention has focused on elucidating the mechanism of TPL in MM (23). A study evaluated several effects of TPL, ranging from antitumor mechanisms to inhibition of signaling pathway activation and regulation of histone expression (24). Few studies examined the effects of TPL on BMMSCs and the BM microenvironment (25,26). Hence, the current study investigated the effects of TPL on BMMSCs obtained from patients with MM. BM from healthy individuals was not included in this investigation and it solely focused on the analysis of a tumor microenvironment. Effects of TPL at varying concentrations were compared with an untreated control group; however, no comparison to a healthy control was made, due to the absence of volunteers. BMMSCs constitute to the microenvironment of hematopoietic cell differentiation and development, and regulate hematopoiesis by expressing soluble cytokines and the ECM (16). The microenvironment serves an important role in differentiation and proliferation of normal bone tissue and plasma cells in the BM, including regulation of MM cell proliferation.

During the process of cell proliferation, cyclin B promotes the G2 to M transition and cyclin A and D promote the G1 to S transition (27). TPL downregulates cyclin A, B, C and D expression, leading to an obstruction of the cell

cycle and inhibition of cell proliferation (28). It has been demonstrated previously that TPL upregulates p27 and p21 expression and downregulates CDC25A and cyclin A, delaying progression from the S phase (10). In the present study, TPL demonstrated inhibitory effects on BMMSC proliferation. An IC_{50} value of 101.55 ± 2.45 ng/ml was determined. Furthermore, TPL stalled BMMSCs in the S phase and induced apoptosis. A dose-dependency was determined with 1.43 ± 0.15 , 3.70 ± 0.20 , 3.77 ± 0.32 and $5.33 \pm 0.68\%$ apoptosis at 0, 25, 50 and 100 ng/ml TPL, respectively. As the measured apoptotic rates were low, further experiments with higher TPL doses should be performed to validate these observations. The exact mechanism by which TPL affects the cell cycle of BMMSCs requires further verification.

NF- κ B, a polyphenic and multifunctional transcription factor, is present in the cytoplasm and is associated with the regulation of cell proliferation and apoptosis (29,30). It has been reported that NF- κ B and inhibitor (I) κ B proteins exhibited irregular expressions in various tumors and that NF- κ B is activated in various tumor cells (31,32). Furthermore, NF- κ B serves an important role in inhibiting tumor cell apoptosis, promoting tumor cell proliferation and tumor metastasis (31). There are various mechanisms by which NF- κ B promotes tumorigenesis (33): i) Inducing anti-apoptotic genes, including B-cell lymphoma-XL, X-linked inhibitor of apoptosis protein and cellular inhibitor of apoptosis 1 and 2; ii) metastasis regulation, including cell adhesion and migration through intercellular adhesion molecule 1, vascular cell adhesion protein 1, endothelial-leukocyte adhesion molecule 1, **matrix metalloproteinase-9 and urokinase-type plasminogen activator** regulation; iii) angiogenesis regulation via VEGF, IL-1, IL-8 and tumor necrosis factor; iv) proliferation induction via oncogenes, including cyclin D1, c-myc; v) induction of telomerase for unlimited proliferation; vi) inflammation regulation via cyclooxygenase 2 and inducible nitric oxide synthase expression; vii) apoptosis induction via pro-apoptotic genes, including Fas and death receptor 4/5; viii) tumor suppressor gene activation and induction of apoptosis via protein 53 in **human immunodeficiency virus**; ix) anti-proliferation through c-Jun N-terminal kinase-inhibited proliferation, induced by cyclin-dependent kinase inhibitor p21(WAF1/CIP1) **expression**; x) **transformation inhibition**.

Thalidomide is a clinical medication, but TPL is not used as a clinical hematological drug, so thalidomide was used as a positive control in the current study and, as TPL and thalidomide work ultimately through protein expression, we choose the western blot. In the current study, P65 expression in BMMSCs treated with TPL was decreased compared with the untreated control and the thalidomide group. As a result, it is suggested TPL affected BMMSCs through NF- κ B as P65 is in the NF- κ B signaling pathway. In response, inhibition of NF- κ B expression may decrease the release of various above-mentioned inflammatory factors.

In the present study, TPL inhibited the expression levels of cytokines, including IL-6, IL-1 β , SCF and VEGF, which are associated with the constitution of the BM microenvironment (17). The biological behavior of MM primary tumor cells and the clinical outcomes partially depend on the genome and epigenetic abnormality of the MM cells (34). The BM

microenvironment serves a key role in the pathogenesis and development of MM (35-39). MM cells establish complicated interactions with other cellular constituents within the BM microenvironment. Consequences of these interactions are outlined in the following: MM cells primarily interact with BMMSCs and osteoclasts, leading to cell signaling pathway activation, including phosphatidylinositol 3 kinase/protein kinase B, Janus kinase/signal transduction and activating transcription factor 3, RAS/RAF/mitogen-activated protein kinase/extracellular signal-regulated kinase and NF- κ B; and MM cells promote proliferation, survival, metastasis and drug resistance (34,39,40). In addition, MM cells disturb the equilibrium of the BM environment, leading to anemia, immunosuppression and uncoupling (41). The bone remodeling process results in the development of lytic bone lesions, which are characteristic in the development of MM (41). Results of the present study demonstrated that following TPL treatment, IL-6, IL-1 β and SCF mRNA and VEGF protein levels in BMMSCs were significantly decreased compared with the untreated control. The BM microenvironment was improved to regulate the proliferation of MM cells by decreasing IL-6, IL-1 β and SCF mRNA expression (14). Due to a close association of IL-6 and VEGF expression with NF- κ B, decreased levels of IL-6 and VEGF may be associated with the TPL-mediated inhibition of NF- κ B signal transduction. These findings were in accordance with previous results (42).

In conclusion, the current study suggested that in addition to the direct induction of BMMSC apoptosis, TPL indirectly affected proliferation of MM cells by inhibiting IL-6, IL-1 β and SCF expression, and VEGF secretion. TPL further possessed a potential anti-MM effect, which may inspire future treatments for MM.

Acknowledgements

Not applicable.

Funding

The current study was supported by the Zhejiang Provincial Medical and Health Science and Technology Plan (grant no. KYA028), the Zhejiang Traditional Chinese Medicine Science Research Fund (grant no. 2015ZA021), and Zhejiang Provincial Department of Science and Technology (Zhejiang Provincial Natural Science Foundation; grant no. QY19H080001).

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

HW, YW and LR performed the experiments and analyzed the data. HW was a major contribution in writing the manuscript. WZ, YJ and SG screened and examined patients, and obtained the ethical approval. DT, CS, ZC analyzed and interpreted the

data. HJ designed experiments and guided the experimental process. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province (Hangzhou, China) and patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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