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Triptolide inhibits proliferation of Epstein–Barr virus-positive B lymphocytes by down-regulating expression of a viral protein LMP1

Heng Zhou [a], Wei Guo [b], Cong Long [a], Huan Wang [a], Jingchao Wang [a], Xiaoping Sun [a,c,*]

[a] Department of Pathogen Biology, School of Basic Medical Sciences, Wuhan University, Wuhan 430071, PR China
[b] Department of Pathology and Physiology, School of Basic Medical Sciences, Wuhan University, Wuhan 430071, PR China
[c] State Key Laboratory of Virology, Wuhan University, Wuhan 430072, PR China

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ABSTRACT

Epstein–Barr virus (EBV) infects various types of cells and mainly establishes latent infection in B lymphocytes. The viral latent membrane protein 1 (LMP1) plays important roles in transformation and proliferation of B lymphocytes infected with EBV. Triptolide is a compound of *Tripterygium* extracts, showing anti-inflammatory, immunosuppressive, and anti-cancer activities. In this study, it is determined whether triptolide inhibits proliferation of Epstein–Barr virus-positive B lymphocytes. The CCK-8 assays were performed to examine cell viabilities of EBV-positive B95-8 and P3HR-1 cells treated by triptolide. The mRNA and protein levels of LMP1 were examined by real time-PCR and Western blotting, respectively. The activities of two LMP1 promoters (ED-L1 and TR-L1) were determined by Dual luciferase reportor assay. The results showed that triptolide inhibited the cell viability of EBV-positive B lymphocytes, and the over-expression of LMP1 attenuated this inhibitory effect. Triptolide decreased the LMP1 expression and transcriptional levels in EBV-positive B cells. The activity of LMP1 promoter ED-L1 in type III latent infection was strongly suppressed by triptolide treatment. In addition, triptolide strongly reduced growth of B95-8 induced B lymphoma in BALB/c nude mice. These results suggest that triptolide decreases proliferation of EBV-induced B lymphocytes possibly by a mechanism related to down-regulation of the LMP1 expression.

1. Introduction

Epstein–Barr virus (EBV), a γ-herpes virus, has infected with more than 90% adults of all human population. EBV infection is associated with various human malignancies including both B-cell and epithelial cell sources, such as Burkitt and Hodgkin lymphoma, post-transplate lymphoproliferative diseases, nasopharyngeal carcinoma (NPC), and gastric cancer [1]. *In vivo*, EBV causes three kinds of latent infection. In Burkitt's lymphoma diseases, EBV causes including type I or type III latent infection [22]. Type II latent infection is found in NPC or Hodgkin's disease cells. In type III latent infection, the resting B lymphocytes are induced to continuous proliferate when infected with EBV [2].

The EBV-encoded protein latent membrane protein 1 (LMP1) is abundantly expressed in cells with the type II and type III latent infection. LMP1expression is essential for oncogenic transformation of rodent fibroblasts and plays an important role in B cell immortalization [1]. LMP1 interacts with tumor necrosis factor receptor-associated factors or tumor necrosis factor receptor-associated death domain protein to trigger the non-canonical nuclear factor-κB, the extracellular signal-regulated kinase and p38 by mimicking action of CD40 [3,14]. Under the background, LMP1 contributes to development of malignancies, including resisting to cell apoptosis, promoting tumor invasion and metastasis, and increasing angiogenesis [4,15]. In addition, LMP1 increases production of virus genome and promotes virion release to supernate during lytic infection [6]. In the type II infection epithelial cells, LMP1 transcription is mediated by an up-stream STAT-regulated promoter, named TR-L1, which is a TATA-fewer promoter [7]. However, in the type III infection B lymphocytes, expression of LMP1 mRNA is regulated by EBV-encoded EBNA2, EBNA-LP, and the cellular protein C/EBP encoded from an ED-L1 promoter [8–10]. The TR-L1 promoter is located in approximately 600 bp upstream of the ED-L1 promoter [7].

Triptolide is a diterpene epoxide of *Tripterygium* extracts, showing anti-inflammatory, immunosuppressive, anti-fertility, anti-cystogenesis, and anti-cancer activities [11]. The anti-cancer role of triptolide has been found for more than a decade [11]. Triptolide

* Corresponding author at: Department of Pathogen Biology, School of Basic Medical Sciences, Wuhan University, 185 Eastlake Road, Wuhan 430071, PR China.
E-mail address: xsun6@whu.edu.cn (X. Sun).

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has been reported to be effective against a variety of malignancies *in vivo* experiments, including pancreatic cancer, ovarian cancer, breast cancer, and neuroblastoma [18]. It has been reported that triptolide covalently binds to XPB, a subunit of the transcription factor TFIIH, and inhibits transcription of its down-stream genes [16]. Triptolide induces apoptosis of pancreatic tumor cells through decreasing the expression of O-GlcNac transferase to change distribution of transcription factor specificity protein 1 (Sp1) [12]. Triptolide inhibits proliferation of prostate cancer cells by decreasing expression of SUMO-Specific Protease 1 [16]. In clinic trial, triptolide shows a better efficacy as anti-cancer and anti-inflammatory agents when compared with adriamycin and aclacinomycin [18]. However, it has not been examined if triptolide affects EBV-induced malignancies.

Here we demonstrate that triptolide inhibits cell proliferation of EBV-positive B lymphoma cells via decreasing the transcriptional expression of LMP1. Triptolide down-regulates the LMP1 mRNA by inhibiting the activation of LMP1 promoter in type III infection cells, ED-L1. Furthermore, triptolide inhibits proliferation of EBV-induced B lymphoma in BALB/c nude mice.

2. Materials and methods

2.1. Cell lines and reagents

EBV-positive B lymphoma cell lines B95-8 and P3HR-1 were kindly provided by Prof. Y. Cao (Central South University, Changsha, China). HONE1/Akata and C666-1 (gifts from Prof. S.W. Tsao, the University of Hong Kong, Hong Kong, China) are EBV-positive NPC cell lines. HeLa (a malignant human epithelial cervical cancer cell line) was given by Prof. H. Li (Wuhan University, Wuhan, China). Human renal embryonic 293T cells were obtained from Prof. Z. Yang (Wuhan University, Wuhan, China).

All cell lines were cultured at 37 °C with a humidified atmosphere of 5% CO_2 in growth RPMI-1640 media (Hyclone, USA) supplemented with 10% FBS (Gibco, USA). For maintaining the recombinant EBV genomes, G418 (400 ng/ml) was added into the medium of HONE1/Akata. Triptolide (Sigma, USA) was dissolved in DMSO.

2.2. Plasmids

The plasmid pSG5-LMP1 was constructed by inserting the full LMP1 sequence into the BglII/BamHI of SG5 plasmid (YouBio, ShangHai, China). PGL3.0-basic was purchased from Promega (Wisconsin, USA). PRL-TK, a plasmid expresses the Renilla Luciferase, was obtained from Prof. D. Guo (Wuhan University, Wuhan, China). All the promoter sequences were amplified from the B95-8 genome. The primers used in this study were given as follows: ED-L1_Forward, 5′-TAGCCTCGAGTGAATCCGCCACCTCATTC; ED-L1_Backward, 5′-AATCTTCCATGGTCAGGGCAGTGTGTC; TR-L1_Forward, 5′-ATCGCTCGAGCCGCGCCAGCCAAGCCCCCAAG; and TR-L1_Backward, 5′-GCTACCATGGCCCGCAGCCCCCGGAGCCCGC. The amplified sequences were digested with XhoI and NcoI (NEB, USA) and inserted into the Xho/NcoI sites of the pGL3.0-basic. All the plasmids were purified using a kit (Axygen, San Francisco, USA) as described by the manufacturer.

2.3. Cell viability

B95-8 and P3HR-1 cells (1×10^4/ml) were placed in 96-well plates as a 100-μl cell suspensions and treated with triptolide (50, 100, 200, or 400 nM) for 2, 3, and 4 days. Ten microliters of the Cell Counting Kit-8 (DOJINDO, Tokyo, Japan) reagent were then added to each well and cells were incubated at 37 °C for 4 h as indicated. The absorbance was detected at a 450 nM wave-length using a microplate reader (BioTek, EXL800, Vermont, USA).

2.4. Cell transfection

For transfection, 4×10^5 cells were placed in 6-well plates. All cells were transiently transfected using X-tremeGENE HP DNA Transfection Reagent (ROCHE, Basel, Switzerland) as the manufacturer's indication. After 4 h transfection, cells were treated with triptolide for 44 h.

2.5. Cell cycle analysis

B95-8 cells (1×10^6) were harvested and washed with phosphate buffered saline (PBS) solution. After resuspended with 1 ml of PBS, 3 ml of 100% ethanol were mixed with the cells suspension. Cells were incubated at −20 °C for 24 h, centrifuged at 1000 rpm for 5 min and resuspended in 5 ml of PBS. After 15 min, cells were centrifuged at 1000 rpm for 5 min and resuspended with reagent A (Multisciences, ShangHai, China). Following incubation in dark at room temperature for 30 min, the propidium iodide-stained nuclei were analyzed by a Beckman Coulter system (EPICS ALTRA II, Fullerton, USA).

2.6. Dual luciferase reportor assay

HeLa cells (1×10^5/well) were placed in 24-well plates. pGL3.0-EDL1/pRL-TK or pGL3.0-TRL1/pRL-TK were co-transfected into the cells. Then these cells were treated with triptolide (25, 50 or 100 nM) or DMSO (0.01%) for 24 h. Total protein was prepared using 1 × Passive Lysis Buffer (Promega, Wisconsin, USA) and luciferase activity was measured by the GLO-MAX 20/20 system (Promega, Wisconsin, USA) according to the protocol of Dual-luciferase reportor assay (Promega, Wisconsin, USA). The firefly luciferase activity was normalized to the Renilla luciferase activity.

2.7. Real-time PCR

The total RNA was extracted using the TRIzol Reagent (Life, USA) and reverse transcripted into cDNA using Reverse Transcription kit (TaKaRa, Tokyo, Japan) according to the manufacturers' instructions. The LMP1 mRNAs were quantified by the CFX96 Real-Time PCR Detection System using a SYBR Premix Ex Taq kit (TaKaRa, Tokyo, Japan). The primers were as follows: LMP1 5′-CTATTCCTTTGCTCTCATGC and 5′-TGAGCAGGAGGGTGATCATC. GAPDH 5′-GGTGGCTTCTGACTTCAACA and 5′-GTTGCTGTAGCCAAATTCGTTGT. The LMP1 levels were normalized to the housekeeping gene GAPDH.

2.8. Western blotting

Cell lysates were prepared by resuspending cells with RIPA lysis buffer (Beyotime, ShangHai, China). Followed by storing on ice for 10 min, total proteins were sonicated for 15 s. After centrifugation at 12,000×*g* for 10 min, the supernatants were transferred into new tubes, mixed with 5 × loading buffer (250 nM Tris–HCl (PH 6.8), 10% SDS, 0.5% BPB, 50% glycerol, 5% β-mercaptoethanol) and boiled for 5 min. Total protein was separated on 10% SDS–PAGE gels and subjected to immunoblot analyses. The primary antibodies used as follows: LMP1 (Cat No. ab78113, 1:4000; abcam, Cambridge, UK) and GAPDH (Cat no, 10494-1-AP, 1:100,000; proteintech, Peking, China). Secondary antibodies are horseradish-peroxidase-conjugated secondary anti-mouse IgG (1:10,000; Kerui tech, Wuhan, China), anti-rabbit IgG (1:100,000; Kerui tech, Wuhan, China). The blot was detected using ECL system (BIO RAD, USA). Western blot gray values were determined by ImageJ.

2.9. Animal studies

B95-8 cells (1×10^7) were inoculated subcutaneously into both flanks of 4-week-old male BALB/c nude mice (purchased from the ABSL-3 animal lab at Wuhan University). After 7 days, when tumor had become to be palpable, mice (five per group) were treated with a single intraperitoneal injection of triptolide at 0.4 mg/kg or DMSO daily. Mice were sacrificed after 21 days treatment by cervical dislocation. Tumor size was measured and tumor volume was calculated as $0.5 \times \text{length} \times \text{width}^2$.

2.10. Statistical analysis

Data were shown as the mean ± standard deviation and analyzed using GraphPad Prism for Windows version 5.0 (GraphPad Software, La Jolla, USA). A Student's *t*-test was used to determine the significance of the difference between any two samples. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Triptolide inhibits proliferation of EBV-positive B cells

To determine if triptolide affects cell viability of EBV-positive B cell lines, B95-8 and P3HR-1 cells were treated with triptolide in a dose- and time-dependent manner. The cells treated with DMSO (0.01%) were used as a control. As shown in Fig. 1A and B, triptolide significantly inhibited cell viability of B95-8 and P3HR-1 cells in comparison with the cells treated with DMSO only. To determine if triptolide affects cell cycles, B95-8 cells were treated with DMSO (0.01%) or triptolide (100, 200, and 400 nM) for 48 h. The cell cycle distribution was analyzed by flow cytometry (Fig. 1C). The B95-8 cells retarded cell cycling with a reduction in S phase (Fig. 1D), which was 33.1% for the control and 22.0% for treatment group (400 nM). The fractions of G0/G1 cells were increased from 53.3% in DMSO group to 63.2% in the 400 nM triptolide group. These results suggest that triptolide decreases cell proliferation of EBV-positive B lymphocytes *in vitro*.

3.2. Triptolide decreases expression of LMP1 in EBV-positive B cells

To determine whether triptolide affects expression of LMP1 in EBV-positive cells, two different EBV-positive B cell lines (B95-8, P3HR-1) and two different EBV-positive NPC cell lines (HONE1/Akata and C666-1) were treated with vehicle control (0.01% DMSO) or triptolide (100 or 200 nM) for 48 h. Cells were harvested and the total proteins were subjected to Western blot analyses. As shown in Fig. 2A, LMP1 expression in the 200 nM triptolide treatment group were reduced to 17.1% when compared to the expression levels in the untreated B95-8 cells. In P3HR-1 cells, expression of LMP1 was down-regulated to 33.4% in 200 nM treatment group when compared with the control group (Fig. 2B). However, in the two NPC cell lines, the levels of LMP1 expression were not significantly decreased (Fig. 2C and D). These results suggest that triptolide decreases LMP1 expression in EBV-positive B cell lines, but possibly not in the NPC cell lines.

To determine whether triptolide down-regulates transiently expression of LMP1, pSG5-LMP1 plasmid was constructed using a commercial SV40 promoter SG5. pSG5-LMP1 plasmids were transfected into 293T and HeLa cells. After 4 h, cells were treated with

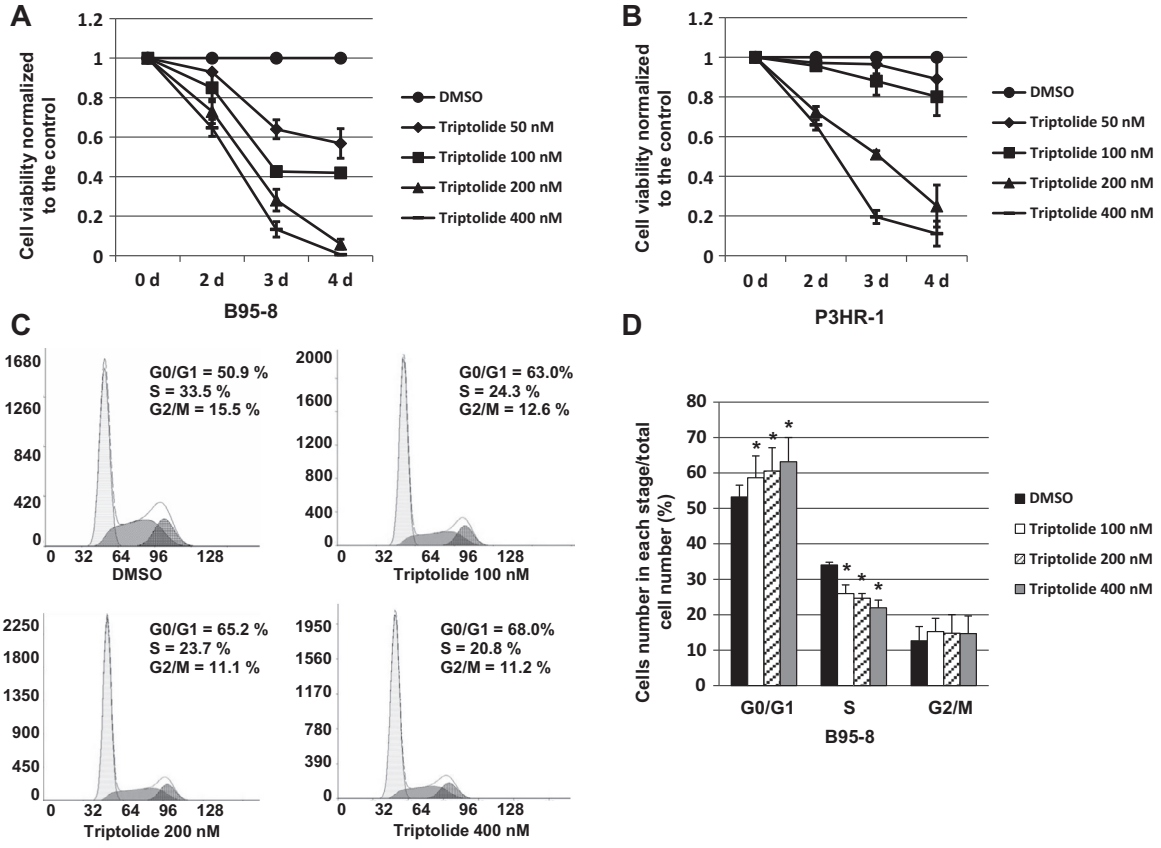

Fig. 1. Triptolide inhibits proliferation of EBV-positive B lymphocytes *in vitro*. (A) B95-8 and (B) P3HR-1 cells (1×10^4/ml) were placed in 96-well plates and treated with DMSO control (0.01%) or triptolide (50, 100, 200, or 400 nM). Cell viability assays were detected by a CCK-8 kit. (C) B95-8 cells were treated with DMSO (0.01%) or triptolide (100, 200, or 400 nM) for 48 h. The distribution of cell cycle was determined by flow cytometry. (D) The cell cycle distribution is quantified by the bar graphs.

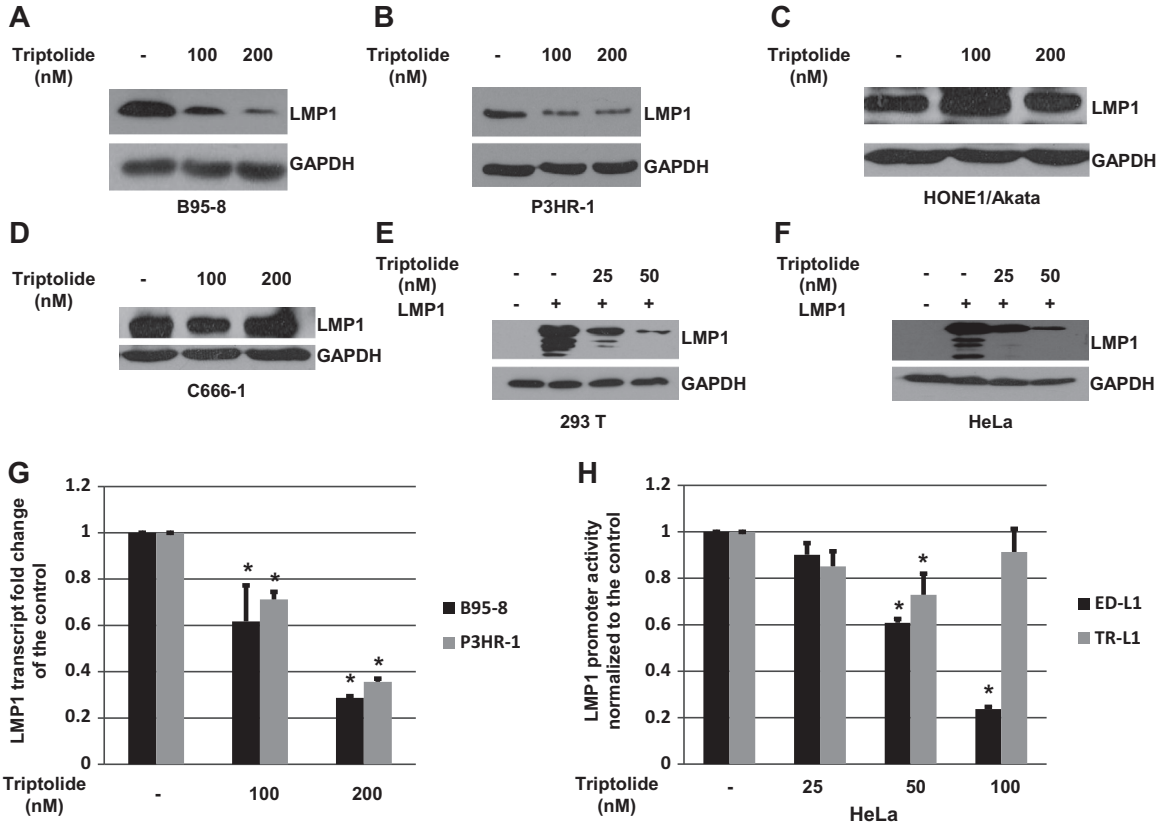

Fig. 2. Triptolide decreases expression of LMP1 protein and mRNA. B95-8 (A or B) P3HR-1 cells (4×10^5) were placed in 6-well plates and treated with DMSO (0.01%) or triptolide (100 or 200 nM) for 48 h. (C) HONE1/Akata or (D) C666-1 cells were placed in 6-well plates. The cells were treated with DMSO (0.01%) or triptolide (100 or 200 nM) for 48 h. (E) 293T or (F) Hela cells were transfected with pSG5 or pSG5-LMP1 as indicated, followed by 44 h treatment with triptolide (25 or 50 nM) beginning at 4 h after transfection. Whole-cells extracts were prepared and subjected to Western blot analysis. (G) B95-8 or P3HR-1 cells were treated with DMSO (0.01%) or triptolide (100 or 200 nM) for 48 h. Expression of the LMP1 mRNA transcript was examined by real time-PCR. (H) HeLa cells were co-transfected with pGL3.0-EDL1/pRL-TK or pGL3.0-TRL1/pRL-TK, followed by 24-h treatment with DMSO (0.01%) or triptolide (25, 50 or 100 nM) beginning at 4 h post-transfection. Cell lysates were prepared and the promoter activity was determined by a Dual-luciferase reportor assay system.

triptolide (25 or 50 nM) for 44 h. As shown in Fig. 2E and F, triptolide decreased significantly LMP1 expression in 293T and HeLa cells.

Decreased protein levels may be resulted from the down-regulated mRNA levels. To determine if triptolide affects LMP1 transcript levels, B95-8 and P3HR-1 cells were treated with DMSO (0.01%) and triptolide (100 and 200 nM) for 48 h. The total RNAs were harvested and subjected to real-time PCR. As shown in Fig. 2G, triptolide significantly down-regulated LMP1 mRNA levels to 61.7% in 100 nM and 28.8% in 200 nM treatment group when compared with control group in B95-8cells. In P3HR-1 cells, the LMP1 mRNA expression was decreased to 71.2% in 100 nM dose and 35.7% in 200 nM dose by triptolide treatment, respectively. Altogether, the above results show that triptolide decreases expression of viral LMP1 in EBV-positive B lymphocytes by affecting protein and mRNA levels.

3.3. Triptolide inhibits ED-L1 promoter activity

The LMP1 transcription is initiated from the TR-L1 in type II infection or the ED-L1 promoter in type III infection, respectively. To determine if triptolide could decrease the activity of LMP1 promoter, pGL3.0-EDL1 or pGL3.0-TRL1 were co-transfected with pRL-TK into HeLa cells. The cells were treated with DMSO or triptolide (25, 50, and 100 nM) for 24 h after 4 h. The promoter activities were analyzed by the Dual-GLU system. The activity of ED-L1 promoter was decreased significantly to 23.7% in 100 nM dose when compared with control group (Fig. 2H). In contrast to the effect on ED-L1, triptolide did not affect the TR-L1 activity. These results suggest that triptolide may inhibit the promoter activity of LMP1 in type III infection.

3.4. Over-expression of LMP1 attenuates the inhibitory effect of triptolide

LMP1 is an oncogene with function of promoting proliferation, survival, and transformation of B lymphocytes (4, 16). To determine if cell viability decreases in the triptolide treatment group are primarily caused by the reduced expression of LMP1, pSG5-LMP1 or vehicle control (pSG5) were transfected into B95-8 or P3HR-1. The cells were treated with DMSO or triptolide (200 nM) 4 h later. Cell viabilities were detected at day 2 and day 4. LMP1 expressions were increased by transient expression of LMP1 and decreased by triptolide obviously (Fig. 3A and B). As shown in Fig. 3C and D, triptolide decreases both B95-8 and PEHR-1 cell viabilities significantly. Followed by transfection, cell viabilities were enhanced by 285.1% in B95-8 and 44.0% in P3HR-1 respectively on day 4. The over-expression of LMP1 increased cells viability and attenuated the inhibition efficiency of triptolide (Fig. 3C and D). These results suggest that triptolide inhibits proliferation of EBV-induced B cells possibly through a mechanism related to LMP1.

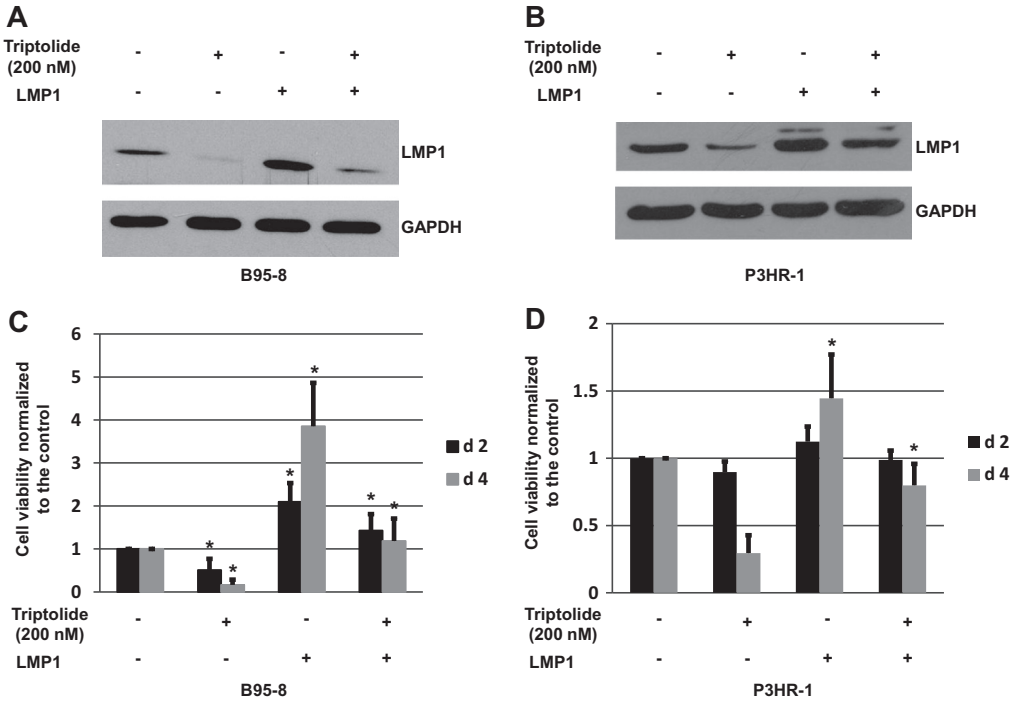

Fig. 3. Over-expression of LMP1 attenuates the effects of triptolide that inhibits cell proliferation of EBV-positive B lymphocytes. B95-8 and P3HR-1 cells were transfected with an empty vector (pSG5) or pSG5-LMP1. Cells were transfected and then treated with 200 nM triptolide. (A and B) Total proteins were prepared from B95-8 and P3HR-1 cells after a 44 h-treatment and immunoblot analysis was performed. Antibodies against LMP1 and GAPDH were used. (C and D) Cell viabilities of B95-8 and P3HR-1 cells were measured at day 2 or day 4, respectively.

3.5. The effect of triptolide on B lymphoma in vivo

To study if triptolide influences growth of the B lymphoma in BALB/c mice, 1×10^7 B95-8 cells were injected subcutaneously into flanks of the mice. When tumor had grown to be palpable (7 days later), the mice were treated with 0.4 mg/kg triptolide or DMSO daily. After 21 days, mice were euthanized and tumors were measured and weighed. As shown in Fig. 4A–C, tumor volumes, tumor weights and representative photos were given. These results suggest that triptolide inhibits proliferation of B95-8 cells-induced tumors *in vivo*.

In order to determine the mechanism that triptolide suppresses growth of tumor, expression of LMP1 in the tumor tissues were detected by Western blot analyses (Fig. 4D). The average LMP1 expression level of treatment group was decreased to 25.1% when compared to the control group. Total mRNAs were extracted from tumor tissues. The mRNA levels were analyzed by real time-PCR. As shown in Fig. 4E, the mRNAs of LMP1 of treatment groups were decreased to 40.0% when compared to the control group. These results support the above data that triptolide decreases cell proliferation of EBV-induced B cells through a LMP1-related mechanism.

4. Discussion

LMP1 is a major oncogene of EBV latent infection for promoting proliferation and transformation of resting B cells [5,17,19,24]. Therapeutics targeting LMP1 provides a novel strategy of EBV-induced tumor. This study has shown that triptolide effectively inhibited proliferation of EBV-positive B lymphocytes by decreasing expression of LMP1 *in vitro* and *in vivo*. The LMP1 promoter activity during EBV latency III infection was reduced by triptolide in HeLa cells. Furthermore, over-expression of LMP1 attenuated the suppressive effects of triptolide in EBV-positive B lymphocytes. These findings supported that triptolide reduces proliferation of EBV-positive B lymphoma cells possibly through a LMP1-related mechanism.

It was found that triptolide induced cell apoptosis in pancreatic cancer cells via inhibition of Hsp70 [13]. Our previous study has found that an Hsp90 inhibitor 17-DMAG increased expression of LMP1 [20] and Hsp70 (not published) in lymphoblasts cell lines. Our findings suggest that triptolide decreases the transcriptional levels of LMP1 in EBV-positive B cells. Interestingly, triptolide did not inhibit expression of LMP1 in NPC cells. LMP1 transcription is controlled by the TR-L1 promoter in type II latent infection or ED-L1 promoter in type III latent infection, respectively [9]. It is possible that the effect of triptolide on LMP1 transcription may be due to the fact that transcription is derived from different LMP1 promoters. Our results suggest that triptolide decreases the activity of ED-L1 rather than the activity of TR-L1. The suppressed activity of ED-L1 leads to reduction in the LMP1 transcription levels.

The function of LMP1 has been reported to promote proliferation of B lymphocytes [17,19]. Consistently, over-expression of LMP1 increased the viabilities of B95-8 and P3HR-1. As our speculation, over-expression of LMP1 attenuated the inhibitory effect of triptolide. These results suggest that the reduced proliferations of EBV-positive B cells by triptolide treatment were due to the decrease in LMP1 levels. Consistent with the ability of Hsp90 inhibitors (such as 17-AAG and Radicicol) to repress the tumors in NOG mice by decreasing the LMP1 expression [21], triptolide shows a significant effect on inhibition of the tumor growth caused by B95-8 cells on BALB/c nude mice. Triptolide decreases the LMP1 expression of treatment group at both the protein and mRNA levels. Targeting LMP1-mediated glycolysis also increases NPC cells sensitivities to radiation therapy [23].

In conclusion, it is demonstrated here that triptolide shows an ability of reducing proliferation of EBV-positive B cells. Over-expression of LMP1 attenuates effects of triptolide treatment in

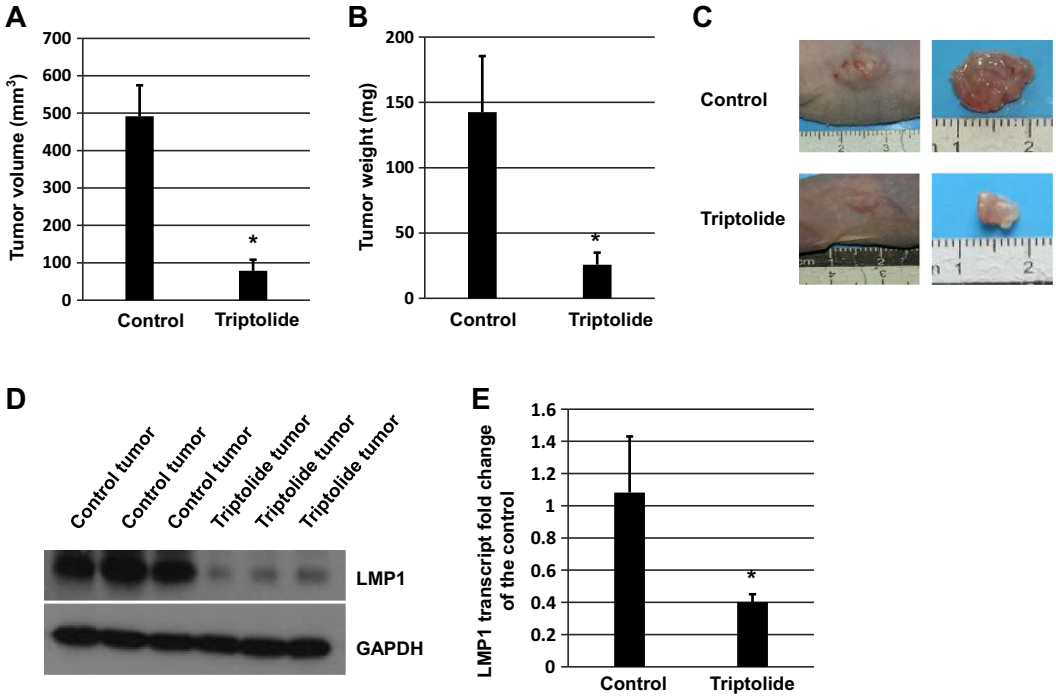

Fig. 4. Triptolide inhibits proliferation of B lymphocyte-induced tumor *in vivo*. Nude mice with the B95-8 cell-induced tumors were treated with DMSO or 0.4 mg/kg triptolide (n = 5 animals per group) for 21 days. (A) Tumor volumes and (B) weigh are given. (C) Representative photos of the non-dissected (left) and dissected (right) tumors were shown. (D) Effects of triptolide on protein level of LMP1 in tumor tissues. (E) The LMP1 mRNA expression of tumor tissues. Expression of LMP1 was normalized against the housekeeping gene GAPDH.

EBV-positive B cells. The inhibited activity of ED-L1 promoter can provide a clue for the decrease of LMP1 transcription levels induced by triptolide. It is hoped that triptolide may be useful as anti-cancer drug against EBV-associated B cell lymphoma.

Conflict of interests

No conflict of interests is stated by authors.

Acknowledgments

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