Triptolide decreases expression of latency-associated nuclear antigen 1 and reduces viral titers in Kaposi's sarcoma-associated and herpesvirus-related primary effusion lymphoma cells

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Abstract. Kaposi's sarcoma-associated herpesvirus (KSHV) can establish a life-long persistence in the host after primary infection and is associated with certain malignancies, which are resistant to conventional chemotherapeutic agents with a poor prognosis. Latency-associated nuclear antigen 1 (LANA1) encoded by KSHV is essential for segregation, replication and maintenance of viral genome. In addition, LANA1 upregulates the transcriptional activity of signal transducer and activator of transcription 3 (STAT3), which plays an important role in promoting survival of KSHV-associated primary effusion lymphoma (PEL) cells. Furthermore, LANA1 mediates transcriptional modulation of KSHV and host genome in host cells. In the present study, the antitumor effect of triptolide was assessed. CCK-8 assays were performed to demonstrate that the proliferations of PEL cells were efficiently inhibited by triptolide in a dose- and time-dependent manner. Flow cytometric results indicated that triptolide induced cell cycle arrest and apoptosis. Western blot results suggested that triptolide downregulated LANA1 expression and reduced half-life of LANA1 in the KSHV-infected malignant cells. Viral titer experiments indicated that triptolide treatment impaired the number of viral DNA copies and the production of virions in BCBL-1 cells. Triptolide also suppressed STAT3 activity and inhibited secretion of IL-6 in PEL cells. In a mouse xenograft model of primary effusion lymphoma by BCBL-1 cells, triptolide treatment significantly inhibited ascites formation and diffused organ infiltration. These results indicate that triptolide impairs the expression of LANA1 and shows antitumor activity against PEL *in vitro* and *in vivo*. Triptolide may be a potential agent for treatment of PEL.

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Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), a gammaherpesvirus, is associated with Kaposi's sarcoma (KS) and several lymphoproliferative diseases, including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (1). PEL, which was originally identified in patients with advanced AIDS, belongs to a variant of non-Hodgkin's B lymphoma showing serious lymphomatous effusion in body cavities. Although treated with cytotoxic chemotherapeutic agents, prognosis of patients is extremely poor upon diagnosis, with a median survival times of 3-6 months (2). Similar to the other herpesviruses, KSHV infection can be divided into latent and lytic stages and can establish a life-long persistence in the host after primary infection (3). During the latent infection stage, only a limited set of genes are expressed, including open reading frame *(Orf) 71*, *Orf 72*, *Orf 73*, viral-encoded microRNAs. After reactivating and entering lytic stage, most viral genes are expressed in an orderly fashion based on the time of expression, defined as immediate-early, early and late genes, which facilitate genomic DNA replication and mature virion production (4,5).

Currently, candidate drugs for management of KSHV-related diseases can be divided in two groups, which include compounds targeting viral DNA polymerase or not. DNA polymerase inhibitors are mainly involved in nucleoside analogs, acyclic nucleoside phosphonates, pyrophosphate analogs and non-nucleoside analogs. Non-DNA polymerase inhibitors, which target viral or cellular proteins, include compounds isolated from plants (6). For example, resveratrol, a non-flavonoid polyphenol derived from *Polygonum cuspidatum*, was demonstrated to decrease ERK1/2 activity and expression of Egr-1 in KSHV-infected cells, resulting in inhibition of KSHV reactivation from latency (7). Additionally, DD2, a benzyl-substituted 4-(pyridine-2-amido) benzoic acid, trapped an inactive monomeric conformation, resulting in disruption of KSHV proteasomal dimerization (8). Despite the fact that various antiviral agents are known to inhibit KSHV replica-

tion, studies on these pre-drugs, mainly *in vitro*, are limited due to toxic effect or incomplete efficiency. Moreover, these antiviral agents can not eliminate persistent latent KSHV. To date, there are no standard therapeutic guidelines for the treatment of KSHV-infected diseases, and little progress has been made since the discovery of KSHV 20 years ago.

Latency-associated nuclear antigen 1 (LANA1), encoded by KSHV *Orf 73*, is a viral oncoprotein that contributes to tumorigenesis (9). LANA1 is a protein expressed in all KSHV-infected cells and is required for viral genomic establishment and maintenance in the nucleus (10). As a master regulator of KSHV latency, LANA1 can also be considered as a transcriptional modulator for host cell genes, resulting in dysregulation of several pathways (11). For example, LANA1 can regulate the Wnt pathway by nuclear trapping of glycogen synthase kinase 3β (GSK3β), resulting in stabilizing of β-catenin (12). LANA1 is essential for KSHV-induced angiogenesis and can activate phosphorylation of survivin, which contributes to cell proliferation and viral latent replication (13,14). LANA1 has also been shown to enhance transactivation of human telomerase reverse transcriptase (hTERT) promoter by interacting with transcription factor specificity protein 1 (Sp1), therefore, contributing to immortalization of PEL cells (15). Additionally, LANA1 targets degradation of the mitotic checkpoint kinase Bub1, resulting in chromosomal instability, and facilitates establishment of viral latency through interacting with Krüppel-associated box domain associated protein-1 (KAP1) (16,17). LANA1 also interacts with tumor suppressors, such as p53 and Rb proteins, leading to disruption of their tumor-suppressive functions (18,19). Moreover, LANA1 enhances transcriptional activity of signal transducer and activator of transcription 3 (STAT3) via physical and functional interaction with STAT3 (20). Collectively, the biological insights into LANA1 make it a promising rational target for therapeutic strategies.

Triptolide, a diterpenoid triepoxide, is purified from the roots of Chinese herb *Tripterygium wilfordii* (21). Triptolide displays a broad-spectrum bioactivity profile, including antitumor, anti-inflammatory, immunosuppressive and antifertility effects. It has been used for treatment of autoimmune diseases such as rheumatoid arthritis (22). In addition, triptolide has been reported to inhibit proliferation and induce apoptosis against a variety of tumor cell lines *in vitro*, including cholangiocarcinoma, melanoma, breast, gastric cancer and non-small cell lung carcinoma (23). Triptolide can also induce pancreatic cancer cell death via inhibition of Hsp70 and O-GlcNAc modification of transcription factor Sp1 (24,25). Currently, triptolide has entered clinical trials for treatment of diseases including psoriasis vulgaris, diabetic nephropathy and nephritic syndrome. Minnelide, a water-soluble pre-drug of triptolide, has also entered clinical trials for treatment of pancreatic cancer showing good patient toleration profile (26). However, little is known about effect of triptolide on KSHV-associated PEL.

Previously, our laboratory found that Hsp90 inhibitors inhibit outgrowth of Epstein-Barr virus (EBV, another gammaherpesvirus closely related to KSHV)-infected malignant cells via downregulation of EBV nuclear antigen 1 (EBNA1) (27). A small molecule pre-drug 17-DMAG (an Hsp90 inhibitor) also decreases expression of conserved herpesvirus protein kinases in EBV-infected malignant cells (28). Recently, we found that triptolide inhibited proliferation of latency III type infected EBV-positive B lymphocytes through the virus latent membrane protein 1 (LMP1)-dependent mechanism (29). In the present study, our results demonstrate that triptolide can effectively inhibit cell proliferation and induce cell apoptosis in the PEL cells. Furthermore, triptolide specifically decreases LANA1 expression without reducing the LANA1 transcript level. LANA1 accumulates after treatment with proteasomal inhibitor MG-132 in the absence or presence of triptolide. It is also indicated that triptolide reduces the half-life of LANA1. In addition, the number of viral DNA copies and the production of virions are reduced by triptolide. Moreover, triptolide inhibited expression of the phosphorylated STAT3 and secretion of IL-6. Finally, we also show that the KSHV-induced lymphoproliferative diseases in non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice are strongly inhibited by a non-toxic dose of triptolide.

Materials and methods

Cell lines and reagents. PEL cells (BCBL-1, JSC-1 and BC-3) and a Burkitt lymphoma cell line, BJAB, were gifts kindly provided by Professor K. Lan (Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China). P3HR-1 cells (EBV-positive and KSHV-negative) were kindly provided by Professor Y. Cao (Central South University, Changsha, China). Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by using Ficoll-Paque Premium (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Human renal embryonic 293T cells and HeLa cells, which were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), were obtained from Professor H. Li (Wuhan University, Wuhan, China).

BJAB, P3HR-1, BCBL-1, BC-3, JSC-1 cells and PBMCs were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA). HeLa and 293T cells were cultured in DMEM containing 10% FBS. The whole cell lines were cultured at 37˚C with 5% CO_2 and 100% humidity.

Triptolide (Sigma, St. Louis, MO, USA), 12-O-Tetradecanoylphorbol-13-Acetate (TPA; Sigma), cidofovir (CDV; Selleck Chemicals, Shanghai, China), cycloheximide (CHX; Sigma), and MG-132 (Calbiochem, Billerica, MA, USA) were dissolved in dimethylsulfoxide (DMSO). Sodium butyrate (NaB; Sigma) was dissolved in PBS.

Plasmids. Plasmid DNA was purified through columns (Axygen Scientific Inc., Union City, CA, USA) as described by the manufacturer. The plasmid pSG5-LANA1 was constructed by inserting the full LANA1 sequence into the *Mfe*I/*Eco*RI of pSG5 vector (YouBio, Shanghai, China).

Cell viability assay. The viability of cells was determined by the Cell Counting kit-8 assay (CCK-8; Dojindo Laboratories, Kumamoto, Japan). In brief, cells were seeded in 96-well plates at a density of $1x10^4$/100 μl culture medium. All the cells were treated with vehicle control (DMSO; 0.006%, vol/vol) or increasing concentrations of triptolide for 24, 48 and 72 h.

After different treatments, 10 μl of tetrazolium substrate was added into each well of the plate. The plates protected from light were incubated at 37˚C for 1 h. The optical density (OD) was determined at an absorbance of 450 nm using an ELx800 microimmunoanalyser (BioTek Instruments, Inc., Winooski, VT, USA).

Cell transfection. For transfection, KSHV-negative HeLa cells were transiently transfected using X-tremeGENE HP DNA Transfection reagent (Roche, Basel, Switzerland) with pSG5 (the empty vector) or pSG5-LANA1 according to the manufacturer's protocol. At 4 h post-transfection, cells were treated with vehicle control (DMSO; 0.006%, vol/vol) or triptolide for further 44 h before cell harvesting. In some experiments, cells were treated in the absence or presence of MG-132 (10 μM) or CHX (25 μg/ml).

Immunoblot analysis. Briefly, cells treated in different conditions were collected and washed with ice-cold PBS twice. Whole cell lysates were prepared in RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with 0.5% cocktail protease inhibitor (Roche) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates harvested were sonicated for 15 sec, followed by centrifugation at 12,000 x g for 10 min. The supernatants were collected and transferred into new tubes. Protein concentrations were measured by the bicinchoninic acid method using bovine serum albumin as a standard. Based on the measurement of protein concentrations of all samples in the same experiment, the loading volumes of samples were accordingly adjusted. Therefore, equal amounts of proteins mixed with 5X loading buffer [250 nM Tris-Hcl (pH 6.8), 10% SDS, 0.5% BPB, 50% glycerol, 5% β-mercaptoethanol] were subjected to 10% SDS-PAGE gels. The gels were run at 100 V, followed by transfer to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skim milk in TBST, followed by incubation with primary antibodies overnight at 4˚C. After 3x5-min washes in TBST, the membranes were incubated with appropriate horseradish-peroxidase-conjugated secondary antibodies for 1 h. The primary antibodies used were as follows: GAPDH (cat. no. 10494-1-AP, 1:5,000; Proteintech, Wuhan, China), β-actin (cat. no. A5441, 1:5,000; Sigma), LANA-1 (cat. no. 13-210-100, 1:4,000; Abionline, Cambridge, UK), vIL-6 (cat no. 13-214-050, 1:1,000; Abionline), v-Cyclin (cat no. F105P, 1:1,000; Exalpha Biological, Inc., Boston, MA, USA), STAT3 (cat no. 9139, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), phospho-STAT3 (cat. no. 4113, 1:1,000; Cell Signaling Technology) caspase-3 (cat. no. 9668, 1:1,000; Cell Signaling Technology), cleaved caspase-3 (cat. no. 9664, 1:1,000; Cell Signaling Technology), RTA (cat. no. 251345, 1:500; Abbiotec LLC, San Diego, CA, USA). A rabbit polyclonal antibody against v-Flip was raised in our laboratory. The secondary antibodies were horseradish-peroxidase-conjugated secondary anti-mouse IgG (1:10,000; Wuhan Kerui Technology Co., Ltd., Wuhan, China), anti-rabbit IgG (1:10,000; Wuhan Kerui Technology), or anti-rat IgG (1:5,000; Wuhan Kerui Technology). Antibody-bound proteins were detected using the ECL system (Bio-Rad Laboratories). Image quantifications were performed using ImageJ software.

RNA extraction, cDNA preparation and quantitative real-time PCR. PEL cells treated with vehicle control (DMSO; 0.006%, vol/vol) or triptolide for 24 and 48 h were harvested and the total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration of total RNA was determined by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), and all isolated RNA samples (A260/A280 ratio >1.8) were stored at -80˚C. Total RNA (1 μg) was reversely transcribed using a PrimeScript RT reagent kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Tokyo, Japan). Genomic DNA elimination reactions were performed in a volume of 10 μl containing 2 μl of 5X gDNA Eraser buffer, 1 μl of gDNA Eraser, and 7 μl of RNase-free dH_2O and the total RNA (1 μg), followed by storage at room temperature for 30 min. The reverse-transcription reaction mixture (20 μl) included 4 μl of 5X PrimeScript buffer 2, 1 μl of PrimeScript RT Enzyme Mix I, 1 μl of RT Primer Mixture containing oligo dT primer and random 6 mers, 4 μl of RNase-free dH_2O, and 10 μl of genomic DNA elimination reaction solution as mentioned above. The condition for reverse-transcription reaction was 15 min at 37˚C and 5 sec at 85˚C. Expression of viral LANA1 mRNAs was quantified by quantitative PCR using a SYBR-Green PCR kit (cat no. RR420A; Takara Bio). Quantitative PCR reaction mixtures (20 μl) included 1X SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.4 μM each primer and 1 μl cDNA reaction mixture (equivalent to 50 ng of input RNA). Reaction was performed by a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) and included an initial 30 sec denaturation step at 95˚C, followed by 40 cycles of 10 sec at 95˚C, 10 sec at 60˚C and 15 sec at 72˚C. Melting curve analysis was performed between 65 and 95˚C (with 0.5˚C increments) to verify amplicon specificity. The average cycle threshold (CT) was determined by three independent samples. Template-negative (quantitative PCR reaction mixtures without cDNA) and RT-negative (RNA after genomic DNA elimination) conditions were used as controls. The relative amounts of LANA1 transcript were normalized to the housekeeping gene GAPDH and quantitative PCR data for the relative quantification were calculated with the $^{\Delta\Delta}$Ct method. The level of LANA1 transcript in cells treated with vehicle control (DMSO; 0.006%, vol/vol) was set as 1. The primers were as follows: LANA1 forward, 5'-CGAGAGGAAGTTGTAGGAAACG-3' and LANA1 reverse, 5'-CTTCCAGGTATAGGCAAGGTG-3' (30); GAPDH forward, 5'-ACATCGCTCAGACACCATG-3' and GAPDH reverse, 5'-TGTAGTTGAGGTCAATGAAGGG-3' (30). All experiments were repeated from three independent cultures.

Flow cytometric analysis. PEL cells were seeded in 6-well plates and treated with vehicle control (DMSO; 0.006%, vol/vol) or triptolide (50 or 100 nM) for 24 h, respectively. For apoptosis analysis, PEL cells were evaluated with Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Multisciences, Shanghai, China). Briefly, $2x10^5$ cells were washed 3 times with PBS and resuspended in 500 μl of 1X binding buffer, followed by adding 5 μl of Annexin V-FIFC and 10 μl of PI. The whole cells were analyzed immediately by a Beckman-Coulter system (EPICS Altra II; Beckman-Coulter, Fullerton, CA, USA).

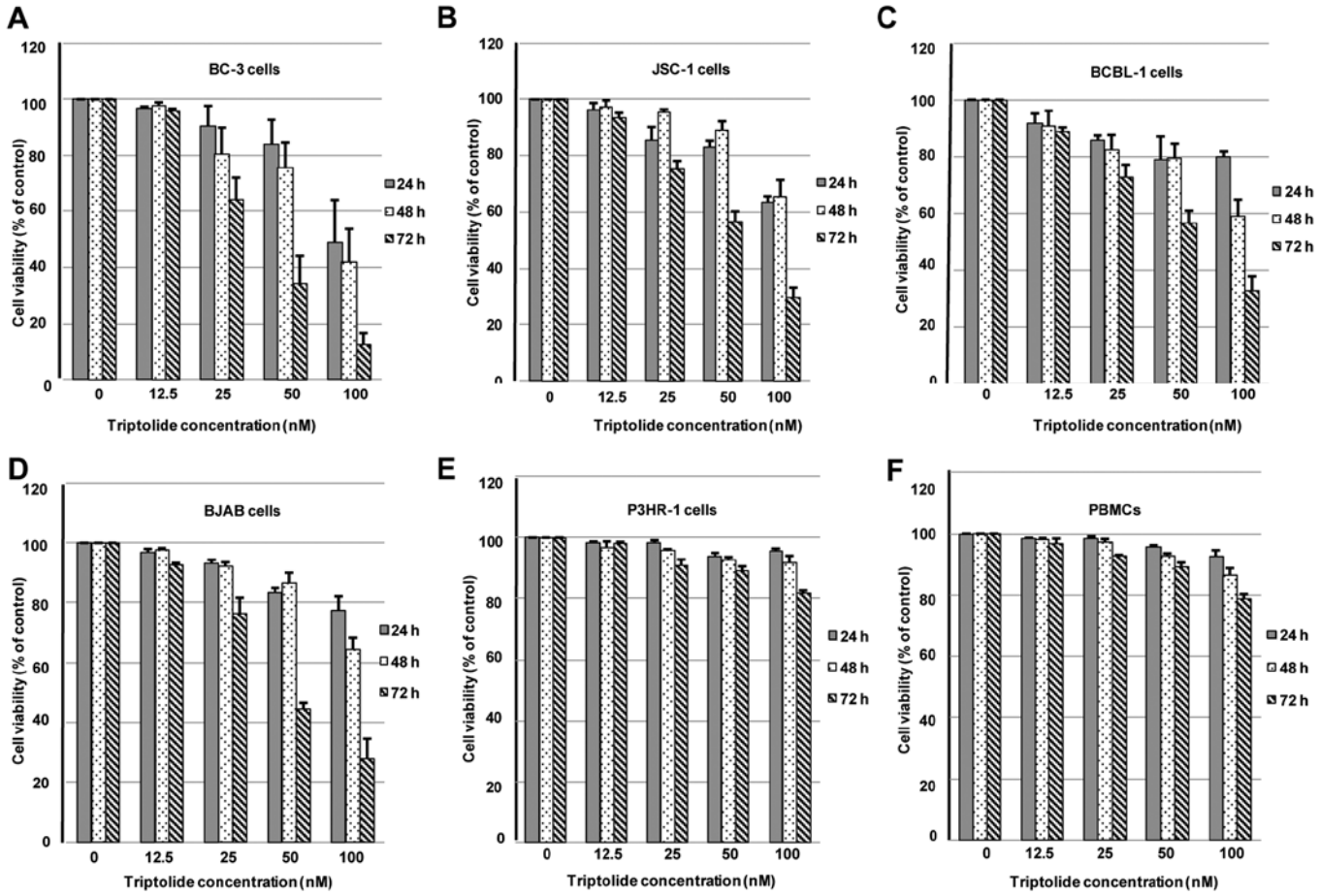

Figure 1. Triptolide inhibits cell proliferation *in vitro*. The effects of triptolide on cell viability. (A) BC-3, (B) JSC-1, (C) BCBL-1, (D) BJAB, (E) P3HR-1 cells, and (F) PBMCs were treated with vehicle control or a series of increasing concentrations of triptolide for 24, 48 and 72 h, respectively. The cell viabilities were measured by CCK-8 assay. The values shown are mean ± SD of 3 independent experiments carried out in triplicate.

Progeny virus analysis. TPA (20 ng/ml) and NaB (1.2 mM) were used to induce BCBL-1 cells entering lytic replication. At 3 h post-induction, cell medium was replaced and cultured in the absence or presence of triptolide for further 48 h. KSHV virion-associated DNA derived from the supernatants was collected according to the method previously described (31). Equal amounts of the viral lysate were used for the quantitative real-time PCR assay of the KSHV specific region, *Orf K9*. The primer pair for *Orf K9* was: *Orf K9* forward, 5'-GTCTCTGCGCCATTCAAAAC-3' and *Orf K9* reverse, 5'-CCGGACACGACAACTAAGAA-3'. Intracellular viral DNA in BCBL-1 cells was extracted with Phenol-Chloroform method according to the manufacturer's instructions. The copy numbers of KSHV genome were evaluated by a quantitative real-time PCR assay.

Enzyme-linked immunosorbent assay (ELISA). PEL cells were treated with or without 100 nM triptolide for 24, 48, and 72 h, respectively. Cell supernatants were collected and subjected to ELISA. IL-6 level of the cell supernatants was measured using human IL-6 ELISA kit (Multisciences) according to the manufacturer's instructions.

In vivo xenograft model. The animal protocol used in the present study was approved by the Medical Ethics Committee of Wuhan University. NOD/SCID mice were maintained under specific pathogen-free conditions in the Animal Experiment Center of Wuhan University, Animal Biosafety Level-III Laboratory.

BCBL-1 cells were inoculated intraperitoneally (i.p.) into the flank of 4-week-old female NOD/SCID mice (purchased from Beijing HFK Bioscience, Co., Ltd., Beijing, China). Twelve mice were injected with BCBL-1 cells at $1x10^7$ per mouse on day 0. Three days post-injection, the mice were randomly divided into vehicle and treatment groups (six mice per group), and daily i.p. injection of triptolide (0.4 mg/kg) or PBS was performed. After 21 days of treatment, all the mice were sacrificed by cervical dislocation. Tumor burden was evaluated by measuring body weight and volume of ascites. Tissue samples were collected and fixed in 10% neutral buffered formalin, embedded and sectioned at 5 μm. Hematoxylin and eosin staining for histological analysis and immunohistochemistry analysis were performed as previously described (32).

Statistical analysis. Data were presented as the mean ± standard deviation (mean ± SD) of three separate experiments. The statistical significance of the difference was determined by the Student's t-test, and P-values <0.05 were considered to indicate a statistically significant result.

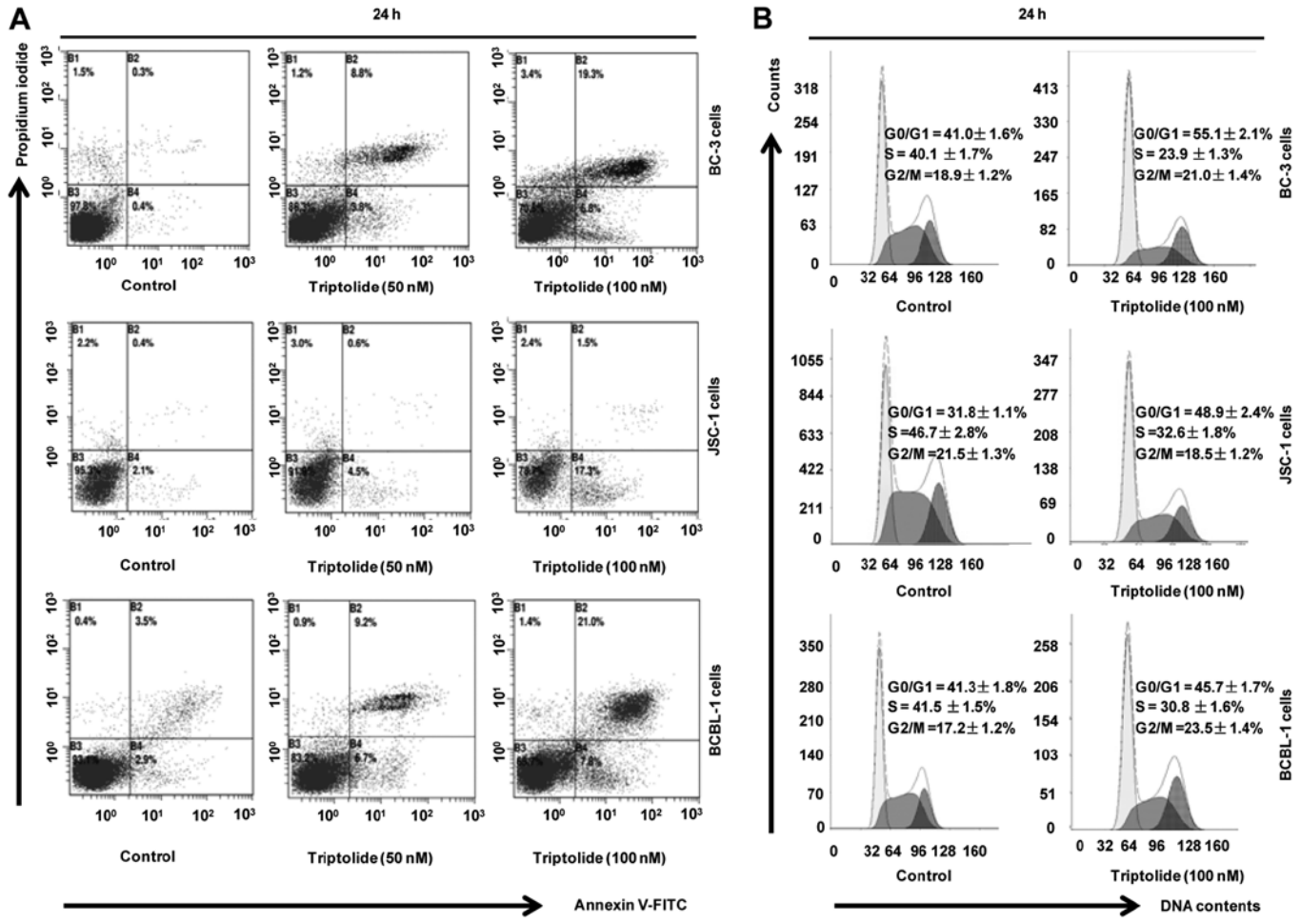

Figure 2. Triptolide induces cell cycle arrest and apoptosis in the KSHV-associated PEL cells. (A) The effects of triptolide on apoptosis. BC-3, JSC-1 and BCBL-1 cells were treated with vehicle control or indicated triptolide for 24 h, followed by straining with Annexin V-FITC/PI. Apoptotic cells were assessed by flow cytometry. (B) The effects of triptolide on cell cycle. BC-3, JSC-1 and BCBL-1 cells were treated with vehicle control or indicated triptolide for 24 h, respectively, followed by straining with PI. Cell cycle analysis was performed using flow cytometry.

Results

Triptolide inhibits proliferation of KSHV-associated PEL cells. To determine whether triptolide specifically targets cell viability in the KSHV-associated PEL cells, BC-3, BCBL-1 and JSC-1 cells, together with KSHV-negative BJAB cells were treated with vehicle control or a series of increasing concentrations of triptolide for 24, 48 and 72 h, respectively. Cell viability was determined by CCK-8 assays. Triptolide inhibited cell viability of BC-3 cells in a dose- and time-dependent manner, with 50% inhibitory concentration (IC_{50}) values of 108.3, 89.5 and 46.4 nM by a 24-, 48- and 72-h triptolide treatment, respectively (Fig. 1A). Similar experiments were conducted in the other two PEL cells. The IC_{50} values were calculated as 143.3, 128.3 and 67.9 nM in JSC-1 cells and 153.9, 119.6 and 69.1 nM in BCBL-1 cells by 24-, 48- and 72-h triptolide treatment, respectively (Fig. 1B and C). In addition, triptolide inhibited cell proliferation to a similar degree in BJAB cells, and the IC_{50} values were calculated as 146.6, 115.6 and 63.8 nM by 24-, 48- and 72-h triptolide treatment, respectively (Fig. 1D).

To further evaluate the antiproliferative effects of triptolide on KSHV-uninfected B cell line, P3HR-1 cells and PBMCs isolated from healthy donors were treated with vehicle control or a series of increasing concentrations of triptolide for 24, 48 and 72 h, respectively. Our previous results indicated that 100-nM triptolide treatment for 4 days decreased cell viability by <20% in P3HR-1 cells (28). As shown in Fig. 1E, a series of increasing concentrations of triptolide treatment for 24, 48 and 72 h showed slight proliferation inhibition in P3HR-1 cells. Similar results were also found in PBMCs treated by a series of increasing concentrations of triptolide (Fig. 1F). These results suggested that triptolide efficiently inhibited cell viability in PEL cells, but not significantly in PBMCs and P3HR-1 cells.

Triptolide induces cell cycle arrest and apoptosis in the KSHV-associated PEL cells. To examine the effect of triptolide on induction of cell cycle arrest and apoptosis, BC-3, JSC-1 and BCBL-1 cells were treated with vehicle control or indicated triptolide for 24 h. Cell cycle arrest and apoptosis were analyzed by flow cytometry. As shown in Fig. 2A, treatment of PEL cells with triptolide resulted in apoptosis in a dose-dependent manner, which was confirmed by staining with Annexin V-FITC/PI. In addition, treatment with 100 nM triptolide for 24 h could induce cell apoptosis in BJAB cells,

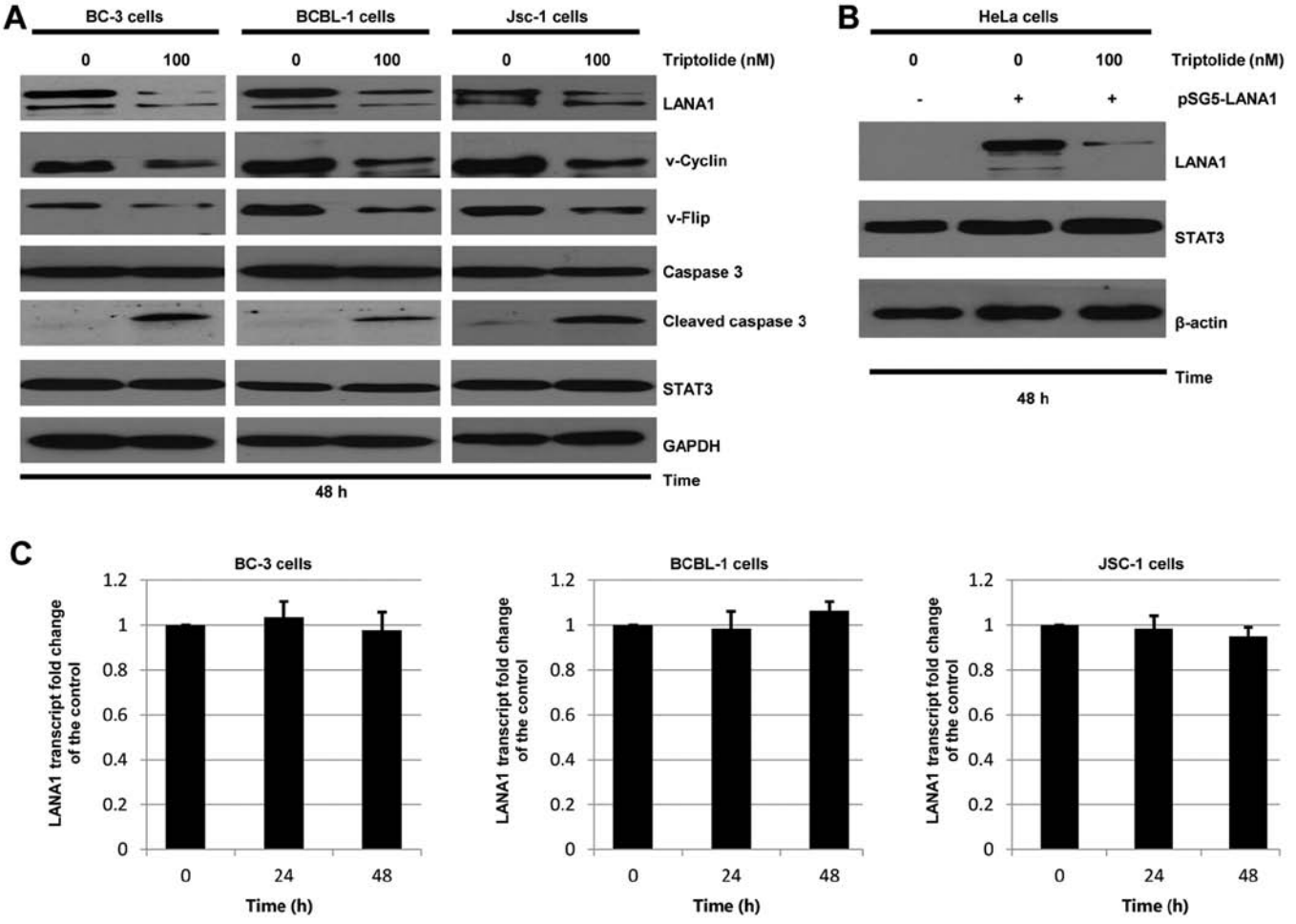

Figure 3. Triptolide downregulates LANA1 expression. (A and B) LANA1 degradation induced by triptolide. (A) BC-3, BCBL-1 and JSC-1 cells were treated with vehicle control or triptolide (100 nM) for 48 h, respectively. (B) HeLa cells were transfected with empty vector (pSG5) or pSG5-LANA1 as indicated, followed by a 44-h treatment with 100 nM triptolide beginning at 4 h after transfection. Whole-cell extracts were prepared and western blotting was performed to analyze expression of LANA1 (20 μg), v-Cyclin (20 μg), v-Flip (20 μg), caspase-3 (10 μg), cleaved caspase-3 (20 μg), STAT3 (10 μg), β-actin (5 μg) or GAPDH (5 μg). (C) BC-3, BCBL-1 and JSC-1 cells were treated with vehicle control or triptolide (100 nM) for 24 and 48 h. The levels of LANA1 transcript were determined by qRT-PCR. The level of LANA1 transcript in cells treated with vehicle control is set as 1.

while not in P3HR-1 cells (data not shown). For cell cycle analysis, treatment of BC-3 and JSC-1 cells with triptolide resulted in G0/G1 arrest as observed by a marked increase in the number of cells in the G1 phase (Fig. 2B). In contrast, treatment of BCBL-1 cells with triptolide resulted in cell cycle arrest in G2/M phase as observed by an increase in the percentage of cells in the G2/M phase (Fig. 2B). These results suggest that triptolide induces cell cycle arrest and apoptosis in the KSHV-associated PEL cells.

Triptolide decreases LANA1 expression without reducing LANA1 transcript level in KSHV-associated PEL cells. To determine whether triptolide alters expression of LANA1, three different latently infected KSHV-associated PEL cells were treated with vehicle control or triptolide for 48 h. Whole-cell extracts were prepared and subjected to western blot analysis. In addition to inhibition of LANA1 expression, two other KSHV-encoded latent proteins, v-Cyclin and v-Flip, were reduced after triptolide treatment in the three KSHV-associated PEL cells (Fig. 3A). When compared to the vehicle control, triptolide reduced the LANA1 expression level to 9.8, 16.8 and 23.4% in BC-3, BCBL-1 and JSC-1 cells, respectively. When compared to the vehicle control, triptolide reduced the v-Cyclin expression level to 3.4, 17.4 and 19.6%, the v-Flip expression level to 14.2, 22.3 and 24.6% in BC-3, BCBL-1 and JSC-1 cells, respectively. Furthermore, consistent with the results of cell apoptosis assays, triptolide resulted in increased caspase-3 cleavage in the three KSHV-associated PEL cells. In addition, expression of STAT3, which is one of transcription factors, remained unchanged with triptolide treatment, suggesting that the effect of triptolide on LANA1 was a selective event. To further determine if triptolide specifically decreases LANA1 expression, KSHV-negative HeLa cells were transiently transfected with pSG5-LANA1, followed by treatments with or without triptolide beginning at 4 h after transfection. As shown in Fig. 3B, triptolide treatment significantly decreased the expression of transfected pSG5-LANA1 in HeLa cells.

Since triptolide reduced expression of LANA1, a real-time quantitative reverse transcript polymerase chain reaction (qRT-PCR) analysis was used to determine if triptolide suppressed LANA1 at the mRNA level. BC-3, BCBL-1

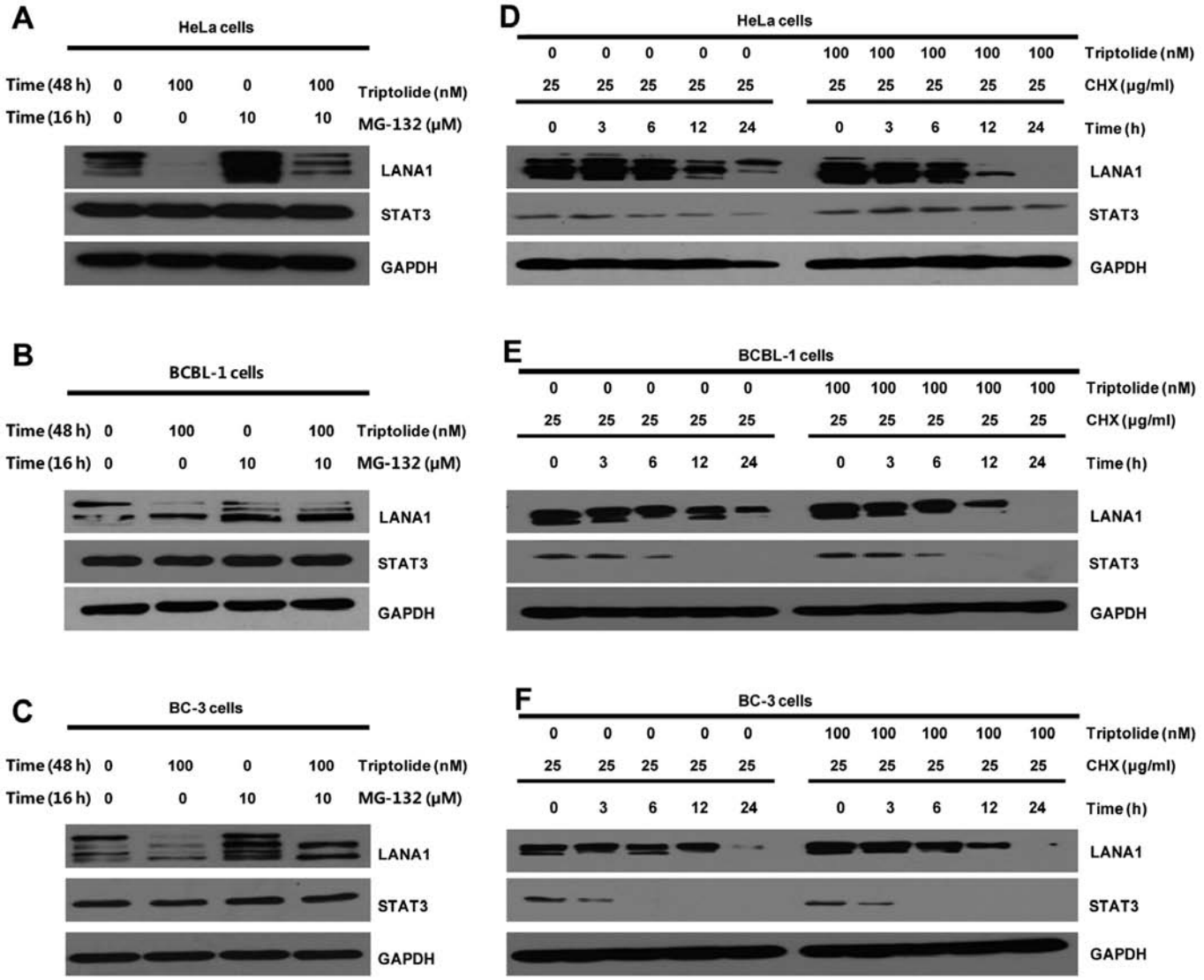

Figure 4. Triptolide induces proteasomal degradation of LANA1, and reduces half-life of LANA1. (A-C) Proteasomal degradation of LANA1. (A) HeLa cells were transiently transfected with pSG5-LANA1, followed by a 44-h treatment with vehicle control or triptolide (100 nM) beginning at 4 h after transfection in the absence or presence of proteasome inhibitor MG-132 (10 μM) for the last 16 h. (B) BCBL-1 and (C) BC-3 cells were treated for 48 h with vehicle control or triptolide (100 nM) in the absence or presence of proteasome inhibitor MG-132 (10 μM) for the last 16 h. (D-F) Half-life analysis of LANA1 in HeLa, BC-3 and BCBL-1 cells. (D) HeLa cells were transiently transfected with pSG5-LANA1 overnight, followed by incubation with vehicle control or triptolide (100 nM) in the presence of CHX (25 μg/ml) for 0, 3, 6, 12 and 24 h, respectively. (E) BCBL-1 and (F) BC-3 cells were treated with vehicle control or triptolide (100 nM) in the presence of CHX (25 μg/ml) for 0, 3, 6, 12 and 24 h, respectively. Whole-cell lysates were immunoblotted with anti-LANA1, anti-STAT3 or anti-GAPDH antibody.

and JSC-1 cells were treated with vehicle control or triptolide (100 nM) for 24 and 48 h, respectively. The total RNAs were harvested and subjected to qRT-PCR. The relative expression was detected by comparison to the housekeeping gene GAPDH. It was indicated that the mRNA levels of LANA1 in BCBL-1, BC-3 and JSC-1 cells were undetectable upon triptolide treatment (Fig. 3C).

Triptolide induces proteasomal degradation of LANA1. To examine whether triptolide decreased expression of LANA1 via the proteasome pathway, HeLa cells were transiently transfected with the pSG5-LANA1 and treated with vehicle control or triptolide in the absence or presence of proteosomal inhibitor MG-132. As shown in Fig. 4A, MG-132 obviously attenuated the effect of triptolide on LANA1 expression. Similarly, MG-132 also resulted in endogenous LANA1 protein level accumulation in BCBL-1 (Fig. 4B) and BC-3 (Fig. 4C) cells after treatment with triptolide.

Triptolide reduces stability or half-life of LANA1. Previous studies had indicated that triptolide decreased the expression of LANA1 and induced proteasomal degradation of LANA1. To determine whether triptolide affect LANA1 stability through some other mechanisms, HeLa cells were transiently transfected with the pSG5-LANA1 and treated with vehicle control or triptolide in the presence of cycloheximide (CHX). As shown in Fig. 4D, triptolide reduced the half-life of LANA1 when compared to vehicle control, while not affecting GAPDH levels. Similarly, triptolide also reduced the half-life of endogenous LANA1 protein in the CHX-treated BCBL-1

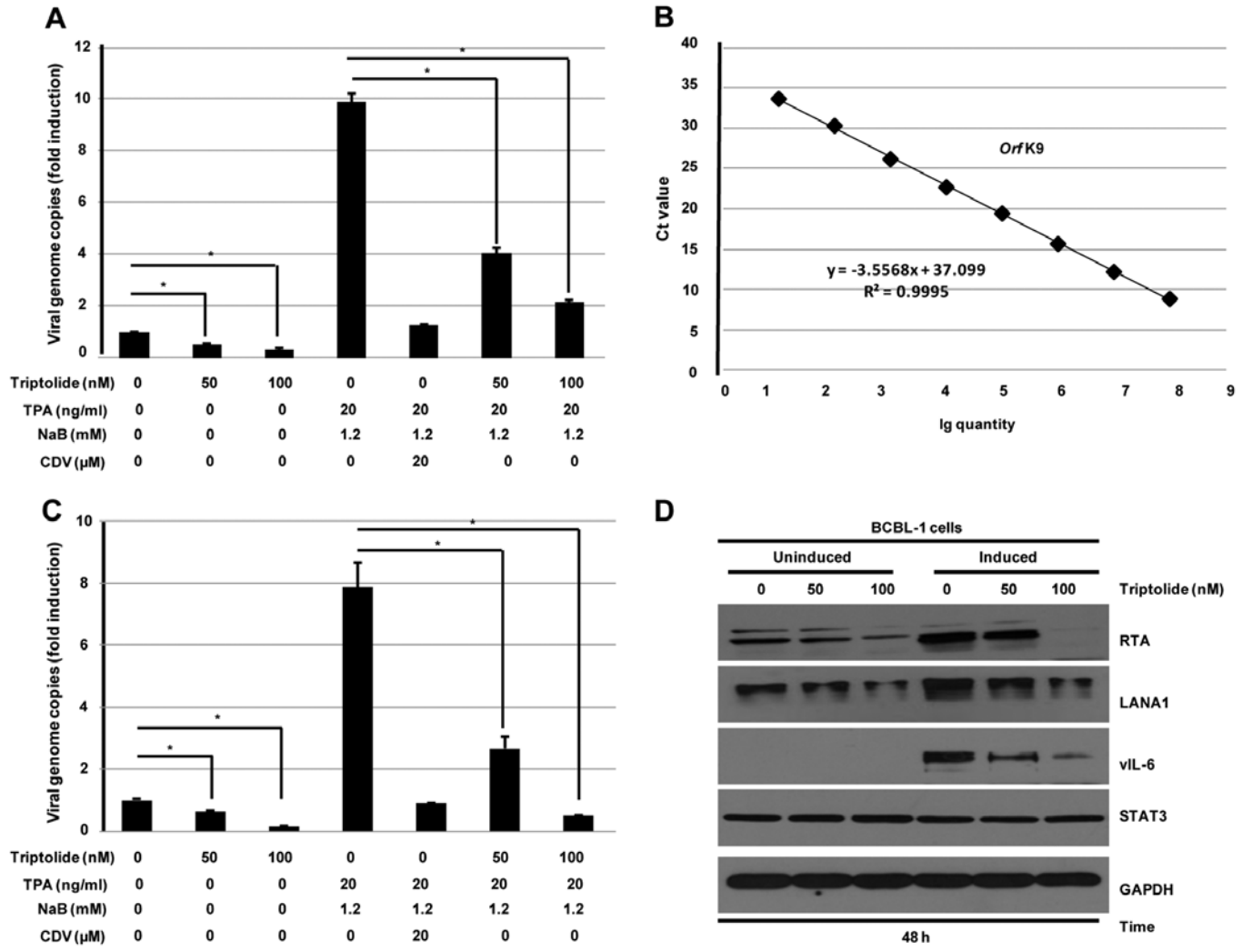

Figure 5. Triptolide reduces lytic and latent replication of KSHV in BCBL-1 cells. Uninduced and induced BCBL-1 cells were treated with vehicle control or indicated concentrations of triptolide for 48 h. The whole cell supernatants, genomic DNA and lysates were prepared. (A) The copy numbers of the intracellular viral DNA were quantified by qRT-PCR, each sample was normalized to the amount of the GAPDH gene. *P<0.05, compared to control. (B) The standard curve for virions quantification used to calculate the number of KSHV virions in the supernatants of BCBL1 cells. (C) The virion production extracted from equivalent supernatants was determined by qRT-PCR, and the viral copy numbers were calculated via the standard curve derived from the *Orf K9* construct. *P<0.05, compared to control. (D) Cells lysates above were immunoblotted with anti-LANA1, anti-RTA (20 μg), anti-vIL-6 (20 μg), anti-STAT3 or anti-GAPDH antibody.

(Fig. 4E) and BC-3 (Fig. 4F) cells. Notably, triptolide did not significantly affect STAT3 stability (Fig. 4D-F).

Triptolide reduces lytic and latent replication of KSHV in BCBL-1 cells. To examine the effect of triptolide on antiviral activity, BCBL-1 cells uninduced or induced by TPA and NaB for 3 h were treated with indicated concentrations of triptolide for 48 h. Whole cell lysates, supernatants, and genomic DNA were prepared at 48 h post-incubation. qRT-PCR and western blotting were used to analyze the effect of triptolide on lytic and latent replication of KSHV. As shown in Fig. 5A, triptolide decreased viral genome copies in both uninduced and induced BCBL-1 cells. When compared to the vehicle control in induced BCBL-1 cells, intracellular viral DNA replication was reduced by 5-fold after triptolide (100 nM) treatment for 48 h. Production of progeny virion extracted from the BCBL-1 cell supernatants was calculated according to the standard curve derived from the *Orf K9* construct (Fig. 5B). Results indicated that the production of progeny virion was dose-dependently reduced after triptolide treatment (Fig. 5C). As expected, CDV, which was used as a positive control, efficiently reduced the lytic replication and virion production of KSHV as previously described (33). Replication and transcription activator (RTA), a lytic switch protein, is necessary for KSHV reactivation and lytic DNA replication. Western blotting was used to detect the effect of triptolide on the expression of RTA in BCBL-1 cells. As shown in Fig. 5D, triptolide reduced expression of LANA1 and RTA in both uninduced and induced BCBL-1 cells. In addition, vIL-6, encoded by KSHV *Orf K2*, contributed to the growth, survival and spread of PEL. Our results as shown in Fig. 5D also indicated that triptolide decreased vIL-6 expression in induced BCBL-1 cells.

Triptolide suppresses STAT3 activity in PEL cells. LANA1, a transcriptional co-activator of STAT3, modulates STAT3 activity in PEL cells (20). STAT3 is constitutively phosphory-

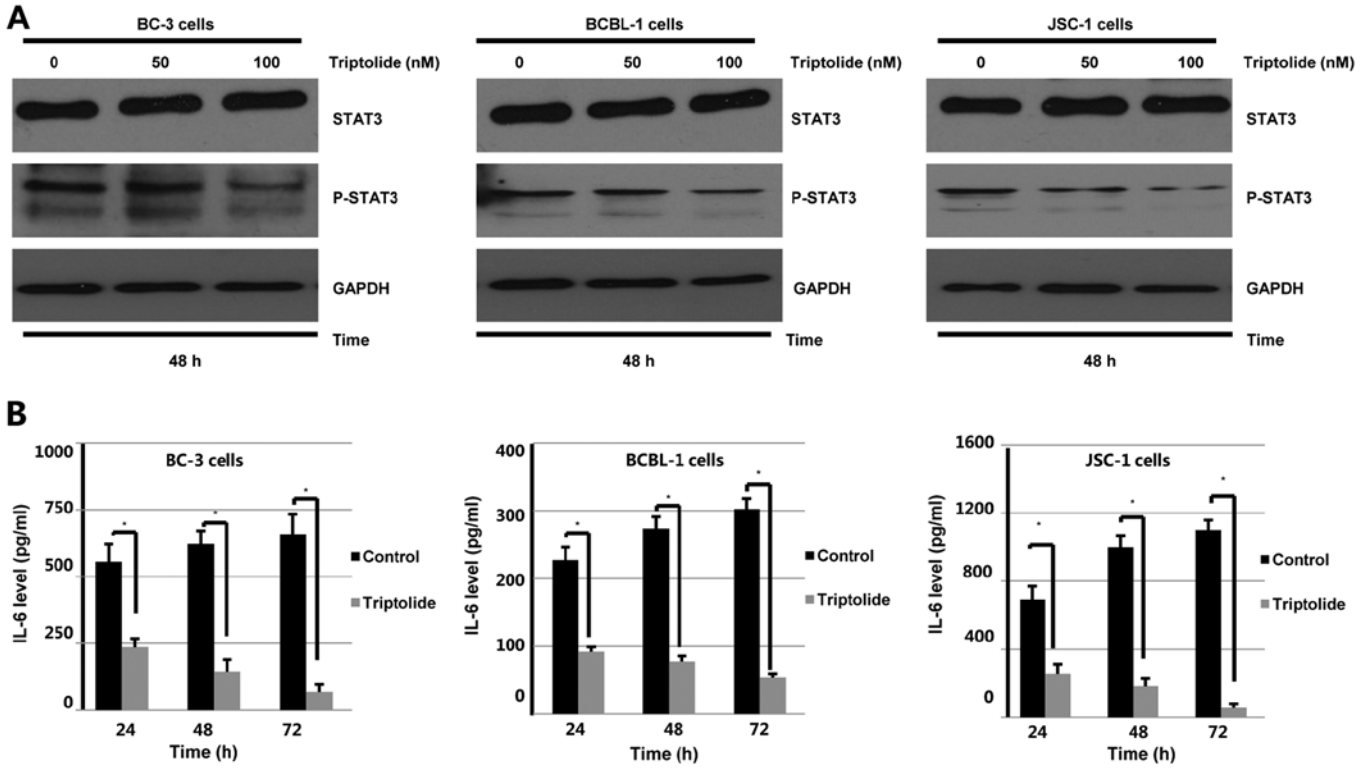

Figure 6. Triptolide suppresses STAT3 activity and secretion of IL-6 in PEL cells. (A) The three kinds of PEL cells were treated with vehicle control or indicated triptolide for 48 h, respectively. Whole-cell extracts were prepared and western blotting was performed to analyze expression of STAT3, p-STAT3 (20 μg), or GAPDH. (B) PEL cells were treated with vehicle control or triptolide (100 nM) for 24, 48 and 72 h, followed by detecting IL-6 levels using ELISA in the supernatants. Values shown are mean ± SD of 3 independent experiments carried out in triplicate. *P<0.05, compared to control.

lated and is essential to cell survival in PEL cells (34). Since STAT3 represents a potential target for treatment of PEL, the STAT3 activity affected by triptolide was determined. Results shown in Fig. 6A indicated that triptolide dephosphorylated STAT3 in BCBL-1, BC-3 and JSC-1 cells. It has been reported that KSHV encoded LANA1 upregulated IL-6 expression by inducing transcription of the IL-6 promoter (35). IL-6 and vIL-6 secreted by KSHV-positive cells are multifunctional cytokines that contribute to the pathogenesis of PEL (35). To determine effect of triptolide on secretion of IL-6, KSHV-associated PEL cells were treated with vehicle control or 100 nM triptolide for 24, 48 and 72 h. Cell supernatants were collected and subjected to ELISA. As shown in Fig. 6B, triptolide resulted in reduced secretion of IL-6 in the supernanants of BCBL-1, BC-3 and JSC-1 cells. These results suggests that downregulation of LANA1 by triptolide results in inhibition of IL-6/STAT3 pathway in PEL cells.

In vivo effects of triptolide on NOD/SCID mice inoculated with BCBL-1 cells. Since our previous results indicated efficacy of triptolide for treatment of PEL *in vitro*, effects of triptolide *in vivo* were also assessed in an immunodeficient xenograft mouse model. NOD/SCID mice were inoculated intraperitoneally with $1x10^7$ BCBL-1 cells. A dose of 0.4 mg/kg triptolide or PBS alone was administrated via intraperitoneal injection daily for 3 weeks from day 3 after cell inoculation. As the dose of triptolide from intraperitoneal injection had already been reported (23), the dosage of triptolide *in vivo* in the present study was expected to be safe. The body weight of the PBS alone treated mice significantly increased compared to that of triptolide treated mice (28.9±1.79 vs. 19.2±2.21 g, n=6, P<0.01; Fig. 7A). Moreover, mice treated with triptolide had a significantly lower volume of ascites (1.45±0.27 vs. 0.17±0.07 ml, n=6, P<0.01; Fig. 7B). In addition, IL-6 level in the supernatants of ascites was significantly decreased in comparison to that of triptolide treatment (Fig. 7C). Western blotting results of ascites cells indicated that triptolide significantly cut down the expression of LANA1 and induced ascites cell apoptosis via caspase-3 pathways (Fig. 7D). Representative photos of mice treated with PBS alone and triptolide treated are shown in Fig. 7E.

Organ invasion by BCBL-1 cells on day 24 was evaluated by hematoxylin and eosin staining, as well as LANA1 immunostaining. As shown in Fig. 7F, spleens of mice treated with triptolide were significantly smaller compared to the group treated with PBS alone. The mice inoculated with BCBL-1 cells exhibited infiltration in the spleen and the number of LANA1 positive cells in triptolide treated mice was significantly reduced (Fig. 7G). These results suggest that triptolide efficiently inhibits growth and infiltration of PEL cells *in vivo*.

Discussion

LANA1 is a pivotal latent protein, which is consistently expressed in all proliferating KSHV-positive cells and is essential for cell survival. Moreover, LANA1 plays an important role in regulating segregation, replication and persistence of viral episome (36), providing an attractive target for the

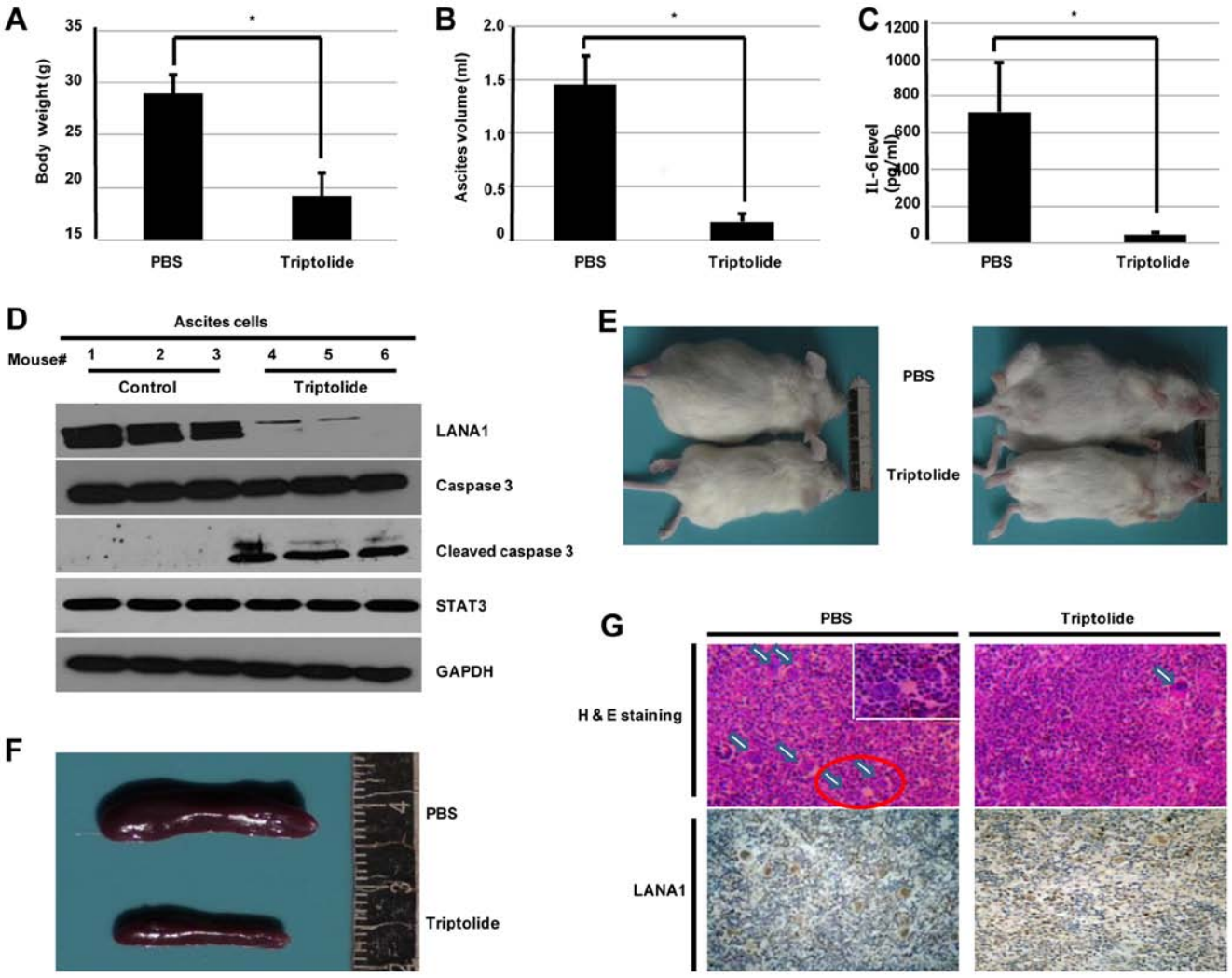

Figure 7. Treatment of NOD/SCID mice with triptolide impairs growth of PEL *in vivo*. NOD/SCID mice with established intraperitoneal BCBL-1 tumors were treated with PBS or 0.4 mg/kg triptolide for 21 days. (A) Body weights, (B) ascites volume, and (C) IL-6 levels of ascites were evaluated in NOD/SCID mice treated with triptolide (0.4 mg/kg/day) or PBS for 21 days. The whole values are shown as mean ± SD of 6 mice. *P<0.05, compared to control. (D) Lysates of ascites cells were subjected to western blot analysis using the indicated antibodies. (E and F) Representative images of mice and spleen from groups of mice treated with either PBS or triptolide are shown. (G) H&E staining was performed to detect infiltrated cells indicated with white arrows, and an enlarged image of infiltrated cells is shown in the upper right. Immunohistochemical staining using anti-LANA1 were performed to detect BCBL-1 cells in the spleen.

treatment of KSHV-induced diseases. In the present study, the direct effects of triptolide on KSHV-associated PEL cells *in vitro* and *in vivo* were evaluated. Firstly, it was demonstrated that triptolide effectively inhibited KSHV-associated PEL cells proliferation with a nanomolar concentration. Moreover, triptolide induced cell cycle arrest and apoptosis in the KSHV-associated PEL cells.

Our previous study demonstrated that triptolide reduced the expression of LMP1 in latency III type infected EBV-positive B lymphocytes (29). Since LANA1 is reported to be a client protein of Hsp90, 17-DMAG is efficacious against KS by promoting the degradation of LANA1 (30). Of note, our findings indicate that triptolide decreases LANA1 expression in a variety of cell types, including three different KSHV-associated PEL cells and KSHV-negative HeLa cells transiently transfected with pSG5-LANA1. Further study indicated that triptolide did not reduce LANA1 expression at the mRNA levels. In addition, LANA1 accumulated after treatment with MG-132 in the presence of triptolide. Triptolide also decreased the stability or half-life of LANA1. In addition to LANA1, v-Cyclin and v-Flip are two KSHV-encoded latent proteins. KSHV-encoded v-Cyclin contributed to tumorigenesis through inhibiting cell cycle inhibitor p27Kip1 in PEL (37). KSHV-encoded v-Flip contributed to activation of NF-kappaB pathway, which is essential for survival in a majority of PEL cells (38). Our results also indicated that triptolide inhibited expression of v-Cyclin and v-Flip in KSHV-associated PEL cells. These results suggest that triptolide reduces the expression of three major latent proteins, together with inducing proteasomal degradation of LANA1 and impairing its stability.

Lytic replication and gene expression of KSHV are essential to promote tumorigenesis (39). Previous studies have demonstrated that higher KSHV viral load has been associated with increased risk of KSHV-induced malignancies (40). Consistent with the ability of triptolide to decrease LANA1 expression in KSHV latency infected cells, our results demonstrated that triptolide impaired lytic replication of KSHV, as well as virion

production released from induced BCBL-1 cells, suggesting that triptolide treatment is efficacious against KSHV replication. Triptolide also reduced the expression of lytic genes RTA, and inhibited their expression induced by TPA and NaB, thereby allaying these safety concerns. Essentially similar effects of BIIB021 and 17-AAG on the expression of RTA have been reported recently (38,41). In addition, triptolide reduced expression of vIL-6, which contributed to pathogenesis of PEL, in TPA and NaB induced BCBL-1 cells. At present, the exact mechanism for the inhibition of lytic replication and gene expression of KSHV by triptolide is not clear and need further study. Taken together, triptolide can efficiently induce apoptosis and cell cycle arrest, together with down-regulation of LANA1 expression and viral replication in the KSHV-associated PEL cells.

STAT3 activity plays an essential role in the survival of PEL cells. STAT3 is constitutively active and inhibition of STAT3 results in apoptosis in PEL cells (33). Previous reports have indicated that LANA1, a transcriptional co-activator of STAT3, modulates STAT3 activity in PEL cells (20). Overexpression of LANA1 enhanced STAT3 activity, and inhibition of LANA1 expression decreased the STAT3-dependent transcription (20). Consistent with the effects of AG490, a tyrosine kinase inhibitor, on STAT3 activity in PEL cells (42), triptolide reduced STAT3 activity in PEL cells. In addition, IL-6 is a pivotal cytokine contributing to cell survival. LANA1 enhanced IL-6 expression by inducing transcription of the IL-6 promoter (34). Our results demonstrated that triptolide reduced secretion of IL-6 in BCBL-1, BC-3 and JSC-1. Therefore, inhibition of STAT3 activity and IL-6 secretion may result from the downregulation of LANA1 by triptolide.

In addition to the effect of triptolide on the KSHV-positive malignant cells *in vitro*, the present study also evaluated the therapeutic effect of triptolide against PEL *in vivo* using a mouse xenograft model. PEL xenograft mice are characterized by lymphomatous effusions in body cavities and rarely present with a definable tumor mass, which are similar to the clinical feature of human PEL patients (43). Consistent with the therapeutic effect of other drugs against PEL xenograft mouse models (44), triptolide treatment significantly repressed the formation of malignant ascites and lymphomatous effusions caused by BCBL-1 cells in NOD/SCID mice. Moreover, triptolide treatment decreased the expression of LANA1 in ascites cells and induced apoptosis of ascites cells in a caspase-dependent manner.

Although KSHV was discovered 20 years ago, there is little progress in the management of KSHV-related diseases. Due to the toxic effect or incomplete efficiency of classical antiviral agents, such as acyclovir, ganciclovir and adefovir, antiviral treatments for KSHV-infected diseases are limited. To date, standard therapeutic guidelines are not drawn for the treatment of KSHV-associated malignancies. Currently antiviral drugs mainly target lytic replication of virus, which is essential to disease progression and virus dissemination. Nevertheless, the majority of KSHV-infected tumor cells are in latency, and antiviral drugs targeting KSHV lytic replication have not shown consistent results for the treatment of KSHV-associated KS in clinic (6). Therefore, targeting viral latency may be a novel strategy for the treatments of KSHV-infected diseases. In this study, our results have demonstrated the ability of triptolide *in vitro* and *in vivo* to inhibit cell proliferation and induce cell apoptosis in the KSHV-associated PEL cells. In addition, triptolide can efficiently reduce the expression of LANA1, inhibit viral replication, and impair production of progeny virion in the KSHV-associated PEL cells. Our results show that triptolide may be a promising compound for the treatment of KSHV-associated diseases.

Acknowledgements

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