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IL-10 knockdown with siRNA enhances the efficacy of Doxorubicin chemotherapy in EBV-positive tumors by inducing lytic cycle via PI3K/p38 MAPK/NF-kB pathway

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ABSTRACT

High levels of IL-10 expression in Epstein–Barr virus (EBV) associated tumors have been reported and it is likely to be important for maintaining EBV latency and EBV-associated tumors. The switch from the latent form of EBV to the lytic form in tumor cells can lead to tumor cell lysis. Here, we found that knockdown of IL-10 induced EBV lytic replication. Subsequently, we demonstrated that IL-10 knockdown activated *BZLF1* promoter through PI3K-p38 MAPK-NF-κB signaling pathway. Interestingly, we verified that VEGF-A was required for IL-10 knockdown to activate PI3K signaling and the accompanying EBV lytic induction. Exogenous recombinant human VEGF-A induced PI3K activation and EBV lytic infection, and inhibition of VEGF-A signaling prevented the PI3K/AKT phosphorylation and EBV reactivation responded to IL-10 knockdown. Most importantly, IL-10 knockdown synergized with chemotherapeutic agent Doxorubicin to kill EBV associated tumor cells *in vitro* and repress EBV-positive tumor growth *in vivo*. Our results suggest that inhibition of IL-10 has the potential to serve as a new supplemental strategy for the treatment of EBV-associated tumors.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human gamma herpes virus, replicating in B lymphocytes and some epithelial cells. It can establish a lifelong infection in more than 90% of the human population. EBV infection is associated with the development of various malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, NK/T cell lymphoma, gastric carcinoma, and undifferentiated nasopharyngeal carcinoma, etc. [1–3].

The life cycle of EBV is divided into latent and lytic phases, and primary lytic infection often proceeds into long-term latent EBV infection [4,5]. EBV only expresses limited genes during latency, including latent membrane protein LMP1, LMP2A and small RNA EBERs [6]. The reactivation of EBV from latent to lytic phase can be triggered by expression of the two viral immediate-early gene products, BZLF1 (also called ZTA or Z) and BRLF1 (also known as RTA or R) [7]. *BZLF1* and *BRLF1* are transcribed from two different immediate early genes, *BZLF1* and *BRLF1*, respectively. Subsequently, BZLF1 and BRLF1 activate both their own and one another's promoters, *BZLF1* promoter (Zp) and *BRLF1* promoter (Rp), to greatly amplify their lytic-inducing effects [7]. They then cooperatively activate the promoters of early (E) lytic genes that encode the viral replication proteins. Following viral genome replication, the late (L) viral genes are expressed. The expression of BZLF1 and BRLF1 is tightly regulated to maintain latency, and a number of cellular and viral factors play important roles in Zp and Rp regulation [1,8–20].

EBV-positive tumors are composed almost exclusively of cells with latent EBV infection. Switching the latent EBV infection into lytic form can induce tumor cell death [21,22]. Thus, strategies for inducing the lytic form of EBV infection in tumor cells are investigated as potential therapies for EBV-positive tumors [21,23–25].

IL-10 is a multifunctional cytokine produced by various cells. Previous studies have observed high levels of IL-10 expression in EBV-associated tumor lesions [26–35]. The high level of IL-10 in tumor

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microenvironment is likely to be important for maintaining EBV latency and EBV-associated tumors. First, IL-10 could inhibit T cell proliferation and IFN-γ secretion induced by both mitogen and recall Ag, and the protective Th1 and cytotoxic responses against latent protein LMP1 [36]. Secondly, IL-10 is an autocrine growth factor for EBV-infected B cell lines [37,38], and neutralization of IL-10 can significantly inhibit the proliferation of these cells *in vitro* [38]. Moreover, IL-10 may be responsible for the establishment and maintenance of EBV latency in EBV-infected cells through the induction of LMP1 protein, which is required for the transformation and proliferation of B cells *in vitro* [39].

Given the significant role of IL-10 in maintaining EBV latent infection, we hypothesize that inhibition of IL-10 can induce lytic infection and thereby inhibit EBV-associated tumor cell proliferation. Here, we demonstrated that knockdown of IL-10 induced EBV reactivation via PI3K/AKT-p38 MAPK-NF-κB pathway. Interestingly, we found that VEGF-A played an important role in the activation of PI3K/AKT signaling pathway and the accompanying EBV reactivation induced by IL-10 knockdown. Importantly, IL-10 knockdown synergized with chemotherapeutic agent Doxorubicin (DOX) to kill EBV associated tumor cells *in vitro* and repress EBV-positive tumor growth *in vivo*. Our results suggest that IL-10 knockdown has the potential to serve as a new supplemental strategy for the treatment of EBV-associated tumors.

2. Materials and methods

2.1. Cell lines

Gastric carcinoma cell line AGS was obtained from American Type Culture Collection. AGS-EBV cell line was created by infecting AGS cells with the wild-type B-95-8 strain. AGS and AGS-EBV cells were maintained in Ham's F-12 medium with 10% fetal bovine serum (FBS). LCL cell line was a gift from Dr. Xiongwen Wu (Tongji Medical College, China). EBV positive primary effusion lymphoma cell line JSC-1 was a gift from Dr. Ke Lan (Wuhan university, China). Burkitt's lymphoma cell line Raji was obtained from China Infrastructure of Cell Line Resource. LCL, JSC-1, and Raji cells were maintained in RPMI 1640 with 10% FBS. All cells were cultured in an incubator with 5% CO_2 at 37 °C.

2.2. Reagents and siRNAs

Doxorubicin, VEGF receptor inhibitor axitinib, and PKC inhibitor GF109203X were purchased from Sigma. Signal pathway inhibitors (p38 MAPK inhibitor SB203580, c-Jun N-terminal kinase (JNK) inhibitor SP600125, NF-κB inhibitor BAY11-7082, and JAK inhibitor Ruxolitinib) were purchased from Enzo Life Science. PI3K inhibitor LY294002, ERK inhibitor PD98059, mTOR inhibitor Rapamycin, STAT1 inhibitor Fludarabine, and IL-10 inhibitor AS101 were purchased from Selleck Company. Human VEGF-A165 was purchased from CST. Human VEGFR2 blocking antibody was purchased from R&D. siRNAs targeting human IL-10 and VEGF-A were purchased from Santa Cruz Biotechnology.

2.3. Cell viability assays

AGS-EBV and AGS cells in 96-well plates were grown to 70–80% confluence and then transfected with 50 nM siIL-10. At 24 h later, Doxorubicin (0.05 μM) or equal volume of DMSO as control was added to cells. Cells were cultured for another 48 h, and then the culture medium was replaced with fresh medium containing 20 μl of 5 mg/ml MTT (3-(4,5)-dimethylthiazol)-z-y1)-3,5-di-phenytetrazoliumromide) (Promega). After washing thrice with PBS, cells were further cultured for 4 h at 37 °C. Cells were subsequently washed carefully and 150 μl of DMSO (per well) was added to dissolve the crystals for 10 min. The resulting absorbance of each well was recorded at 490 nm using a plate reader.

LCL cells transfected with siIL-10 (50 nM) were plated in 96-well plates and then treated with Doxorubicin (0.02 μM) 24 h post-transfection. At 4 days post treatment with Doxorubicin, the percentage of survival cells was analyzed by Cell Titer 96® AQueous One Solution Cell Proliferation Assay (MTS assay) (Promega), which is used to detect suspension cell viability. 20 μl of Cell Titer 96® AQueous One Solution Reagent was added into each well of the 96-well plates, followed by culturing cells for 4 h at 37 °C. The resulting absorbance of each well was recorded at 490 nm using a plate reader.

2.4. ELISA for IL-10 analysis

IL-10 in the supernatants of cell cultures was determined by ELISA kit (Human IL-10 Quantikine ELISA Kit, R&D) according to the manufacturer's instructions.

2.5. Western-blots

Whole-cell extracts were prepared with RIPA lysis buffer (Biyuntian, Shanghai, China) supplemented with protease inhibitors and phosphatase inhibitors. Total proteins were quantified using Thermo Scientific Pierce BCA Protein Assay. Similar amounts of proteins for each sample were separated by 10% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk diluted in PBST (1 × phosphate-buffered saline with 0.1% Tween 20) for 1 h at room temperature. After blocking, membranes were incubated overnight at 4 °C with primary antibodies diluted in 5% non-fat milk in PBST. Primary antibodies were diluted as bellows: anti-BMRF1 antibody (1:500; Mouse mAb, Capricorn), anti-BZLF1 antibody (1:500; Mouse mAb, Argene), anti-BRLF1 antibody (1:500; Mouse mAb, Argene), anti-Tubulin antibody (1:5000; Rabbit pAb, CST), anti-phospho-(Thr/Tyr) p38 MAPK Thr180/Tyr182 antibody (1:1000; Rabbit mAb, CST), anti-phospho-(Ser/Thr)AKT -antibody (1:1000; Rabbit pAb, CST), anti-phospho-IκB-antibody (1:1000; Rabbit mAb, CST), anti-IκB-antibody (1:1000; Rabbit mAb, CST), and anti-VEGFA (1:1000, Rabbit pAb, Proteintech). The membranes were then incubated with the appropriate secondary antibody for 1 h (1:5000) at room temperature. The antibodies were visualized by use of the ECL reagent according to the manufacturer's instructions.

2.6. RNA extraction and Real-Time PCR

Total RNA was extracted using Trizol Reagent (Invitrogen) and 500 ng RNA was used for cDNA synthesis using M-MLV (Promega). Real-time PCR was performed on ABI viiA7 Real-Time PCR System using the Real-Time SYBR master mix kit (TAKARA) following the manufacturer's introductions. Relative expression levels were analyzed using the $\Delta\Delta C_T$ method, and *GAPDH* mRNA was set up as an endogenous control. All PCR experiments were done in triplicate. All the specific primers used were shown in Table 1.

2.7. Determination of EBV DNA level

EBV DNA was determined as previously described [40]. Briefly, total genomic DNA was extracted using DNeasy Blood & Tissue Kit (QIGEN), and then determined by Real-Time PCR analysis. Relative levels of viral DNA were determined by the $\Delta\Delta C_T$ method using cell viral DNA without lytic induction as control. The reference gene *GAPDH* was used for data normalization. The *BALF5* primers used for Real-Time PCR were described previously [41] and shown in Table 1.

2.8. Indirect immunofluorescent assay

Cells were fixed with cold methanol-acetone (1:1) for 10 min at 4 °C, washed with phosphate-buffered saline (PBS), and then blocked with 5% normal goat serum for 30 min at room temperature. After blocking,

Table 1
Primers used for Real-Time PCR.

Target	Forward primer	Reverse primer
IL-10	ACCAAGACCCAGACATCAA	CATTCTTCACCTGCTCCAC
BZLF1	AATGCCGGGCCAAGTTTAAGCAAC	TTGGGCACATCTGCTTCAACAGGA
BRLF1	TGGCTTGGAAGACTTTCTGAGGCT	AATCTCCACACTCCCGGCTGTAAA
BMRF1	GCCGCCGTGTCATTTAGAAACCTT	TGTGGTGGCTCTTGGACACCTTAT
BALF5	CGTCTCATTCCCAAGTGTTTC	GCCCTTTCCATCCTCGTC
CERS6	ACATCCTGGAGCTGTCGTTT	ACCCAGTCGTGTGGTGATAA
PANK1	CGAAGAGGAGCAAGAGGAA	TGCCCATCTGAATGAACC
ROCK1	TGCTGCTGGATAAATCTGG	AATAACCATCACCACCTTGG
ROCK2	TGCGGATTCACTTGTAGGA	GCTGTCTGATTTCTTCCACC
LIF	AACTGGCACAGCTCAATGGC	TTGGTCTTCTCTGTCCCGTTG
TRIM32	CCGGAGCTGTGGTTTGGT	AGTTCCCGCAGACGAGTT
F2RL1	AGATGGAGTCTCGCTGTG	TGAGGCAGGAGAATCACT
CST1	TGATGAGTGGGTACAGCGTG	TGATGAGTGGGTACAGCGTG
GTGF	CGGCCAACCGCAAGATCG	TCGGGAAGGGGCAGTCAGG
GABRA3	AGTTTCATTTAGGGGATC	ATTTGAGCAGGAAGTTAG
HSPB1	TGACGGTCAAGACCAAGGATGG	TGTATTTCCGCGTGAAGCACC
LMAN1	TCTTTTCTGACTTTCCAGT	GTGGCCCTTCTGGAATTCCTCTTTT
CDC25B	AGCCGTGGAGCACTCACA	TGAAGGATGGACCAAGACC
SKIL	CAAGTAGCTGGGACCACA	ACTGGGTAAAGACAACTAACAT
SOCS3	ATGGTCACCCACAGCAAGTTT	TCCAGTAGAATCCGCTCTCCT
VEGFA	GCACCCATGGCAGAAGGAGG	TATCACCGCCTCGGCTGTC
GAPDH	ATCATCAGCAATGCCTCCT	CATCACGCCACAGTTTCC

cells were stained with anti-BZLF1 monoclonal antibody (1:50; Mouse mAb, Santa Cruz), or anti-BMRF1 monoclonal antibody (1:50; Mouse mAb, Santa Cruz) for 60 min at 37 °C. Cells were then washed and incubated with Alexa Fluor Plus 555 Goat anti-Mouse IgG (H + L) (1:1000, Thermofisher scientific) for 60 min at 37 °C. After three washes in PBS, cell nuclei were counter-stained with DAPI and examined by fluorescence microscopy. BZLF1 or BMRF1 positive cells (red) was counted by Image J software, and the percentage of BZLF1 or BMRF1 positive cells was calculated against DAPI positive total cells (blue).

2.9. Construction of EBV IE promoter plasmids

EBV IE gene *BZLF1* promoter sequence from −1729 to +39 and *BRLF1* promoter sequence from −1064 to +15 were cloned into pGL3-basic plasmid (Sigma) between NheI and BglII sites to create luciferase reporter Zp (Zp-Luc) and luciferase reporter Rp (Rp-Luc), respectively. The NF-κB binding site mutant (Zp-NFκB-mut) or deletion (Zp-NFκB-dele) Zp was generated by PCR using primers shown in Table 2. Endo-free Plasmid Mini Kit (QIGEN Biotek) was used to purify DNA plasmids according to the manufacturer's protocol.

2.10. Luciferase reporter assay

AGS cells seeded in 24-well plates were transfected with different luciferase reporter vectors using Jetprime transfection Reagent (PolyPlus). At 24 h later, cells were transfected with siIL-10 or NC. At the indicated time points post transfection, luciferase activities were analyzed using the Dual-Glo luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.11. Tumor studies in-vivo

All animal experiments were approved by the Beijing Association for Science and Technology (approval ID SYXK (Beijing) 2007–0023) and complied with the guidelines of Beijing Laboratory Animal Welfare and Ethics of the Beijing Administration Committee of Laboratory Animals. All animal studies were also performed in accordance with the China Agricultural University Institutional Animal Care and Use Committee guidelines (ID: SKLAB-B-2010-003) and approved by animal welfare committee of China Agricultural University.

LCL-1 cells (5×10^7/each side) were implanted subcutaneously into both sides of the flanks of 6-week-old SCID mouse. When tumors were palpable, mice (4 mice with 8 tumors/each group) were treated with no drug, one dose of Doxorubicin (5 mg/kg of body weight) intra-peritoneally (i.p.), siIL-10 alone (1 mg/kg of body weight once every 6 days for 2 times) or the combination of one dose of Doxorubicin (5 mg/kg of body weight, i.p.) and siIL-10 (1 mg/kg of body weight once every 6 days for 2 times). siIL-10 mixed with Invivofectamine 3.0 (Thermofisher scientific) according to the manufacturer's instructions was injected into the tumors. Tumor volumes were measured every day after treatment. Mice were euthanized when tumor size exceeded 1 cm^3.

2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, and differences in data were evaluated by Student's *t*-test. Differences were considered to be statistically significant if the *P* value is less than 0.05. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

3. Results

3.1. Knockdown IL-10 induces EBV reactivation

To demonstrate whether reducing IL-10 level regulates EBV lytic replication, we utilized siRNA to knockdown IL-10 in AGS-EBV cells.

Table 2
Primers used for cloning of Zp and Rp.

Target	Forward primer	Reverse primer
Zp-1768	CGGCTAGCTTTCTAAATGATGAATGTCTGC	GAAGATCTATAGCAAAGGTGGCCGGCAA
Zp-NFkB-mut	TCCGATCCTCGTGTCCGGAGCCG	GTTCAAACTGCCTTGGACTTGGTTGACAG
Zp-NFkB-dele	TCCTCGTGTCCGGAGCCG	ACTGCCTTGGACTTGGTTGACAG
Rp-1079	CGGCTAGCTTGTGTCCTGTATGGTATTCT	GAAGATCTCTCTTCGGGAAATTGGAAGGTG

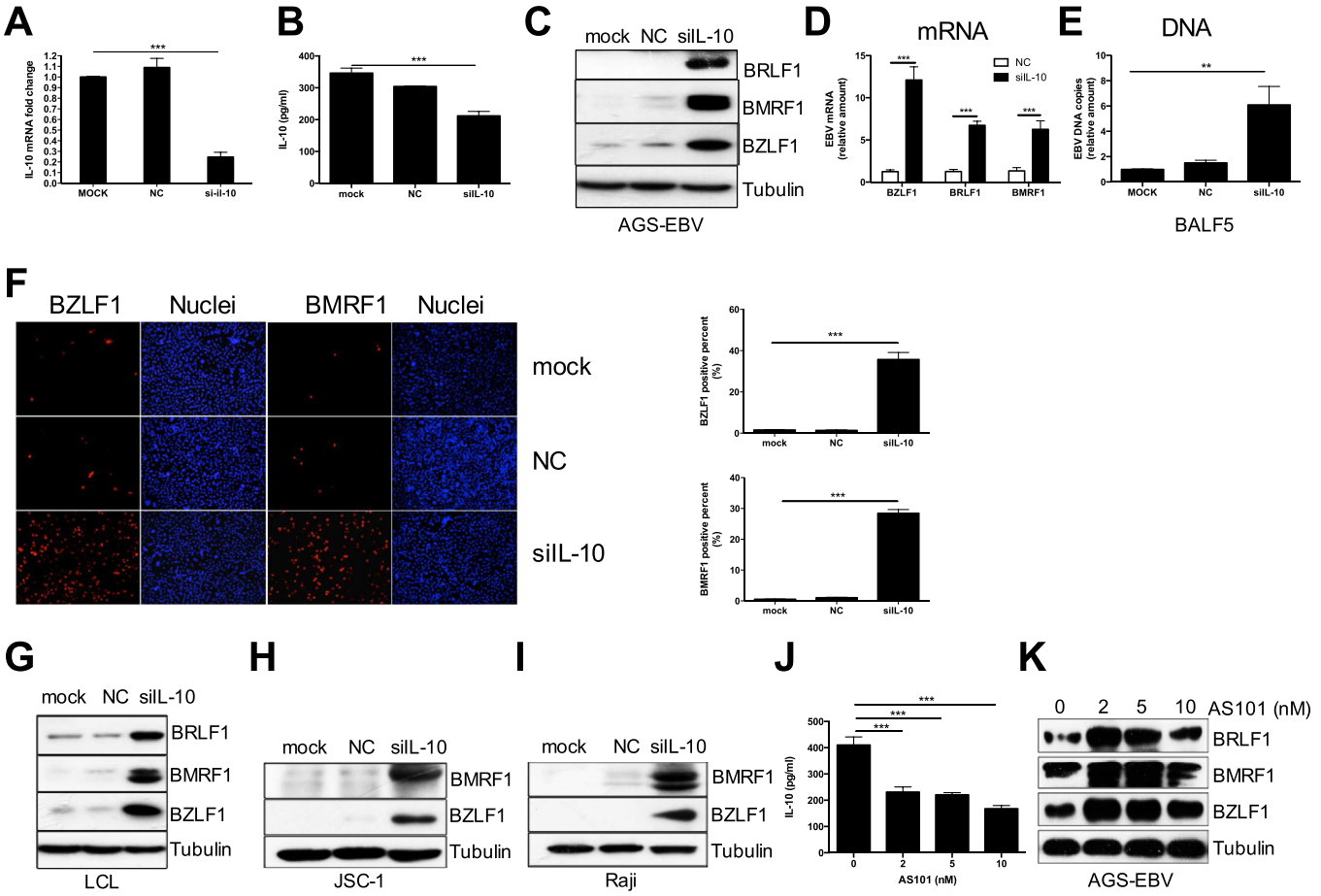


Fig. 1. IL-10 knockdown activates EBV in AGS-EBV and LCL cells. RT-qPCR (A) and ELISA (B) were performed to analyze the knockdown efficiency by siIL-10 compared with the non-targeting control siRNA (NC). (C) IL-10 knockdown induces EBV lytic activation in AGS-EBV cells. AGS-EBV cells were transfected with either NC or siIL-10 (50 nM). At 48 h post-transfection, EBV lytic infection was assessed by Western blot using antibodies against BRLF1, BMRF1 and BZLF1. Tubulin was included as loading control. (D) RT-qPCR was conducted to detect EBV *BZLF1*, *BRLF1* and *BMRF1* mRNA expression upon IL-10 knockdown. (E) Induction of EBV replication upon IL-10 knockdown. The EBV DNA level was evaluated by qPCR using primers specific to EBV *BALF5*. (F) Detection of EBV BZLF1 and BMRF1 protein expression upon IL-10 knockdown by IFA. AGS-EBV cells were transfected with siIL-10 (50 nM), and BZLF1 and BMRF1 protein expression were detected by IFA 48 h post-transfection. Representative images were shown at 200× magnification. BZLF1 or BMRF1 positive cells (red) were counted by Image J software, and the percentage of BZLF1 or BMRF1 positive cells was calculated against DAPI positive total cells (blue) (n = 6). (G–I) IL-10 knockdown activates EBV in LCL cells (G), JSC-1 cells (H) and Raji cells (I). Cells were transfected with either NC or siIL-10 (50 nM). At 48 h post-transfection, protein extracts were analyzed by Western blot using antibodies against BRLF1, BMRF1 and BZLF1. Tubulin was included as loading control. (J–K) IL-10 inhibitor AS101 decreases IL-10 production and induces EBV lytic protein expression. AGS-EBV cells were treated with AS101 at various concentrations for 48 h, and cell supernatant was collected to analyze IL-10 production by ELISA (J). EBV lytic protein expression was verified by Western blot (K). The results were presented as mean ± standard deviation. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Compared with non-targeting control (NC), the siRNA targeting IL-10 (siIL-10) efficiently reduced IL-10 mRNA expression in AGS-EBV cells (Fig. 1A) and protein level in cell supernatant (Fig. 1B). Interestingly, knockdown of IL-10 resulted in elevated levels of the EBV latent-to-lytic switch proteins BZLF1 and BRLF1, and early protein BMRF1 (Fig. 1C). We then examined the level of lytic RNA transcripts in siRNA-transfected cells. As expected, knockdown IL-10 dramatically up-regulated the expression of immediate early genes (*BZLF1* and *BRLF1*) and the early gene *BMRF1* (Fig. 1D). To test whether knockdown IL-10 promotes EBV replication, we measured intracellular EBV genome copies and found that viral DNA replication was significantly increased upon IL-10 down-regulation (Fig. 1E). We need to indicate that infectious EBV particles are generated by IL-10 knockdown (data not shown). To further investigate whether the up-regulation of EBV lytic protein was due to more cells entering the lytic cycle, we detected the expression of BZLF1 and BMRF1 using indirect immunofluorescence assay (IFA). The results indicated that siIL-10 transfection induced more cells expressing BZLF1 and BMRF1 as compared with mock and NC transfected cells, and the percentage of BZLF1 or BMRF1 positive cells increased to about 35% and 28%, respectively (Fig. 1F). We further investigated whether knockdown of IL-10 could induce EBV lytic infection in other EBV associated tumor cell lines. As shown in Fig. 1G–I, down-regulation of IL-10 also induced lytic EBV infection in EBV positive B cell line LCL cells (Fig. 1G), primary effusion lymphoma cell line JSC-1 cells (Fig. 1H) as well as Burkitt's lymphoma cell line Raji cells (Fig. 1I). These results suggest that the reactivation of lytic EBV infection by IL-10 knockdown is independent of cell lines. To further verify that the observed phenomenon is not due to off-target effects, we utilized the nontoxic immunomodulator ammonium trichloro (dioxoethylene-o,o') tellurate (AS101), which directly inhibits IL-10 production [42], to treat AGS-EBV cells. Our results showed that inoculation with AS101 significantly blocked IL-10 secretion (Fig. 1J). Accordingly, EBV lytic infection was significantly induced by AS101 (Fig. 1K).

These results suggest that knockdown of IL-10 acts as a positive regulator for EBV lytic reactivation.

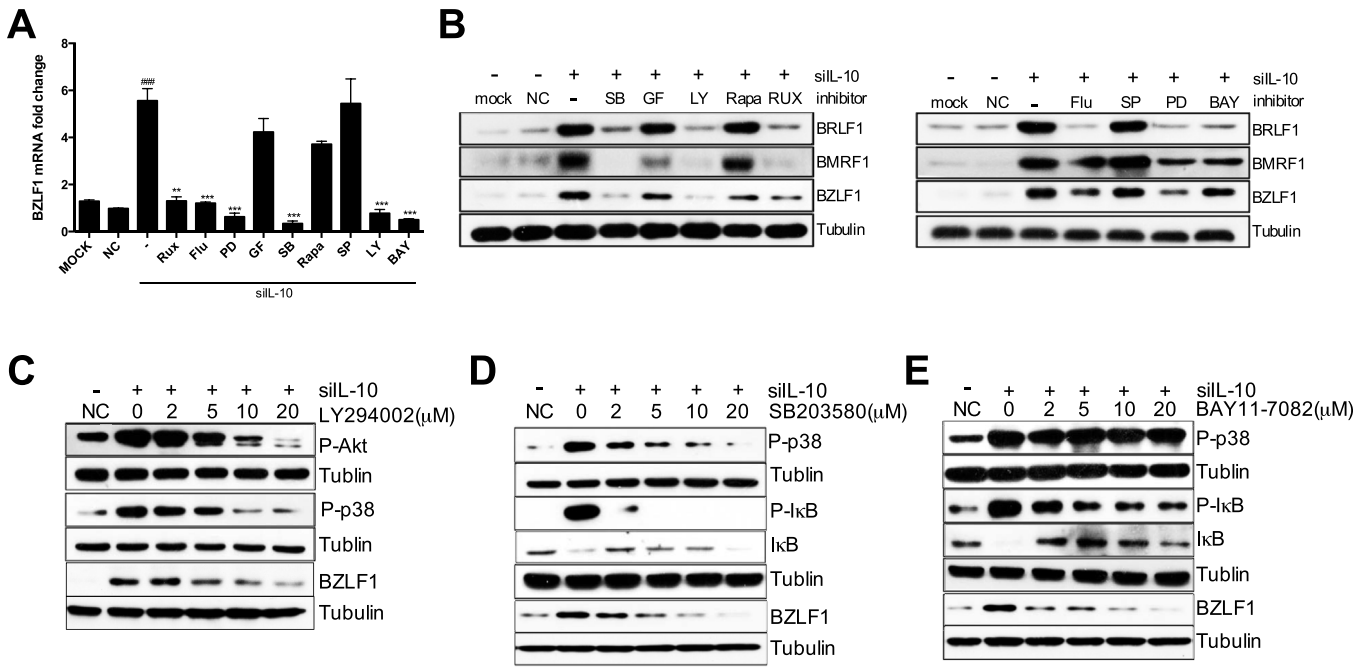


Fig. 2. PI3K, p38 MAPK, and NF-κB pathways are involved in EBV activation upon IL-10 knockdown. AGS-EBV cells were pre-treated with specific inhibitors of p38 MAPK (SB203580), PKC (GF109203X), PI3K (LY294002), mTORC1/2 (Rapamycin), JAK (Ruxolitinib), STAT1 (Fludarabine), JNK (SP600125), ERK (PD98059) or NF-κB (BAY11-7082) individually for 1 h, and then transfected with either NC or siIL-10 (50 nM). At 48 h post transfection, EBV *BZLF1* mRNA was quantified by RT-qPCR (A) and EBV protein expression was analyzed by Western blot (B). AGS-EBV cells were pretreated with various concentrations of either PI3K inhibitor LY294002 (C), P38 inhibitor SB203580 (D) or NF-κB inhibitor BAY11-7082 (E) for 1 h, and then transfected with either NC or siIL-10 (50 nM). Phosphorylation of AKT, p38 MAPK and IκB as well as the BZLF1 expression were analyzed by Western blot at 3 h, 6 h, 12 h, and 48 h post transfection, respectively. Tubulin was included as loading control. The results were presented as mean ± standard deviation. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

3.2. PI3K-p38 MAPK-NF-κB signal pathway is involved in EBV reactivation induced by IL-10 knockdown

To investigate the signal transduction pathways involved in the activation of lytic EBV infection upon IL-10 knockdown, AGS-EBV cells were pre-treated with specific inhibitors of p38 MAPK, PKC, PI3K, mTORC1/2, JAK, STAT1/2, JNK, ERK or NF-κB individually for 1 h, and then transfected with siIL-10. At 48 h post transfection, we examined the *BZLF1* mRNA expression in the presence of various inhibitors. As shown in Fig. 2A, up-regulation of *BZLF1* mRNA by siIL-10 was significantly repressed by p38 MAPK, PI3K, JAK, STAT1, ERK and NF-κB kinase inhibitors. To further confirm these results, we examined EBV lytic proteins using Western blot. As shown in Fig. 2B, inhibition of p38 MAPK, PI3K, JAK, STAT1, ERK and NF-κB potently blocked EBV lytic reactivation induced by IL-10 knockdown. Then, we focused on PI3K, p38 MAPK, and NF-κB signaling pathways. As inhibition of PI3K, p38 MAPK or NF-κB impaired EBV reactivation, we assessed whether P38 MAPK and NF-κB were in the downstream of PI3K. We pretreated cells with inhibitors against PI3K, p38 MAPK or NF-κB before the transfection of siIL-10, and then detected the kinase phosphorylation and EBV lytic infection at various time-points by immunoblot analysis. As shown in Fig. 2C–E, siIL-10 potently activated PI3K, p38 MAPK and NF-κB pathways, while IL-10 knockdown-induced EBV lytic infection was blocked by the inhibitors of PI3K, p38 MAPK, and NF-κB dose-dependently. As expected, PI3K inhibitor LY294002 blocked the phosphorylation of AKT and p38 MAPK in a dose-dependent manner (Fig. 2C), while p38 MAPK inhibitor SB203580 down-regulated the phosphorylation of p38 MAPK and IκB induced by siIL-10 dose-dependently (Fig. 2D). The inhibitor of NF-κB was unable to block p38 MAPK activation, although it potently abolished the phosphorylation of IκB upon IL-10 knockdown (Fig. 2E). Collectively, these results suggest that knockdown of IL-10 induces EBV lytic replication via PI3K-p38 MAPK-NF-κB signaling pathways.

3.3. NF-κB binding site is essential for IL-10 knockdown to activate EBV BZLF1 promoter

To determine whether knockdown of IL-10 directly activates *BZLF1* and/or *BRLF1* immediate-early gene promoters (Zp and Rp), AGS cells were transfected with siIL-10 and *BZLF1* or *BRLF1* luciferase promoter. Our results showed that down-regulation of IL-10 significantly activated *BZLF1* and *BRLF1* promoters (Fig. 3A). Since knockdown of IL-10 induces lytic EBV activation via PI3K-p38 MAPK-NF-κB signaling pathways, we used bioinformatics to analyze *BZLF1* and *BRLF1* promoters. Interestingly, we found that there was one putative NF-κB binding site on Zp. To further confirm the forecasting result, we constructed Zp luciferase reporter plasmids with deletion or mutation of the putative NF-κB binding site (Fig. 3B). Mutation or deletion of the NF-κB binding-site nearly abolished Zp activation induced by IL-10 knockdown (Fig. 3C). These results indicate that IL-10 knockdown activates EBV lytic infection via NF-κB on *BZLF1* promoter.

3.4. VEGF-A is required for IL-10 knockdown to activate EBV lytic activation

Next, to explore the upstream effector responsible for EBV lytic activation upon IL-10 knockdown, we screened the expression of some molecules associated with PI3K signaling. Surprisingly, *VEGF-A* mRNA was dramatically induced upon IL-10 knockdown, with an ~17 times increase compared to the control (Fig. 4A). VEGF-A exerts its angiogenic actions by binding to VEGF receptors 1 (VEGFR1) and 2 (VEGFR2) on the surface of several cells, including endothelial cells [43]. To investigate whether IL-10 knockdown can activate EBV lytic infection via VEGF-A, we treated siIL-10-transfected cells with axitinib, one of the best-characterized VEGF receptor inhibitors that can block the signal transduction from VEGF-A [44]. As shown in Fig. 4B, siIL-10 significantly induced *BZLF1* up-regulation (Fig. 4B), while addition of axitinib blocked the *BZLF1* induction by siIL-10 dose-dependently

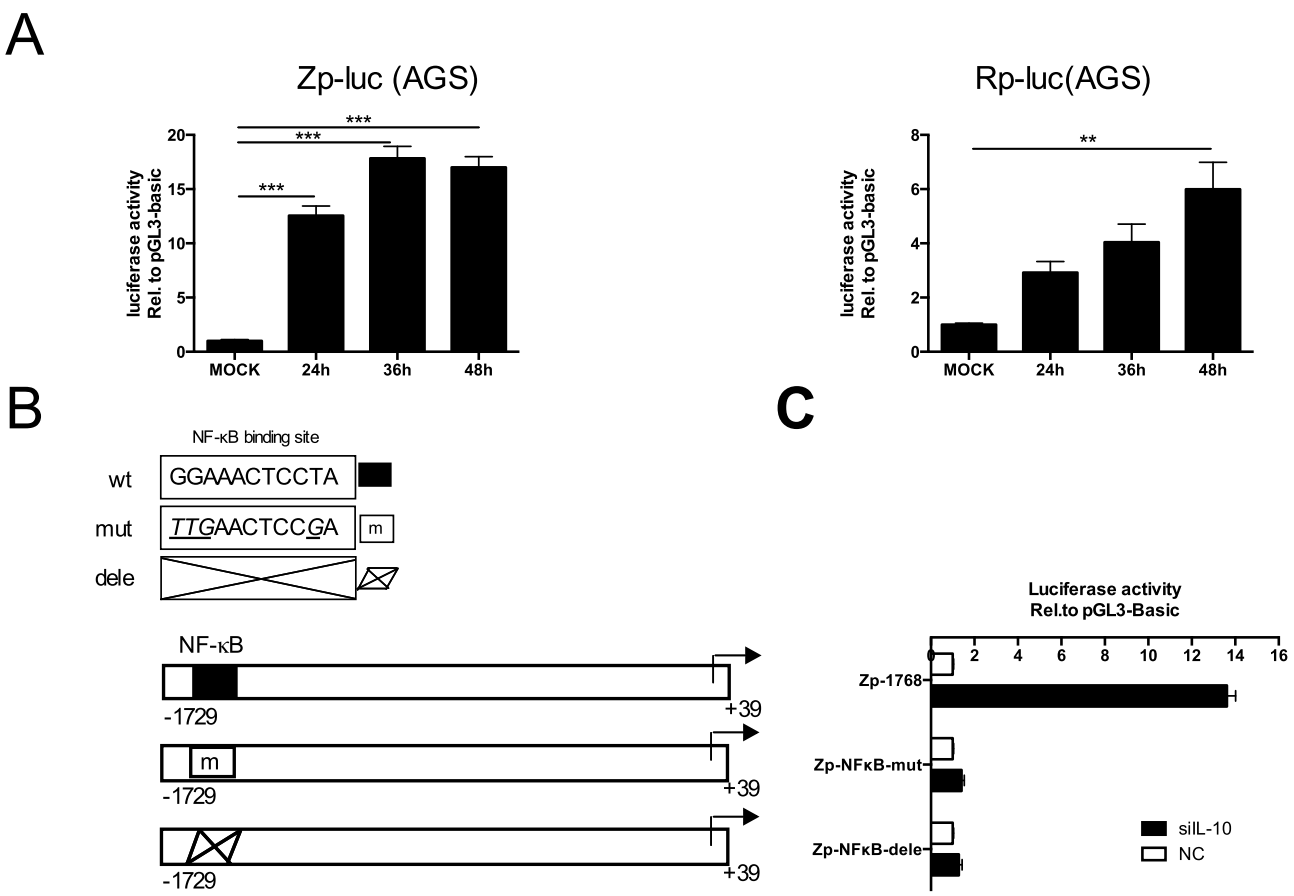


Fig. 3. IL-10 knockdown activates EBV *BZLF1* via NF-κB. (A) *BZLF1* or *BRLF1* promoter construct was transfected into AGS cells with internal reference plasmid 24 h before siIL-10 (50 nM) transfection. Samples were harvested at various time points after siIL-10 transfection for Luciferase analysis. The value at 0 h post siIL-10 transfection was set as 1. (B) Schematic representation of EBV *BZLF1* promoter. ■: predicted NF-κB binding site; ☒: deletion of the predicted NF-κB binding site; m: mutation of the predicted NF-κB binding site. (C) AGS cells were transfected with pGL3-BZLF1 promoter constructs as indicated in (B) 24 h before either NC or siIL-10 (50 nM) transfection. Luciferase assays were performed at 24 h post siRNA transfection. The value of cells transfected with NC was set as 1. The results were presented as mean ± standard deviation. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

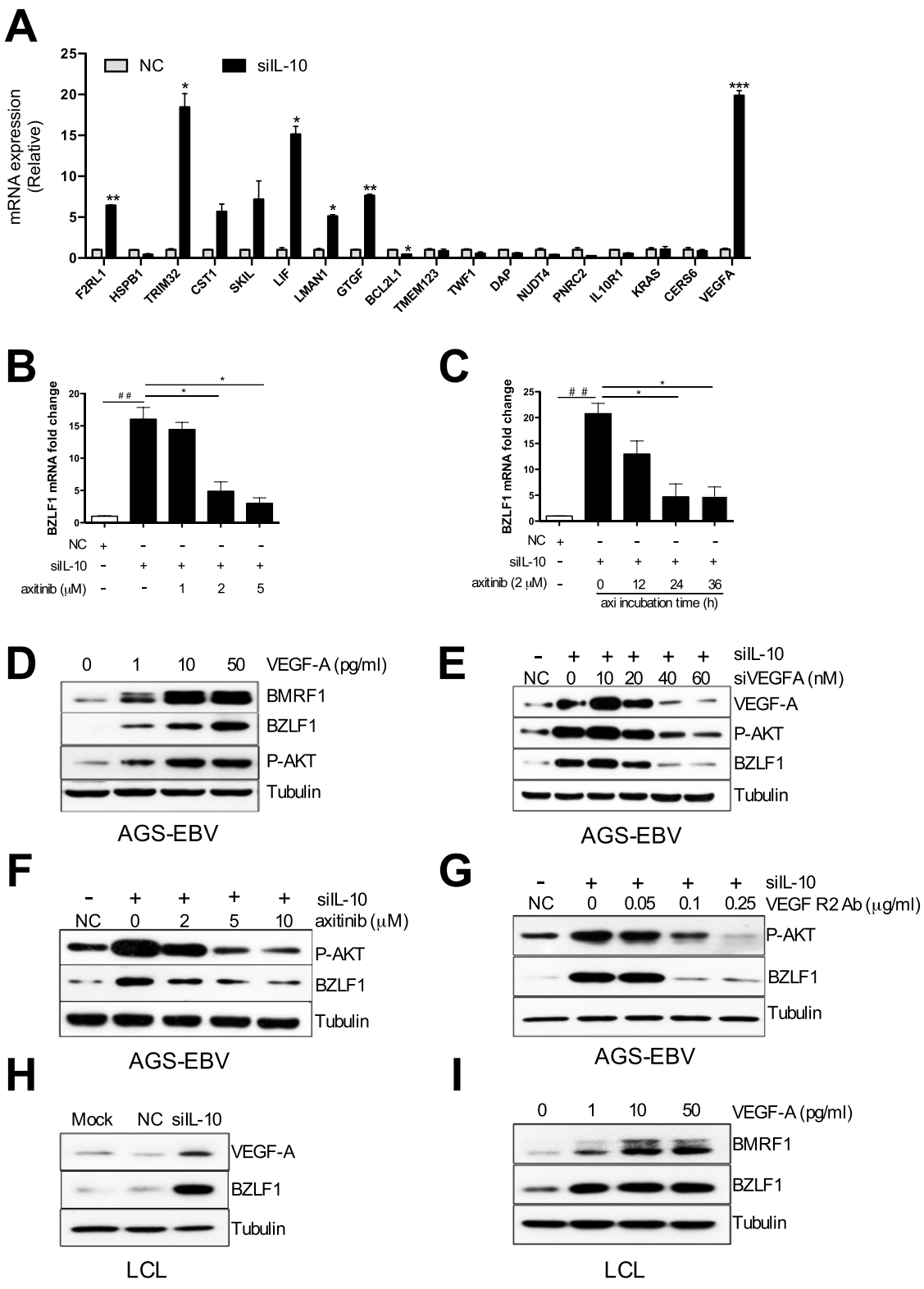
(Fig. 4B) and time-dependently (Fig. 4C). Axitinib at the concentration of 5 μM inhibited siIL-10-induced *BZLF1* expression by ~80%. To further verify whether VEGF-A induces EBV lytic infection, we treated AGS-EBV cells with various concentrations of VEGF-A, and then analyzed EBV lytic infection by Western blot. As shown in Fig. 4D, the addition of VEGF-A into cell culture indeed activated EBV lytic infection. Meanwhile, AKT phosphorylation was also activated by the addition of VEGF-A. Next, we investigated the involvement of VEGF-A in the induction of downstream signaling pathways and EBV lytic activation induced by knockdown IL-10. We transfected AGS-EBV cells with siIL-10 and siVEGF-A, and EBV reactivation and VEGF-A expression were then evaluated at 48 h post siRNA transfection. As shown in Fig. 4E, VEGF-A protein expression was potently up-regulated by IL-10 knockdown compared to the NC. However, IL-10 knockdown-induced VEGF-A expression was inhibited by siVEGF-A transfection. Importantly, knockdown of VEGF-A significantly inhibited AKT phosphorylation, the PI3K effector, and *BZLF1* expression responded to IL-10 knockdown. Similar results were also obtained by using axitinib to inhibit VEGFR signaling (Fig. 4F). We further examined whether blocking VEGF receptor with neutralizing antibody can block the activation of EBV upon IL-10 knockdown. And our results showed that the neutralizing antibody against VEGF R2 effectively impaired AKT phosphorylation and BZLF1 expression induced by siIL-10 (Fig. 4G). We also confirmed the involvement of VEGF-A in the induction of EBV lytic reactivation in LCL cells. As shown in Fig. 4H, siIL-10 transfection up-regulated the expression of VEGF-A protein, along with the induction of BZLF1. Consistently, addition of VEGF-A into the cell culture re-activated EBV lytic infection (Fig. 4I).

These results imply that VEGF-A plays an important role in the activation of PI3K signaling pathway and EBV lytic infection upon IL-10 knockdown.

3.5. IL-10 knockdown synergizes with Doxorubicin to kill EBV$^+$ tumor cells both in vitro and in vivo

We next investigated whether the combination of IL-10 knockdown with Doxorubicin, a chemotherapeutic agent, had synergistic effect on the induction of EBV lytic infection. AGS-EBV cells or LCL cells were treated with Doxorubicin 24 h post transfection of siIL-10, and 24 h later immunoblot analysis was performed to analyze EBV lytic infection. As expected, the induction of lytic protein expression responded to Doxorubicin was significantly enhanced by IL-10 knockdown as compared to siIL-10 or Doxorubicin alone in both AGS-EBV (Fig. 5A) and LCL cell lines (Fig. 5B).

To investigate whether knockdown of IL-10 has a cell killing effect on EBV-positive cells, we transfected AGS-EBV cells and EBV-negative AGS cells with siIL-10. At 48 h post-transfection, cell viability was determined by MTT assay. While siIL-10 was unable to suppress the viability of AGS cells (Fig. 5C), the viability of AGS-EBV cells transfected with siIL-10 was significantly reduced to 83% compared with mock-transfected cells (Fig. 5D). As expected, knockdown of IL-10 in EBV positive LCL cells also impaired cell survival, and the viability of siIL-10



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transfected cells declined to about 70% as compared with the controls (Fig. 5E). Importantly, siIL-10 knockdown potently enhanced EBV-positive cell killing effect of Doxorubicin as compared with Doxorubicin treatment alone (Fig. 5D and E).

We further investigated whether IL-10 knockdown could enhance the efficacy of Doxorubicin for treating EBV-positive tumors *in vivo*. As shown in Fig. 5F, siIL-10 alone slightly inhibited tumor growth when compared to no treatment control group, while Doxorubicin significantly inhibited tumor growth (Fig. 5F). Importantly, combination of siIL-10 with Doxorubicin more effectively inhibited tumor growth as compared with Doxorubicin treatment alone.

These results suggest that combining IL-10 knockdown with Doxorubicin is more effective than Doxorubicin alone in the treatment of EBV-positive tumors in *SCID* mice.

Fig. 4. VEGF-A is important for the activation of PI3K signaling pathway and EBV lytic infection upon IL-10 knockdown. (A) AGS-EBV cells were transfected with siIL-10 (50 nM), and samples were harvested at 48 h post transfection. The expression of selected genes was analyzed by RT-qPCR. (B) AGS-EBV cells were pretreated with various concentrations of axitinib or left untreated for 1 h, and then transfected with either NC or siIL-10 (50 nM). At 24 h post transfection, samples were harvested and *BZLF1* mRNA expression was analyzed by RT-qPCR. (C) AGS-EBV cells were treated with axitinib (2 μM) at 12 h post siIL-10 (50 nM) transfection, and then *BZLF1* mRNA expression was analyzed by RT-qPCR at the indicated time points after axitinib treatment. (D) AGS-EBV cells were treated with different concentrations of recombinant human VEGF-A165, and EBV lytic infection was assessed by Western blot at 48 h post treatment. (E) AGS-EBV cells were transfected with siVEGFA and siIL-10, and protein expression was analyzed by Western blot at 48 h post transfection. (F–G) VEGFR inhibitor axitinib or VEGFR blocking antibody inhibits AKT phosphorylation and EBV BZLF1 protein expression induced by siIL-10. AGS-EBV cells were pretreated with various concentrations of axitinib (F), VEGFR blocking antibody (G), or left untreated for 1 h, and then transfected with siIL-10 (50 nM). At 48 h post transfection, AGS-EBV cells were harvested for AKT phosphorylation and EBV BZLF1 protein expression. (H) IL-10 knockdown induced VEGF-A up-regulation in LCL cells. LCL cells were transfected with either NC or siIL-10 (50 nM), and VEGF-A and BZLF1 were analyzed by Western blot at 48 h post transfection. (I) LCL cells were treated with different concentrations of recombinant human VEGF-A165, and EBV lytic infection was assessed by Western blot at 48 h post treatment. The results were presented as mean ± standard deviation. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

4. Discussion

In this study, we demonstrated that knockdown of IL-10 induced EBV lytic replication via the activation of *BZLF1* promoter through VEGF-A-PI3K-p38 MAPK-NF-κB signaling pathways. Furthermore, knockdown IL-10 synergized with chemotherapeutic agent, Doxorubicin, in EBV activation and treatment of EBV- positive lymphomas in a mouse model.

As induction of EBV lytic activation can induce tumor cell death in EBV-associated malignancies, here we investigated whether knockdown of IL-10 could induce EBV lytic activation, thereby killing tumor cells. We first confirmed the knockdown efficiency of siRNA targeting IL-10 (Fig. 1A and B), and then investigated whether IL-10 knockdown could induce EBV lytic infection. Our results indicated that EBV lytic infection was activated upon IL-10 knockdown in both epithelial and B cell lines (Fig. 1C–I). As siRNA may have off-target effects, we further confirmed the induction activity by inhibiting IL-10 production using IL-10 inhibitor, AS101, which is an anontoxic immunomodulator [42,45].

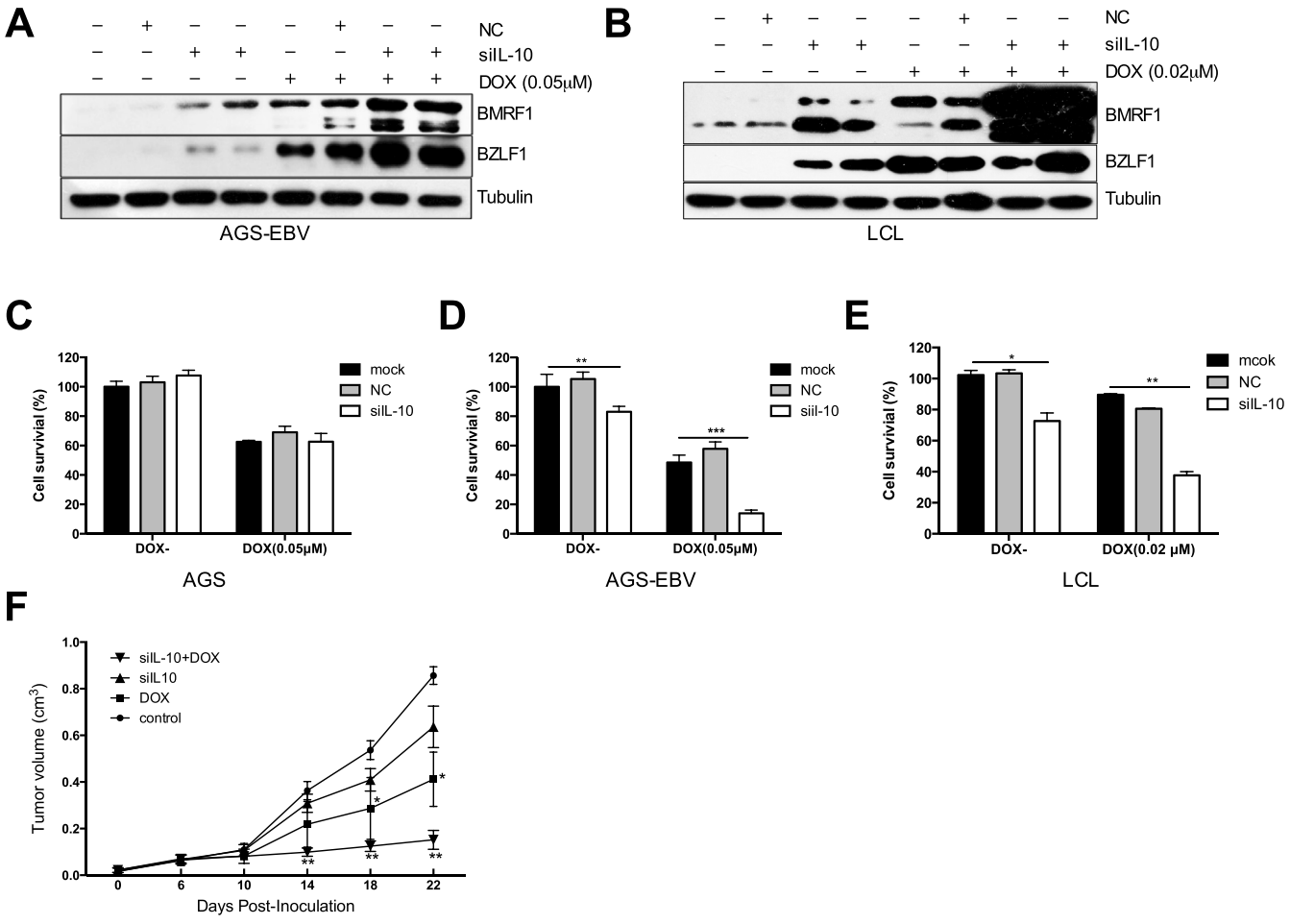

Fig. 5. IL-10 knockdown synergizes with Doxorubicin to activate EBV lytic infection and repress tumor cell growth both *in vitro* and *in vivo*. AGS-EBV (A) or LCL (B) cells were treated with Doxorubicin (DOX) 24 h post transfection of siIL-10, and at 24 h later, EBV lytic proteins were analyzed by Western blot. AGS (C), AGS-EBV (D) or LCL (E) cells were either transfected with NC, siIL-10 (50 nM) or un-transfected 24 h before the addition of DOX. Cell viability was analyzed by MTT (C and D) at 48 h or MTS at 96 h (E) post DOX treatment. (F) IL-10 knockdown enhanced the tumor cell killing effect of DOX *in vivo*. LCL cells were implanted subcutaneously into the flanks of SCID mice. When the tumors were palpable, mice were treated with one dose of DOX (5 mg/kg of body weight) intra-peritoneally (i.p.), siIL-10 alone (1 mg/kg of body weight once every 6 days for 2 times) or combination of one dose of Doxorubicin (5 mg/kg of body weight, i.p.) and siIL-10 (1 mg/kg of body weight once every 6 days for 2 times) or left untreated. Tumor sizes were measured everyday. Mice were euthanized a when the tumor size exceeded 1 cm^3. The tumor size of each group was shown. The results were presented as mean ± standard deviation. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

AS101 has been shown to have beneficial effects in diverse preclinical and clinical studies. Most of its activities have been primarily attributed to the direct inhibition of the anti-inflammatory cytokine IL-10 [42,45,46]. Previous studies have revealed that inhibition of tumor cell-derived IL-10 by AS101 can sensitize the tumor cells to chemotherapy [46,47]. Here we found that AS101 (Fig. 1J) could reactivate EBV in AGS-EBV cell lines (Fig. 1K). Thus, it is reasonable to believe that inhibition of IL-10 production might be a common way to activate EBV lytic infection.

We next investigated the mechanisms used by IL-10 knockdown to activate EBV lytic infection. Protein kinase inhibitor screening assay indicated that multiple kinases were involved in the induction of EBV lytic upon IL-10 knockdown, including PI3K, p38 MAPK, JAK, STAT1, ERK and NF-κB (Fig. 2A and B). These results are in consistent with some other reports, showing that p38 MAPK, PI3K, and ERK signal pathways are involved in EBV reactivation. For example, it is reported that EBV tegument protein BGLF2 promotes EBV reactivation through the activation of the p38 MAPK in EBV-infected lymphoblastoid cells and gastric carcinoma cells [19]. Combining TPA with sodium butyrate (T/B) activates PKCθ-p38 MAPK axis in EBV infected B cells [48]. BZLF1 activates p38 to turn on the ATF2 transcription factor, which binds to the ZII element in Z promoter [49]. BRLF1 induces EBV lytic infection by increasing the levels of phosphorylated p38 MAPK, JNK, and ERK [49,50] and activates AKT phosphorylation through the PI3K pathway [51]. Goswami et al. [52] tested 80 protein kinase inhibitors for their effect on EBV lytic reactivation and found that U-0126 (MEK inhibitor) and wortmannin (PI3K inhibitor) inhibited EBV replication. Thus, it is believed that activation of PI3K/AKT and p38 MAPK signaling pathways is important for EBV reactivation from latency [7,53–55].

NF-κB plays an important and complex role in EBV latent and lytic infections. Recent reports have revealed that some stimuli activate EBV via the activation of NF-κB. For example, Ionizing radiation activates lytic EBV gene expression in the EBV-associated gastric carcinoma cell line SNU-719 mainly through NF-κB activation [56]. NF-κB activation is required for the induction of EBV lytic infection via BCR engagement and TGF-β, likely through effects on MAPKs [7,57]. In the protein kinase inhibitor screen assays performed to test their effect on EBV lytic reactivation, BAY11-7082, which inhibits NF-κB signaling by blocking IκBα phosphorylation, represses EBV replication in response to Ag-bound B-cell receptor (BCR) signaling [52]. Our research here demonstrated that phosphorylation of IκB was significantly induced upon IL-10 knockdown (Fig. 2D and E, line 1 and line 2), while BAY11-7082 blocked the induction of EBV activation responded to IL-10 silencing (Fig. 2A and B). These results suggest that NF-κB signaling is involved in IL-10-knockdown- induced EBV lytic infection. Interestingly, using bioinformatics analysis we found that there was a putative NF-κB binding site (−1202 to −1191) in the Z-promoter but not in R-promoter. To further confirm the predicted results, we performed promoter luciferase assay and our results indicated that mutation or deletion of the predicted NF-κB binding site nearly abolished Zp activation induced by siIL-10. Since PI3K and p38 MAPK inhibitors suppress NF-κB activation and EBV lytic infection induced by IL-10 knockdown, we conclude that knockdown of IL-10 induces EBV lytic infection through PI3K-p38 MAPK-NF-κB signal pathway. In addition, we also found that the inhibitors for ERK, JAK and STAT1 inhibited EBV lytic infection induced by siIL-10. However, how these signaling pathways are involved in IL-10-knockdown induced-EBV lytic infection needs to be investigated in the future.

Vascular endothelial growth factor (VEGF) plays a critical role in tumor growth and angiogenesis [43]. VEGF includes five different isoforms, with VEGF-A165 (VEGF-A) being the dominant regarding the amount and biological activity. VEGF-A exerts its angiogenic actions by binding to VEGF receptors 1 (VEGFR1) and 2 (VEGFR2) on the surface of several cells, including endothelial cells [43]. Interestingly, our results indicate that VEGF-A is significantly induced by siRNA targeting IL-10. And we further demonstrated that VEGF-A was involved in the induction of EBV reactivation in response to IL-10 knockdown, and addition of exogenous VEGF-A activated EBV lytic infection. Previous studies have reported the activation of PI3K/AKT and p38 MAPK pathways responded to VEGF-A stimulation in different cells, such as human monocytes [58], cervical cancer cells [59] and endothelial cells [60]. We here found that VEGF-A was important for the activation of PI3K/AKT signaling pathway stimulated by IL-10 knockdown. Inhibition of VEGFR by its specific inhibitor or blocking the VEGFR using the antibody impaired the activation of PI3K/AKT and thereby EBV lytic infection in response to IL-10 knockdown. These results imply that IL-10 knockdown up-regulates VEGF-A, which in turn possibly activates the downstream signaling pathway PI3K/AKT-p38 MAPK-NF-κB to induce EBV lytic infection.

Finally, we evaluated the tumor cell killing effect of IL-10 knockdown. We found that although IL-10 knockdown had no apparent effect on EBV negative AGS cells, it significantly killed EBV-positive AGS cells as well as LCL cells. More importantly, IL-10 knockdown and Doxorubincin had a synergized effect on EBV-positive tumor cell killing both *in vitro* and *in vivo*. Doxorubicin is one of the most highly used therapies for cancers, including gastric cancer, non-Hodgkin lymphomas and Hodgkin's lymphoma [61]. However, adverse events have limited the use of Doxorubicin in clinical practice, especially for patients with advanced disease requiring dose escalation [62]. Thus, it is important to explore synergistic agents to reduce the clinical dose of Doxorubicin. Here, our results indicate that IL-10 knockdown could significantly synergize with Doxorubicin in killing EBV-positive tumor cells. Besides traditional chemotherapeutic agents, various attempts have been made to utilize EBV lytic activation to treat EBV positive tumors. The FDA-approved HDAC inhibitor romidepsin can significantly induce EBV lytic cycle and mediate enhanced killing of EBV-positive epithelial malignancies via PKC-delta pathway [63]. We have assessed the combination effect of HDAC inhibitors with IL-10 knockdown and found that there is also a synergized effect on inducing EBV lytic cycle (data not shown).

Elevated serum IL-10 levels in various types of cancer patients have been reported, including EBV-associated tumors [26–35]. Indeed, several studies have demonstrated a strong correlation between IL-10 expression and the presence of EBV in tumor samples [64,65]. High IL-10 serum levels have been detected in PTLD patients, which are decreased in those responsive to treatment [27,29]. High circulating IL-10 levels in gastric cancer and Hodgkin's disease patients are positively correlated with poor disease-free survival [26,30,32]. And high level of IL-10 production within EBV associated tumor microenvironment favors the survival of EBV-infected cells *in vivo* and creates a microenvironment required for efficient de novo infection of B lymphocytes by EBV virions [37,39,66].

Due to the significant role of IL-10 in EBV latent infection and EBV associated tumor, we assume that IL-10 could be a target for treating EBV-associated tumors. Indeed, previous studies have reported that blockade of an autocrine IL-10 loop significantly inhibits the proliferation of EBV-positive B-cell lines derived from patients with PTLD [38] and AIDS-related B-cell lymphoma [67]. The immunomodulator AS101 reduces clonogenicity and proliferation ability of tumor cells via inhibition of IL-10, which could be abrogated by exogenous addition of recombinant IL-10 [46]. Moreover, AS101 sensitizes GBM tumor cells to paclitaxel both *in vitro* and *in vivo*, resulting in increased apoptosis [46]. The immu nosuppressant rapamycin has a strong antiproliferative effect *in vitro* on B-cell lines derived from organ transplant recipients with EBV-associated posttransplant lymphoproliferative disorder (PTLD), mostly by inhibition of IL-10 secretion [68].

Although siRNA has the ability to silence target genes with high efficiency and specificity, delivering siRNAs to the tumor cells potently and effectively *in vivo* would be a challenge. siRNA formulations for systemic application face a chain of hurdles *in vivo* before reaching the cytoplasm of the tumor cells. Free siRNA, which is a type of anionic and

hydrophilic double-stranded small RNA, is not readily taken up by cells. Moreover, the hydrophilicity and negative charge of siRNA molecules prevents them from readily crossing biological membranes. This suggests that siRNA needs to be modified or packaged in vesicles in order to enter cells [69]. Various delivery vehicles have been developed for systemic delivery of therapeutic siRNA into solid tumors. The currently developed siRNA delivery systems for cancer therapy mainly include: (i) chemical modifications of siRNA, (ii) lipid based siRNA delivery system, (iii) polymer based siRNA delivery system, (iv) conjugate siRNA delivery systems, (v) co-delivery of siRNA and anticancer drugs, and (vi) inorganic nanoparticles [70]. The rapid progress in nanotechnology has enabled the development of effective nanoparticles as the carrier for siRNA delivery, and nanoparticles can improve stable delivery and also enhance efficacy [71,72]. Previous studies had shown that treatment with PCTAIRE1 siRNA-lipid nanoparticles significantly reduces human HCT116 colorectal cancer tumor volume and weight compared with the scramble-control group. A single dose of PCTAIRE1 siRNA-lipid nanoparticles was found to be highly effective in reducing PCTAIRE1 expression for up to 4 days *in vivo* [73]. Also, next generation lipid nanoparticle carriers are commercially available for *in vivo* delivery, which increases delivery efficacy while minimizes potentially unwanted cytotoxicity. Lipid nanoparticle may also have the benefit of minimizing off-target effects [74]. Our results showed that siIL-10 delivered into LCL tumor cells by lipid nanoparticle carrier indeed suppressed tumor growth. However, further studies are needed to investigate siIL-10 delivery efficiency by different delivery systems.

In summary, our results indicate that knockdown IL-10 is able to induce EBV lytic infection via PI3K-p38 MAPK-NF-κB axis. In addition, we find that VEGF-A is induced by siIL-10, and plays an important role in the activation of PI3K and EBV lytic infection. Importantly, we showed that knockdown IL-10 enhanced the efficacy of Doxorubicin to kill EBV-positive tumor cells both *in vitro* and *in vivo*. Our data and other reports suggest that reducing IL-10 level might have the potential to be used as a supplemental strategy to treat EBV-associated malignances.

Declarations of interest

The authors declare no financial conflict of interest.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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