A p110δ-specific inhibitor combined with bortezomib blocks drug resistance properties of EBV-related B cell origin cancer cells via regulation of NF-κB

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Abstract. Epstein-Barr virus (EBV) infection is closely related to carcinogenesis of various cancers, and is also associated with the development of drug resistance in cancer stem cells. However, in EBV-positive cancer cells, the mechanistic details of the downstream signaling and the connection of PI3K with the NF-κB pathway for development of drug resistance remain controversial. Diffuse large B-cell lymphoma (DLBCL) and multiple myeloma (MM) cells infected by EBV display drug resistance-related proteins (MDR1, MRP1 and MRP2) and stem cell markers (OCT4 and SOX2). EBV-infected HT (HT/EBV) and H929 (H929/EBV) cells activated p110δ expression, but downregulated the expression of p110α and p110β. A combination of CAL-101, a p110δ-specific inhibitor, with bortezomib treatment of HT/EBV cells synergistically suppressed proliferation, reduced levels of drug resistance-related proteins, activated caspase cleavage and recovered expression of p110α/p110β. Additionally, co-treatment with CAL-101 and bortezomib attenuated the expression of OCT4 and SOX2 via inhibition of activated NF-κB. Co-treatment with CAL-101 and bortezomib also attenuated drug resistance and NF-κB activity of EBV-infected H929 cells. Our results provide supportive evidence for the clinical application of CAL-101 and bortezomib to treat EBV-infected hematologic cancer.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL) and accounts for ~30-40% of all newly diagnosed lymphomas among adults (1,2). DLBCL is readily curable with combination chemotherapy, such as R-CHOP (rituximab, cyclophosphamide, adriamycin, vincristine and prednisone) (3,4). Although ~50% of patients with diffuse large B-cell lymphoma can now survive for more than 10 years (5), most patients who do not respond to R-CHOP treatment will eventually die from the disease. As a result, efforts have focused on the development of a more effective and personalized treatment modality.

EBV is associated with a number of malignancies, such as nasopharyngeal carcinoma, NHL and a subset of B-cell lymphoma in immunosuppressed individuals (6). EBV-positive DLBCL in patients older than 50 years is not associated with any known immunodeficiency (7). In addition, EBV-positive DLBCL in the elderly is characterized by activated B-cell phenotypes via NF-κB pathway activation and high expression of latent membrane protein 1 (LMP1) (8). EBV-LMP1 not only activates phosphatidylinositol 3-kinase (PI3K)/Akt pathways (9,10), but also is associated with the development of cancer stem cells (CSC) in nasopharyngeal epithelial cell lines (30). LMP1 also increases the expression of several markers, such as Octamer 4 (OCT4), SRY-related HMG box 2 (SOX2) and ATP-binding cassette (ABC) subfamily G member 2 (ABCG2) (11). The three major multidrug resistance ABC transporter proteins consist of multidrug resistance-related protein-1 (MRP1), multidrug resistance 1 (MDR1; P-glycoprotein) and ABCG2 (12). These proteins specifically allow the transport of various chemical entities, including anticancer drugs (13,14). ABC transporters are also a major route for anthracycline (e.g., adriamycin) elimination (3). Furthermore, the complete remission rates are significantly lower in the group expressing drug resistance-related proteins than in the group not expressing them (15). These results demonstrate that the increased expression of ABC transporters on plasma membranes results in increased efflux of anticancer drugs, leading to multidrug resistance. However, detailed downstream signaling and the role of PI3K in induction of

ABC transporters and survival of EBV-infected B cell cancer are not clear.

B-cell receptor (BCR) ligation activates CD19-related Src family kinases (Lyn, Fyn and Blk) and Syk tyrosine kinase, leading to Lyn- or Syk-dependent phosphoinositide 3-kinase (PI3K) activation (16-18). Several types of B cell malignancies appear to be dependent on the PI3K pathway for survival (19,20). PI3K signaling, especially constitutive expression of p110α, is associated with survival of multiple myeloma (MM) cells (21). Knockdown of p110δ, a PI3K catalytic unit, by small interfering RNA also causes significant inhibition of MM cell growth (22), whereas inhibitors of the p110δ catalytic isoform (idelalisib) fail to suppress aggressive DLBCL (23,24). Furthermore, the catalytic subunit of PI3K association with drug resistance in DLCBL remains unknown.

In the present study, we established EBV-infected DLBCL and MM cell lines as *in vitro* models to characterize EBV-induced drug resistance and identify molecular targets to control refractory B cell cancer. Bortezomib, which is approved for treatment of MM, reduced the level of p50 and p65 components of the canonical NF-κB pathway in EBV-transformed B cells and reduced the level of p52 in the non-canonical NF-κB pathway (25). We also investigated whether a combination of the PI3K inhibitor and bortezomib controls characteristics of EBV-related B cell cancer by blocking NF-κB activity.

Materials and methods

Preparation of EBV infectious culture supernatant and generation of EBV-infected DLBCL and MM cells. EBV supernatant stock was prepared from a B95-8 cell line (Vircell S.L., Granada, Spain). DLBCL or MM cells were added to EBV stock supernatant, and after 2-h incubation at 37˚C, RPMI-1640 media (Corning Inc., Corning, NY, USA) was added ($1x10^6$ cells/ml). The cultures were incubated for 3 weeks. Analysis of surface expression of CD21 was verified by BD Accuri™ C6 (BD Biosciences, San Jose, CA, USA) using PE-conjugated anti-CD21 antibody (BD Biosciences).

Cell lines and reagents. HT cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). NCI-H929 cell lines were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). These cells were maintained in RPMI-1640 media (Corning) supplemented with 10% fetal bovine serum (FBS; RMBIO, Missoula, MT, USA), streptomycin and glutamine at 37˚C in 5% CO_2. Bortezomib was purchased from LC Laboratories (Woburn, MA, USA). Doxorubicin and cyclophosphamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bay11-7082, A66, TGX-221, CAL-101 and LY294002 were purchased from Selleck Chemicals (Houston, TX, USA).

Cell proliferation assay. Cells ($5x10^4$ cells/well) were cultured in media containing 10% FBS in 96-well plates. After 24 h, cell proliferation was measured using an AlamarBlue (Serotec Ltd., Kidlington, Oxford, UK) assay. AlamarBlue was added (10% by volume) to each well, and relative fluorescence units (RFUs) were determined 9 h later with a Wallac 1420 Victor2 multi-label plate reader (Perkin-Elmer, Shelton, CT, USA; excitation, 530 nm; emission, 590 nm). Experiments were performed in triplicate, and relative fluorescence was calculated using mean fluorescence for each culture.

RT-PCR. Total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany) and transcribed into cDNA using oligo (dT) primers and reverse transcriptase. PCR products were amplified using specific primer sets for EBNA1 (upstream primer, 5'-GAGCGGGGAGATAATGTACA; downstream primer, 5'-TAAAAGATGGCCGGACAAGG), EBNA2 (upstream primer, 5'-AACCCTCTAAGACTCAAGGC; downstream primer, 5'-ACTTTCGTCTAAGTCTGCGG), LMP1 (upstream primer, 5'-CACGACCTTGAGAGGGGCCCA; downstream primer, 5'-GCCAGATGGTGGCACCAAGTC), LMP2A (upstream primer, 5'-ATGACTCATCTCAACACATA; downstream primer, 5'-CATGTTAGGCAAATTGCAAA), MDR1 (upstream primer, 5'-TTGCTGCTTACATTCAGG TTTCA; downstream primer, 5'-AGCCTATCTCCTGT G CATTA), MRP1 (upstream primer, 5'-AAGACCAAGACG TATCAGGT; downstream primer, 5'-CAATGGTCACGT AGACGGCAA), MRP2 (upstream primer, 5'-TCTCTCGATA CTCTGTGGCAC; downstream primer, 5'-CTGGAATCCG TAGGAGATGAAGA), OCT4 (upstream primer, 5'-CGACC ATCTGCCGCTTTGAG; downstream primer, 5'-CCCCCT GTCCCCCATTCCTA), and SOX2 (upstream primer, 5'-AGC AACGGCAGCTACAGCA; downstream primer, 5'-TGGGA GGAAGAGGTAACCACAG). A specific primer set for β-actin (upstream primer, 5'-ATCCACGAAACTACCTTCAA; downstream primer, 5'-ATCCACACGGAGTACTTGC) was used as a control, and PCR was performed using Prime Taq Premix (GeNet Bio, Chungnam, Korea). PCR products were analyzed via agarose gel electrophoresis and visualized with ethidium bromide under UV light using the Amersham™ Image 600 (GE Healthcare, Pittsburgh, PA, USA).

Western blot analysis. Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (Elpis Biotech, Inc., Daejeon, Korea) containing a protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Cocktail II; Sigma-Aldrich). Total cell lysates were subjected to SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA), the membranes were blocked with 5% skim milk, and conventional immunoblotting was performed using several antibodies. Chemiluminescence was detected using an ECL kit (Advansta Inc., Menlo Park, CA, USA) and the Amersham™ Image 600 (GE Healthcare). The following primary Abs were used: MDR1, MRP1, MRP2, OCT4, SOX2, phospho-Src (Tyr^{416}), Src, phospho-Syk (Tyr^{323}), phospho-Syk ($Tyr^{525/526}$), Syk, p110α, p110β, p110γ, p110δ, phospho-PI3K (Tyr^{458}), PI3K, phospho-Akt (Ser^{473}), Akt, phospho-Akt (Ser^{473}), caspase-8, caspase-9, caspase-3, PARP, NF-κB p105/p50, p100/p52, c-Rel, Rel-B and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA); EBNA2, LMP2A and Ref-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); β-tubulin was obtained from BD Biosciences; LMP1 was obtained from Abnova (Taipei, Taiwan); and EBNA1 was obtained from Thermo Fisher Scientific (Rockford, IL, USA).

Small interfering RNA (siRNA) transfection. Experimentally verified human SOX2-siRNA duplex, OCT4-siRNA duplex,

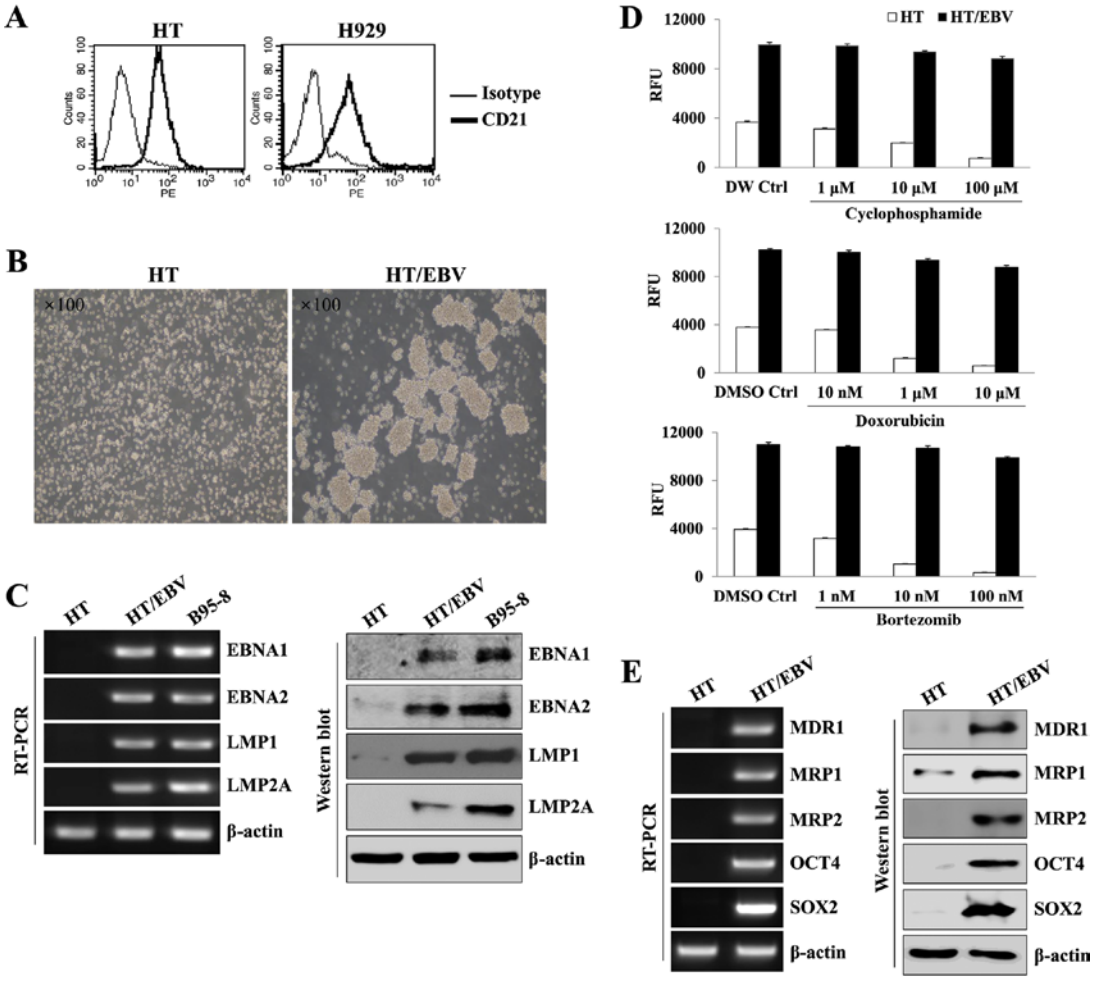

Figure 1. EBV provides DLBCL cells with multidrug resistance. (A) CD21 surface expression on HT and H929 cell lines was analyzed by flow cytometry. The thin line represents isotype control, and the thick line represents CD21 antigen. (B) Phase-contrast images of HT cells with or without EBV. EBV-infected HT cells showed rapid changes in morphology over 3 weeks, resulting in sphere-like clumps. (C) The left and right panels show RT-PCR and western blot analysis of EBV-related gene expression in EBV-infected and uninfected HT cells. B95-8 cells were used as a positive control. (D) HT or HT/EBV cells ($5x10^4$ cells/well) were cultured in 96-well plates and treated with the indicated concentration of cyclophosphamide, doxorubicin, or bortezomib for 24 h. Cell proliferation was determined by AlamarBlue assays. RFU signifies relative fluorescence units. (E) The left and right panels show increased expression of drug resistance markers, including MDR1, MRP1, MRP2, SOX2 and OCT4, at both the mRNA and protein levels, respectively. The data are representative of three independent experiments.

and negative control-siRNA were obtained from Bioneer. Cells were seeded at a concentration of $1x10^6$/well in a 6-well plate and grown overnight prior to transfection with 300 nM siRNA using Viromer Blue reagent (Lipocalyx GmbH, Halle, Germany) according to the manufacturer's instructions. Cells were used for further experiments 48 h after transfection.

Detection of NF-κB translocation by fractionation. Nuclear and cytosol cellular fractions were prepared using a Nuclear/Cytosol Fractionation kit (BioVision Inc., Mountain View, CA, USA), according to the manufacturer's protocol. Briefly, $2x10^6$ cells with or without various treatments were harvested and suspended in 200 μl of cytosol extraction buffer A. After incubation on ice for 10 min, cytosol extraction buffer B was added to the cell suspension and incubated on ice for 1 min. The obtained pellets were re-suspended in 10 μl of nuclear extraction buffer mix and designated nuclear fractions.

Measurement of NF-κB activity by NF-κB DNA-binding ELISA. ELISA and an NF-κB p50/p65 Transcription Factor assay kit (Abcam, Cambridge, MA, USA) were used according to the manufacturer's protocol to quantify the DNA-binding activity of NF-κB. Briefly, nuclear extracts were transferred to a 96-well plate coated with a specific dsDNA sequence containing the NF-κB response element. NF-κB proteins bound to the target sequence were detected with a primary antibody and an HRP-conjugated secondary antibody. Absorbance was measured at 450 nm as a relative measure of protein-bound NF-κB. All fractions were stored at -80˚C until further use.

Statistical analysis. Data are expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of the variance (ANOVA). $P<0.05$ were considered statistically significant.

Results

EBV infection is capable of inducing drug resistance in DLBCL cells. To establish EBV-transformed DLBCL and MM as a model of EBV-related drug resistant B cell cancer, we

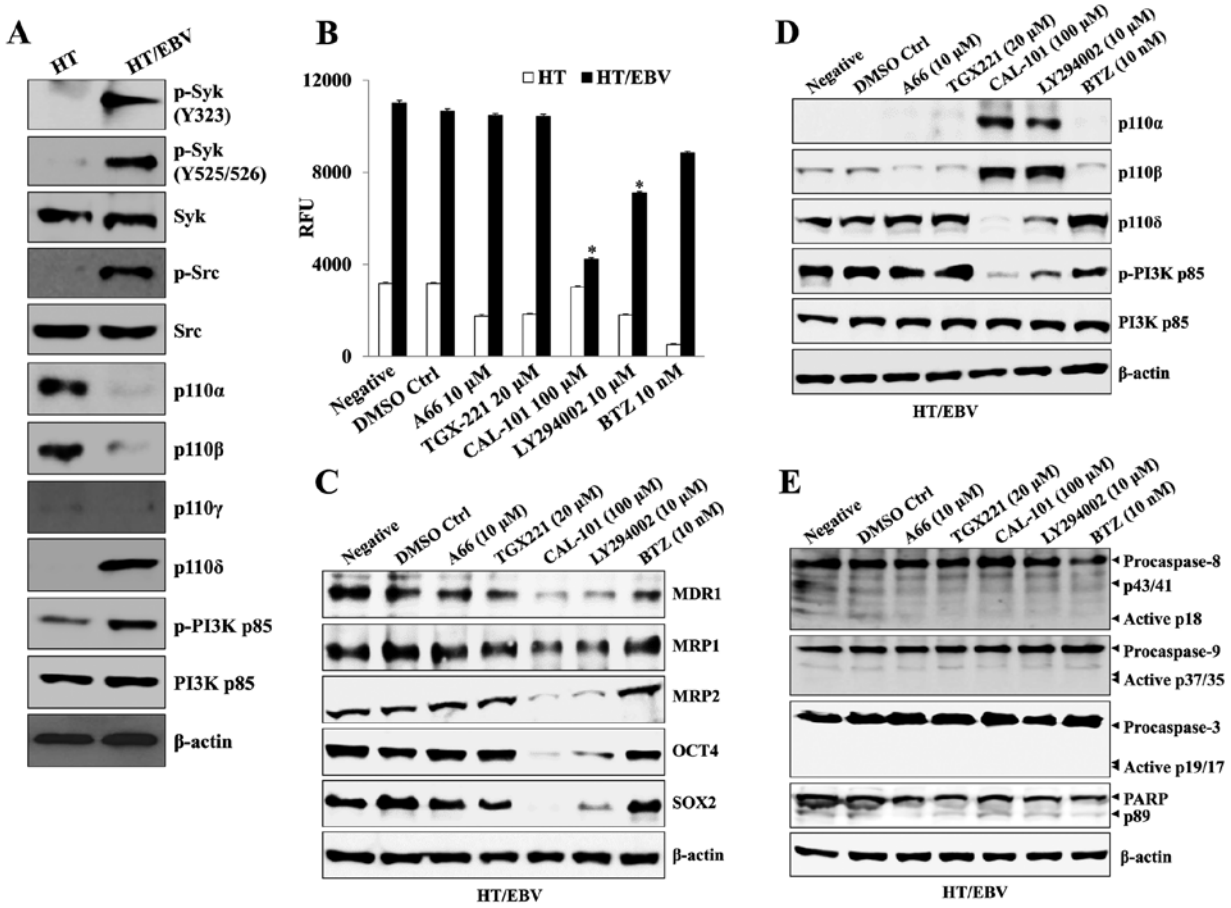

Figure 2. Syk/Src-mediated p110δ/Akt pathway induces drug resistance in HT/EBV cells. (A) The EBV-infected and uninfected HT cells were harvested and subjected to western blot analysis with the indicated antibodies. (B) HT or HT/EBV cells ($5x10^4$ cells/well) were treated with 10 μM A66, 20 μM TGX-221, 100 μM CAL-101, 10 μM LY294002, or 10 nM BTZ for 24 h. Cell proliferation was determined by AlamarBlue assays. RFU signifies relative fluorescence unit. *P<0.01. (C-E) HT/EBV cells ($2.5x10^5$ cells/ml) were treated with 10 μM A66, 20 μM TGX-221, 100 μM CAL-101, 10 μM LY294002, or 10 nM BTZ for 24 h. Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as the internal control. The data are representative of three independent experiments. BTZ, bortezomib.

first examined the expression of CD21, a receptor for cellular infection of EBV, in HT and H929 cell lines. HT and H929 had stable expression of CD21 (Fig. 1A). In addition, HT cells showed characteristic cluster formation and rapid proliferation at 3 weeks after EBV infection (Fig. 1B). At 5 weeks, EBV-infected HT (HT/EBV) cells expressed and maintained EBV-related mRNA and proteins (EBNA1, EBNA2, LMP1 and LMP2A) and levels similar to the protein expression observed in B95-8 cells (Fig. 1C). EBV-infected HT cells also exhibited drug resistance to several approved drugs, including cyclophosphamide, doxorubicin and bortezomib (Fig. 1D). HT/EBV cells upregulated the expression of MDR1, MRP1, MRP2 and stem cell markers, including OCT4 and SOX2 (Fig. 1E). These results suggest that HT/EBV cells display relatively high drug resistance compared with non-infected HT cells.

Syk/Src-mediated p110δ/Akt pathway induces drug resistance in HT/EBV cells. Next, we investigated which p110 isoform is associated with the generation of chemoresistant cells after EBV infection using each inhibitor of the p110 isoform of PI3K. EBV-infected HT cells had enhanced expression of phosphorylated Syk/Src kinase and p110δ compared to that of non-infected HT cells, whereas p110α and p110β activation were significantly inhibited after EBV infection (Fig. 2A). Although treatment with bortezomib resulted in the most effective anti-proliferative action against non-infected HT cells (Fig. 2B), specific inhibitors of p110α and p110β also significantly inhibited the proliferation of non-infected HT cells (Fig. 2B). Whereas, A66 (p110α inhibitor) and TGX-221 (p110β inhibitor) had no effect on the proliferation rate of HT/EBV cells (Fig. 2B), but CAL-101 (a specific inhibitor of p110δ) or LY294002 (p110α/β/δ inhibitor) significantly reduced the growth rate of HT/EBV cells (Fig. 2B). The treatment with CAL-101 or LY294002 of HT/EBV cells suppressed the appearance of drug resistance-related proteins (Fig. 2C) and re-induced the expression of p110α and p110β, whereas the level of p110δ was suppressed (Fig. 2D). However, treatment with high doses of CAL-101 or bortezomib alone had no effect on the cleavage of caspase-9 and -3 in H929/EBV cells (Fig. 2E). These results suggest that the activation of the p110δ isotype of PI3K results in drug resistance and the expression of p110δ has a critical role in the development of drug-resistant cancer cells after EBV infection.

Combination of a p110δ inhibitor with bortezomib induces cell death and reverses the characteristics of HT/EBV cells. Next, we examined whether CAL-101 combined with

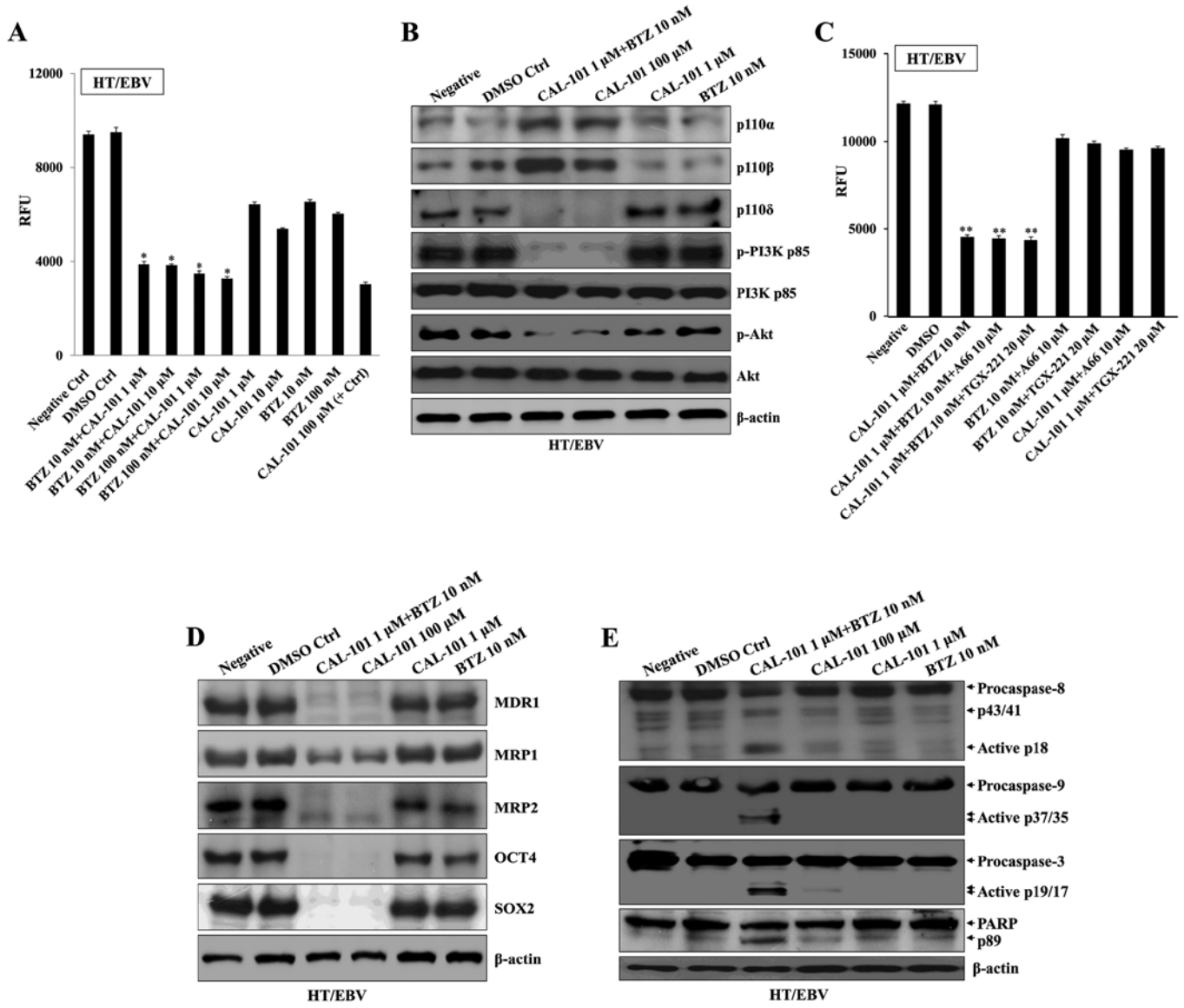

Figure 3. Combination of a p110δ inhibitor with bortezomib elicits cell death and attenuates the characteristics of HT/EBV cells. (A) HT/EBV cells ($5x10^4$ cells/well) were cultured in 96-well plates and treated with the indicated concentrations of bortezomib and CAL-101 for 24 h. For comparison, cells were treated either with BTZ (10 and 100 nM) or CAL-101 (1 and 10 μM) alone for 24 h. Cells treated with 100 μM CAL-101 for 24 h were used as a positive control. Cell proliferation was determined by AlamarBlue assays. RFU signifies relative fluorescence units. *P<0.05. (B-E) HT/EBV cells ($2.5x10^5$ cells/ml) were treated with 1 μM CAL-101 and 10 nM BTZ for 24 h. For comparison, cells were treated either with BTZ (10 nM) or CAL-101 (1 and 100 μM) alone for 24 h. Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as the internal control. (C) HT/EBV cells ($5x10^4$ cells/well) were cultured in 96-well plates and treated with the indicated concentration of bortezomib, CAL-101, A66 and TGX-221 for 24 h. Cell proliferation was determined by AlamarBlue assays. RFU signifies relative fluorescence units. **P<0.01. The data are representative of three independent experiments. BTZ, bortezomib.

bortezomib prevents proliferation and induces cell death of HT/EBV cells. Co-treatment of HT/EBV cells with CAL-101 (1 μM) and bortezomib (10 nM) effectively inhibited proliferation compared with high doses of CAL-101 (100 μM) or bortezomib (100 nM) alone (Fig. 3A). CAL-101 combined with bortezomib prominently restored the expression of p110α and p110β as well as suppression of p110δ activation in HT/EBV cells (Fig. 3B). However, additional combination with A66 or TGX-221 failed to further inhibit the proliferation of HT/EBV cells (Fig. 3C). The expression of MDR1, MRP1, MRP2 and stem cell markers in H929/EBV cells was completely reversed after treatment with CAL-101 and bortezomib (Fig. 3D). In addition, combining CAL-101 with bortezomib activated caspase-9 and -3 synergistically and resulted in cleaved PARP, the target of activated caspase-3 (Fig. 3E). These results suggest that CAL-101 and bortezomib synergistically converted the intractable DLBCL infected by EBV into treatable DLBCL cells.

Combining CAL-101 and bortezomib reverses drug resistance through blockage of NF-κB activation. B-cell-related cancer cells promote downstream signaling through the PI3K and NF-κB pathways for survival and proliferation (26,27). Inhibition of canonical NF-κB signaling leads to apoptosis and suppression of OCT4 expression (28). We next investigated whether co-treatment with CAL-101 and bortezomib affects the expression of SOX2 and OCT4 via regulation of NF-κB activity. Treatment with an NF-κB inhibitor (Bay-11-7082)

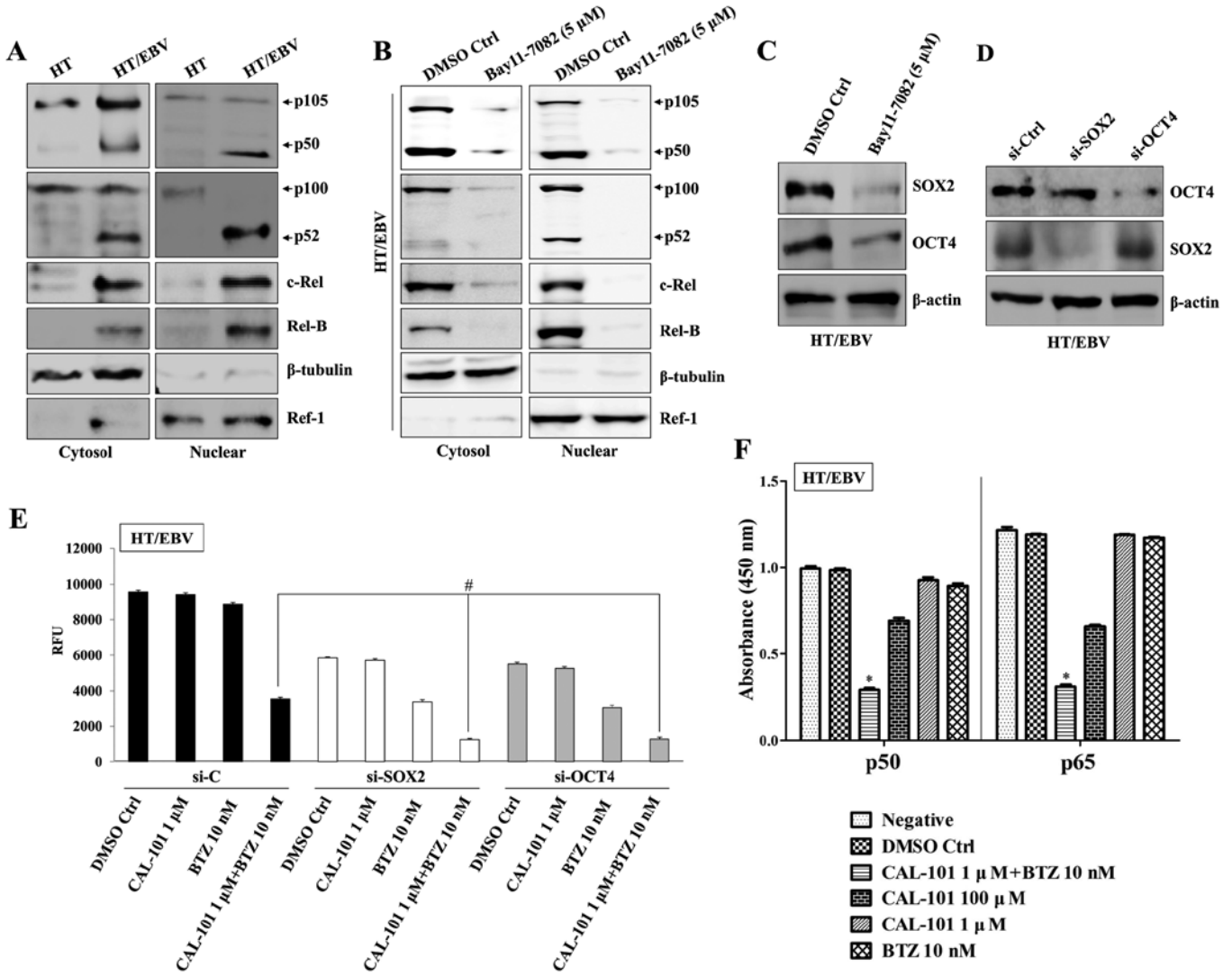

Figure 4. Combined treatment with CAL-101 and bortezomib reverses drug resistance by blocking NF-κB activation. (A) Cytosolic extracts (left panel) or nuclear extracts (right panel) of HT and HT/EBV cells were analyzed by western blots using Abs against p105/p50, p100/p52, Rel-B and c-Rel. A nuclear marker, Ref-1 and cytosol marker, β-tubulin, were used to verify the purity of each fraction. (B) HT/EBV cells ($2.5x10^5$ cells/ml) were treated with the NF-κB inhibitor Bay11-7082 (5 μM) for 8 h at 37˚C. (B) Cells were then washed with PBS and continuously cultured for 24 h. Cells were harvested, and the NF-κB levels in the cytosol and nuclear fractions were determined by western blot analyses. (C) Western blot analysis with antibodies against SOX2 and OCT4. (D) Western blot showing specific knockdown of SOX2 or OCT4. (E) HT/EBV cells ($5x10^4$ cells/well) were cultured in 96-well plates, transfected with SOX2-, OCT4-, or control-siRNA for 24 h, and then treated with the indicated drug combinations for an additional 24 h. Cell proliferation was determined by AlamarBlue assays. RFU signifies relative fluorescence unit. $^{\#}$P<0.01. (F) HT/EBV cells ($2.5x10^5$ cells/ml) were treated with the indicated drugs for 24 h. ELISA was used to measure NF-κB DNA-binding activity in nuclear extracts. Transcription factors NF-κB p50 and p65 (from kits) served as positive controls of NF-κB activity. ELISA results are expressed as relative absorbance. *P<0.005. The data are representative of three independent experiments. BTZ, bortezomib.

resulted in inhibition of activation and nuclear translocation of key components p50, p52 and Rel-B of NF-κB (Fig. 4A and B) in both the cytosol and nuclear fractions of HT/EBV cells and reduced the expression of SOX2 and OCT4 (Fig. 4C). To confirm the direct involvement of OCT4 and SOX2 in drug resistance of HT/EBV cells, OCT4 and SOX2 were silenced with siRNA (Fig. 4D), followed by treatment with CAL-101 and bortezomib in HT/EBV cells. Gene silencing of HT/EBV cells with OCT4 siRNA or SOX2 siRNA effectively suppressed proliferation and increased the sensitivity to bortezomib and co-treatment with CAL-101 and bortezomib (Fig. 4E). In addition, combinational treatment with CAL-101 and bortezomib synergistically attenuated NF-κB activation through inhibition of both canonical (p50) and non-canonical (p65) pathways in H929/EBV cells (Fig. 4F). These results suggest that the p110δ PI3K/NF-κB signaling pathway regulates drug resistance in EBV-infected DLBCL cells.

Suppression of p110δ PI3K and NF-κB activity attenuates drug resistance of EBV-positive MM cells. The EBV genome was also detected in MM (29-31). Furthermore, SOX2 is expressed not only in monoclonal gammopathy of undetermined significance (MGUS), but also in symptomatic MM (11). We next investigated whether co-treatment with CAL-101 and bortezomib blocked the development of drug resistance and generation of stem cell characteristics in EBV-positive MM cells. The H929 cell line, an EBV-negative MM cell line, was infected with EBV, and EBV-infected H929

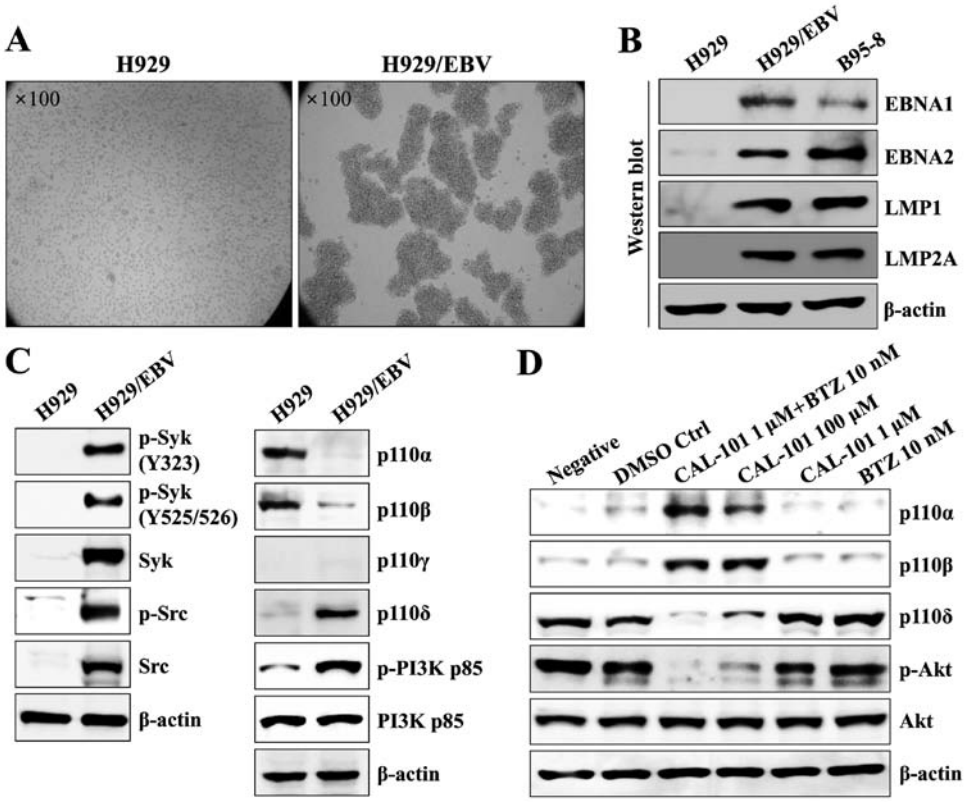

Figure 5. EBV infection increases activation of Syk/Src and p110δ/Akt in multiple myeloma cells. (A) Phase-contrast images of H929 cells with or without EBV. EBV-infected H929 cells showed rapid changes in morphology over 3 weeks, resulting in sphere-like clumps. (B) Western blot analysis of EBV-related gene expression in EBV-infected and uninfected H929 cells. B95-8 cells were used as a positive control. (C) EBV-infected and uninfected H929 cells were harvested and subjected to western blot analysis with the indicated antibodies. (D) H929/EBV cells ($2.5x10^5$ cells/ml) were treated with 1 μM CAL-101 and 10 nM BTZ for 24 h. For comparison, cells were treated either with BTZ (10 nM) or CAL-101 (1 and 100 μM) alone for 24 h. Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as the internal control. The data are representative of three independent experiments. BTZ, bortezomib.

(H929/EBV) cells generated clumps and showed enhanced proliferation (Fig. 5A). EBV-related mRNAs and proteins were detected in H929/EBV cells at similar levels to those of B95-8 cells (Fig. 5B). Although the expression of p110α/p110β was suppressed, the p110δ isoform of PI3K increased along with upregulation of phosphorylated Syk/Src kinase in H929/EBV cells (Fig. 5C). Treatment with CAL-101 and bortezomib efficiently blocked the elevation of p110δ and restored the expression of p110α/p110β in H929/EBV cells (Fig. 5D). Although H929/EBV cells increased the expression of MDR1, MRP1, MRP2, OCT4 and SOX2 (Fig. 6A), a combination of CAL-101 with bortezomib synergistically suppressed the expression of drug resistance-related proteins and stem cell markers (Fig. 6B) and enhanced the expression of cleaved caspases compared with the group treated with single drugs (Fig. 6C) in H929/EBV cells. Furthermore, co-treatment with CAL-101 and bortezomib of H929/EBV cells blocked the activation of canonical and non-canonical NF-κB (Fig. 6D). These results indicate that co-treatment with CAL-101 and bortezomib has a suppressive effect against EBV-positive MM cells.

Discussion

Although EBV genome-carrying cells represent a small fraction of the total population in various EBV-related cancers (23,25,45), EBV infection resulted in drug resistance in DLBCL and MM cells in the present study. Class I PI3K enzymes expressed in B cells are heterodimeric complexes composed of regulatory (p85) and catalytic (p110α, p110β, p110δ or p110γ) subunits (32). While the p110α and p110β catalytic isoforms are ubiquitously expressed, the p110δ and p110γ isoforms are largely restricted to leukocytes (33,34). The *PIK3CA* gene, which encodes p110α, is mutated in many cancers, whereas PI3K/AKT mutations are rarely found, especially in B cell malignancies and multiple myeloma (35-37). Although resistance to chemotherapy is acquired through a variety of mechanisms, including activation of key pro-survival signaling molecules, such as PI3K and NF-κB activation (38), the mechanisms underlying aberrant expression and activity of p110δ in EBV-infected cancer cells remain unclear. From these results, we hypothesize that co-treatment with CAL-101 and bortezomib might reduce the resistance to anticancer drugs by synergistic suppression of NF-κB activity. In this study, EBV-infected cancer cells upregulated Syk/Src-dependent p110δ PI3K activation, leading to promotion of the expression of drug resistance-related proteins, including MDR1, MRP1 and MRP2. A combination of CAL-101 with bortezomib attenuated chemoresistance and the expression of stem cell markers by blocking the nuclear translocation and accumulation of NF-κB in EBV-infected DLBCL and MM cells. These results suggest that the regulation of p110δ/Akt-mediated NF-κB pathway plays a critical role in the generation of EBV-induced chemoresistant cancer cells.

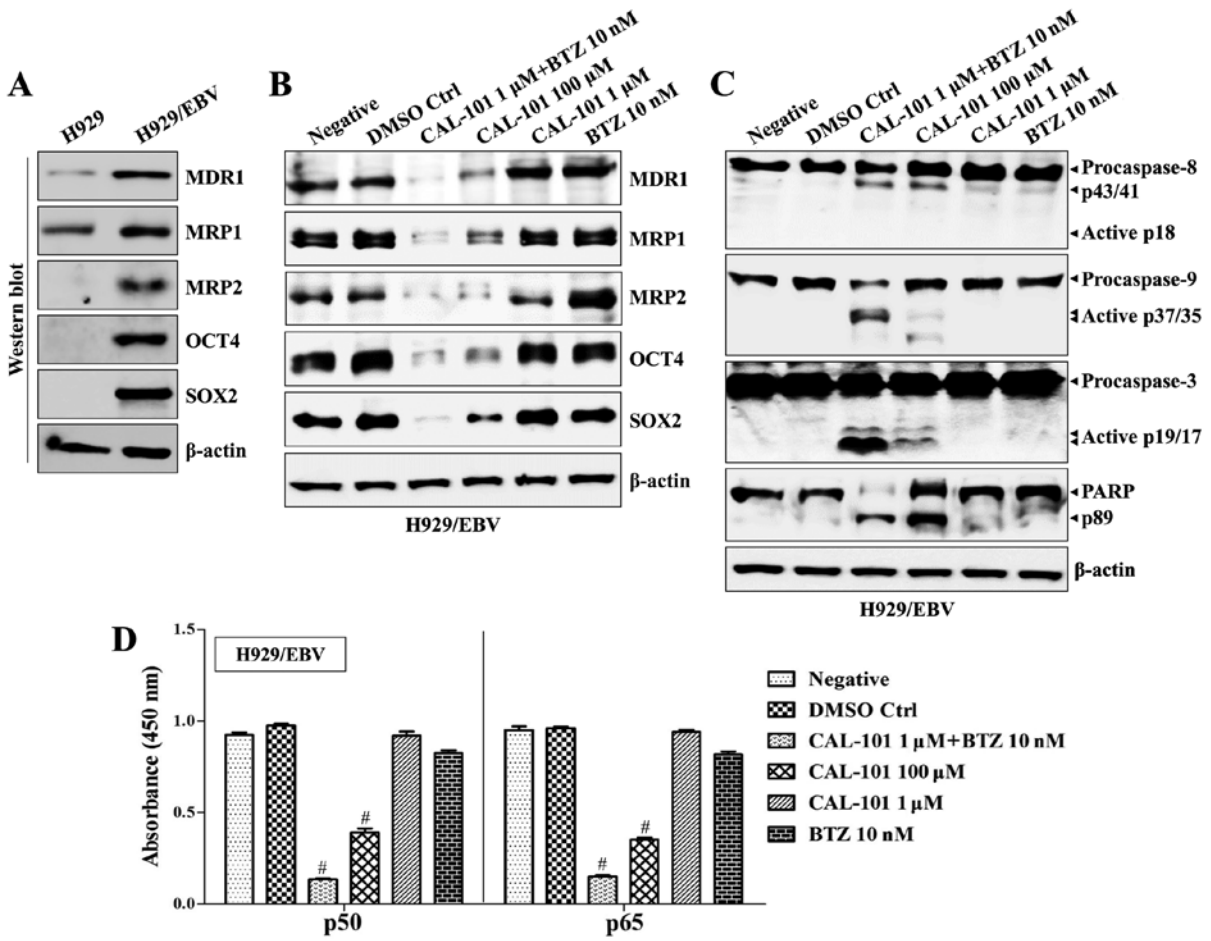

Figure 6. Suppression of p110δ PI3K and NF-κB activity diminishes drug resistance of EBV-positive multiple myeloma cells. (A) Western blot analysis of drug resistance markers including MDR1, MRP1, MRP2, SOX2 and OCT4, in EBV-infected and uninfected H929 cells. (B-D) H929/EBV cells ($2.5x10^5$ cells/ml) were treated with 1 μM CAL-101 and 10 nM BTZ for 24 h. For comparison, cells were treated either with BTZ (10 nM) or CAL-101 (1 and 100 μM) alone for 24 h. (B and C) Total cell lysates were immunoblotted with the indicated antibodies. β-actin served as the internal control. (D) H929T/EBV cells ($2.5x10^5$ cells/ml) were treated with the indicated drugs for 24 h. ELISAs were used to measure NF-κB DNA-binding activity in nuclear extracts. Transcription factors NF-κB p50 and p65 (from kits) served as positive controls of NF-κB activity. ELISA results are expressed as relative absorbance. $^{\#}P<0.01$. The data are representative of three independent experiments. BTZ, bortezomib.

EBV-positive DLBCL in the elderly accounts for 8-10% of DLBCL among Asian patients (39). EBV-positive cells are characterized by postgerminal center B-cell phenotypes and prominent NF-κB activation (8). The prognosis of EBNA2-expressing EBV-positive DLBCL patients is significantly worse compared with EBV-negative cases (40). Most studies have also shown that the outcome of elderly patients with EBV-positive DLBCL treated with drug combinations (CHOP) in the presence of rituximab (R-CHOP) is worse than that of patients who are EBV-negative under the same treatment (41-43). MRP1 has been identified in cell lines showing the typical multidrug resistance phenotypes, but without elevated expression of MDR1 (P-glycoprotein) (17). Expression of MDR1 has a significant effect on response to chemotherapy and prognosis in AIDS-related NHL (18). Since MDR1 and MRP1 are key efflux routes of anthracycline, which is one of the key drugs for treatment of DLBCL, we investigated whether blocking p110δ activation has an effect on the expression of drug resistance-related proteins in EBV-infected cancer cells. Treatment with high doses of CAL-101 or LY294002 significantly suppressed proliferation and expression of MDR1, MRP1 and MRP2 in EBV-infected B cell cancer cells. These results suggest that activation of p110δ plays an important role in development of drug resistance induced by EBV infection.

Bortezomib is widely used to treat both newly diagnosed MM and relapsed or refractory MM (44,45). However, bortezomib treatment appears to generate very short-duration responses and results in rapid development of drug resistance (46). Although bortezomib, a proteasome inhibitor, has been found to induce apoptosis in EBV lymphoblastoid cell lines through suppression of canonical and non-canonical activity of NF-κB (25), the effects of bortezomib on the generation of drug resistance in EBV-infected B cell cancer remain controversial. Inhibition of NF-κB activation results in apoptosis and suppression of OCT4 expression in human embryonic stem cells (28). In this study, treatment with bortezomib alone had no effect on levels of SOX2 and OCT4 in EBV-infected DLBCL and MM cells. Notably, our results showed that CAL-101, in combination with bortezomib, suppressed expression of stem cell markers and induced cleavage of caspase-3 via inhibition of NF-κB activation in EBV-infected DLBCL and MM cells. Based on these results, we confirmed that p110δ/Akt-mediated NF-κB activation is an essential pathway in the generation of EBV-related cancer stem cells, and these

cells might be converted into drug-sensitive cancer cells after treatment with a combination of CAL-101 and bortezomib.

Multiple myeloma (MM) is characterized by uncontrolled proliferation of plasma cells (PCs) in bone marrow (47). Despite the development of novel targeted drugs, including proteasome inhibitors (bortezomib) and immunomodulatory drugs (thalidomide and lenalidomide), the incidence of MM is expected to increase due to aging populations (48,49). In addition, MM is considered treatable, but not curable, even after bone marrow transplantation (50,51), because this disease eventually results in relapse and becomes hopeless due to the development of high malignancy and resistance to first-line anti-MM drugs (52,53). However, the molecular characteristics of drug-resistant refractory MM and the specific method to convert intractable MM to a treatable condition are still unclear and undefined. Although there is still controversy about MM cancer stem cells and drug-resistant condition, we found that p110δ-dependent NF-κB activation in EBV-infected MM cells is responsible for the development of stem cell characteristics and generation of drug resistance. Our results suggest that the combination of a specific p110δ inhibitor and bortezomib may convert incurable refractory hematologic disease into a manageable condition and that this combination can be used to treat DLBCL in elderly patients and relapsed drug-resistant MM-expressing cancer stem cell markers.

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