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Oxidative Stress Induces Reactivation of Kaposi's Sarcoma-Associated Herpesvirus and Death of Primary Effusion Lymphoma Cells[▽]

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Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL) cells are predominantly infected with latent Kaposi's sarcoma-associated herpesvirus (KSHV), presenting a barrier to the destruction of tumor cells. Latent KSHV can be reactivated to undergo lytic replication. Here we report that in PEL cells, oxidative stress induced by upregulated reactive oxygen species (ROS) can lead to KSHV reactivation or cell death. ROS are upregulated by NF-κB inhibition and are required for subsequent KSHV reactivation. Disruption of the intracellular redox balance through depletion of the antioxidant glutathione or inhibition of the antioxidant enzyme catalase also induces KSHV reactivation, suggesting that hydrogen peroxide induces reactivation. In addition, p38 signaling is required for KSHV reactivation induced by ROS. Furthermore, treatment of PEL cells with a higher concentration of the NF-κB inhibitor than that used for inducing KSHV reactivation further upregulates ROS and induces massive cell death. ROS, but not p38 signaling, are required for PEL cell death induced by NF-κB inhibition as well as by glutathione depletion. Importantly, anticancer drugs, such as cisplatin and arsenic trioxide, also induce KSHV reactivation and PEL cell death in a ROS-dependent manner. Our study thus establishes a critical role for ROS and oxidative stress in the regulation of KSHV reactivation and PEL cell death. Disrupting the cellular redox balance may be a potential strategy for treating KSHV-associated lymphoma.

Kaposi's sarcoma-associated herpesvirus (KSHV) is an oncogenic human DNA virus belonging to the gammaherpesvirus family. KSHV causes Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and a plasmablastic subtype of multicentric Castleman disease (MCD) (8, 13, 22). KSHV has two phases in its life cycle, i.e., latency and lytic replication. During lytic replication, most of the viral genes are expressed and new virions are produced to facilitate virus propagation and transmission. In contrast, only a few viral genes are expressed during latency (20, 57), enabling KSHV to evade immune surveillance and promoting virus persistence (3). KSHV persists in its latent form in the majority of KS and PEL tumors (21, 53, 77). Thus, latency presents a barrier to the elimination of KSHV and the treatment of KSHV-associated tumors.

Therapeutic induction of virus reactivation provides an opportunity to target and eliminate KSHV-associated tumor cells (1, 29, 70). A key prerequisite to the success of this approach is to understand how cellular signals regulate KSHV reactivation in order for us to target specific cellular pathways to achieve efficient virus reactivation in tumor cells. KSHV replication and transcription activator (RTA) is the key viral regulator of virus reactivation (49, 61). RTA can activate the transcription of its target genes through direct binding to RTA-responsive elements (RRE) (59, 60) or by using cellular coregulators, such as CSL/RBP-Jκ (44, 51), Oct-1 (55), C/EBPα (68), and AP1 (67). KSHV also encodes negative regulators of viral lytic gene expression. Latency-associated nuclear antigen (LANA), which is encoded by KSHV and expressed at high levels during latency, represses transcription of RTA and several other lytic genes to promote latency (39, 40, 46). As lytic products, vGPCR, K-bZIP, and K1 inhibit virus lytic replication or the expression of certain lytic genes (7, 34, 41, 45), suggesting the possible existence of feedback regulation of viral lytic replication. Chromatin remodeling of the RTA promoter also plays a role in the regulation of KSHV reactivation (48). Recently, several studies showed that KSHV-encoded microRNAs (miRNAs) also regulate KSHV reactivation (2, 42, 47), further highlighting the importance of the regulation of KSHV latency and reactivation. Several cellular factors, such as XBP-1, Ras, Ets-1, PARP-1, hKFC, CBP, the SWI/SNF chromatin remodeling complex, the TRAP/Mediator complex, RBP-Jκ, human Notch intracellular domain, and HMGB1, have been shown to promote KSHV reactivation and/or lytic gene expression (11, 31, 32, 44, 71, 72, 75, 76), suggesting a close link between many cellular processes and KSHV reactivation. Other cellular factors, such as Oct-2, KAP-1, and Hey1, were found to inhibit KSHV reactivation and/or lytic gene expression (12, 19, 25). However, the regulation of KSHV reactivation by cellular signals is still not fully understood.

Reactive oxygen species (ROS) are highly reactive molecules generated by partial reduction of the unpaired electrons of oxygen (23). As products of normal cellular metabolism, ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$). ROS originate from various cellular enzyme systems, such as the mitochondrial electron transport chain, the NADPH oxidase complex, xanthine oxidase, lipoxygenase, cyclooxygenase, and peroxisomes (23). Low to moderate levels of ROS exist under various physiological conditions, with functions ranging from facilitating cellular defense against infectious agents to acting as secondary messengers in a number of cellular signaling systems (64, 65). In

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contrast, high concentrations of ROS can damage various cellular components, including lipids, proteins, and nucleic acids, and can cause oxidative stress (65). Excess levels of ROS can result from the overproduction of ROS and/or deficiency in antioxidants. Major cellular antioxidant systems include antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) and the major nonenzymatic antioxidant glutathione (GSH) (64).

Nuclear factor kappa B (NF-κB) is activated by KSHV protein vFLIP, K15, and KSHV-encoded microRNA (miRNA) in PEL cells (4, 14, 28, 42), where it functions to inhibit KSHV reactivation and lytic gene expression (5, 33, 42, 73). In addition, NF-κB activity is essential for the survival of PEL cells *in vitro* (28, 36) and *in vivo* (35).

Previous studies showed that NF-κB inhibits ROS generation and protects cells from ROS-mediated cell death (37, 52, 56, 62), suggesting that ROS may play a role in KSHV reactivation and PEL cell death. Here we have addressed the function of ROS in KSHV reactivation and PEL cell death induced by inhibition of NF-κB, by disruption of other cellular antioxidant systems, and by anticancer drugs.

MATERIALS AND METHODS

Cell lines and reagents. BC-3 and BCBL-1 cells were kindly provided by E. Cesarman (Cornell Medical College, NY). The establishment of the BC-3-G cell line was previously described (76). Bay 11-7082 (Bay), PD 98059 (PD), SB 203580 (SB), p38 inhibitor III, and p38 inhibitor VIII were purchased from Calbiochem. *N*-Acetyl-L-cysteine, diethyl maleate, 3-amino-1,2,4-triazole (3-AT), phorbol 12-myristate 13-acetate, sodium butyrate, and arsenic trioxide were purchased from Sigma-Aldrich.

Flow cytometry analysis of KSHV reactivation. BC-3-G cells were treated with chemicals or transfected with plasmids. Cells were cultured in conditioned medium to promote cell survival. At different time points posttreatment or posttransfection, cells were spun down, washed with phosphate-buffered saline (PBS), resuspended in PBS, and analyzed by flow cytometry for enhanced green fluorescent protein (EGFP) expression on a FACSCanto II cytometer (BD Biosciences). Cells were also analyzed for red fluorescent protein (RFP) expression if cells were transfected with an RFP plasmid.

Flow cytometry analysis of ROS levels. BC-3 and BCBL-1 cells were loaded with a 5 μM concentration of the ROS dye 5-(and -6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (c-H_2DCFDA) for 30 min at 37°C in Hanks' balanced salt solution (HBSS) containing Ca and Mg. Cells were then washed with HBSS-Ca-Mg once to remove dye, resuspended in growth medium, pretreated with 10 mM *N*-acetyl cysteine (NAC) or the vehicle water for 1 h, and treated with 5 or 20 μM Bay for 2 h. Cells were washed with HBSS-Ca-Mg once, resuspended in HBSS-Ca-Mg, and subjected to flow cytometry on a FACSCanto II cytometer (BD Biosciences). Data were analyzed with software provided by Cytobank (www.cytobank.org).

Western blotting. Protein samples were separated by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% of Tween 20, followed by incubation with a primary antibody and then with anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Cell Signaling Technology). Signals were detected by chemiluminescence detection (ECL Plus system; Amersham Pharmacia Biotech). The α-tubulin antibody was purchased from Santa Cruz Biotechnology. The generation of anti-RTA rabbit serum was described previously (60).The phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182) antibody was purchased from Cell Signaling Technology.

Cell viability assay. Cells were seeded at a density of 5×10^4 or 1×10^5 cells per well on 48-well plates. Cells were then treated with chemicals. At different time points posttreatment, the numbers of live and dead cells were measured with the trypan blue (Sigma-Aldrich) exclusion assay using a hemocytometer. Cell viability was calculated as the percentage of live cells in the total cell population of both live and dead cells.

Reverse transcription-quantitative PCR (RT-Q-PCR) analysis. Cells were washed with PBS twice, followed by centrifugation. RNA was purified using the PureLink RNA minikit (Invitrogen). First-strand cDNA was synthesized using Superscript III polymerase (Invitrogen). Syber green PCR was performed using probes specific for the KSHV RTA and the human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene.

Measurement of virion production. Supernatants from cells treated with chemicals were collected and cleared by centrifugation at $200 \times g$ for 3 min followed by another centrifugation at $3{,}000 \times g$ for 5 min. Cleared supernatants were then treated with DNase I (Invitrogen) at a concentration of 100 U/ml for 1 h. After heat inactivation of DNase I at 65°C for 30 min in the presence of 10 mM EDTA, supernatants were treated with proteinase K (Sigma-Aldrich) at 65°C for 2 h. Virion DNA was extracted with phenol-chloroform, followed by DNA precipitation with ethanol. DNA was air dried, dissolved in 40 μl of TE buffer, and measured by RT-Q-PCR using primers specific for the KSHV major capsid gene.

Luciferase reporter assay. BC-3 cells were cotransfected with the RTA promoter reporter plasmid and the simian virus 40 (SV40)-RL *Renilla* luciferase control plasmid. Cells were treated with chemicals at 12 h posttransfection and analyzed for luciferase activities at 48 h posttreatment. In other experiments, in addition to luciferase reporter plasmids, BC-3 cells were also cotransfected with the indicated short hairpin RNA (shRNA) plasmids and analyzed at 48 h posttransfection. Luciferase activities were measured using the dual-luciferase reporter assay kit from Promega. Firefly luciferase activity from the RTA promoter reporter was normalized to the *Renilla* luciferase activity from the SV40-RL control plasmid. Relative fold activation compared to the control treatment or transfection was then calculated.

Construction of shRNA plasmids. shRNA plasmids were constructed as previously described (43). Briefly, two DNA oligonucleotides containing target sequences were annealed and cloned into the pLKO.1-TRC vector (Addgene). The target sequences, obtained from Sigma-Aldrich, are CGG AGA TTC AAC ACT GCC AAT (shCat) and CAA CAA GAT GAA GAG CAC CAA (shCtrl).

RESULTS

Inhibition of NF-κB upregulates ROS. NF-κB can inhibit the generation of reactive oxygen species (ROS) (37, 52, 56, 62). To determine whether NF-κB suppresses the generation of ROS and prevents oxidative stress in primary effusion lymphoma (PEL) cells, we examined ROS levels with a fluorescent sensor, 5-(and -6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (C-H_2DCFDA) in two types of PEL cells, BC-3 and BCBL-1, treated with Bay 11-7082 (Bay), a specific NF-κB inhibitor (36), in the presence or absence of pretreatment with an antioxidant, *N*-acetyl cysteine (NAC). A 5 μM concentration of Bay has been shown to significantly inhibit NF-κB activity in PEL cells (5, 36) and was used in this ROS assay. After 2 h of treatment with 5 μM Bay, both BC-3 and BCBL-1 cells had higher levels of ROS than untreated (UT) cells (Fig. 1A). Pretreatment of BC-3 and BCBL-1 cells with NAC reduced the levels of ROS to levels slightly lower than those observed in untreated cells (Fig. 1A).

ROS are required for KSHV reactivation induced by inhibition of NF-κB. To examine whether ROS play a role in KSHV reactivation induced by NF-κB inhibition, we utilized a BC-3-derived reporter cell line BC-3-G, which expresses enhanced green fluorescent protein (EGFP) driven by the minimal promoter of the KSHV lytic gene, polyadenylated nuclear RNA (PAN) (76). The minimal PAN promoter responds specifically to RTA with a high sensitivity (59), enabling us to monitor KSHV reactivation through EGFP expression, although EGFP expression in BC-3-G cells likely underestimates the percentage of cells in which KSHV is reactivated since EGFP needs proper folding and assembly into a tetramer to be fluorescent. Previous studies have shown that brief inhibition of NF-κB by Bay can induce KSHV reactivation (5), whereas prolonged NF-κB inhibition results in apoptotic cell death

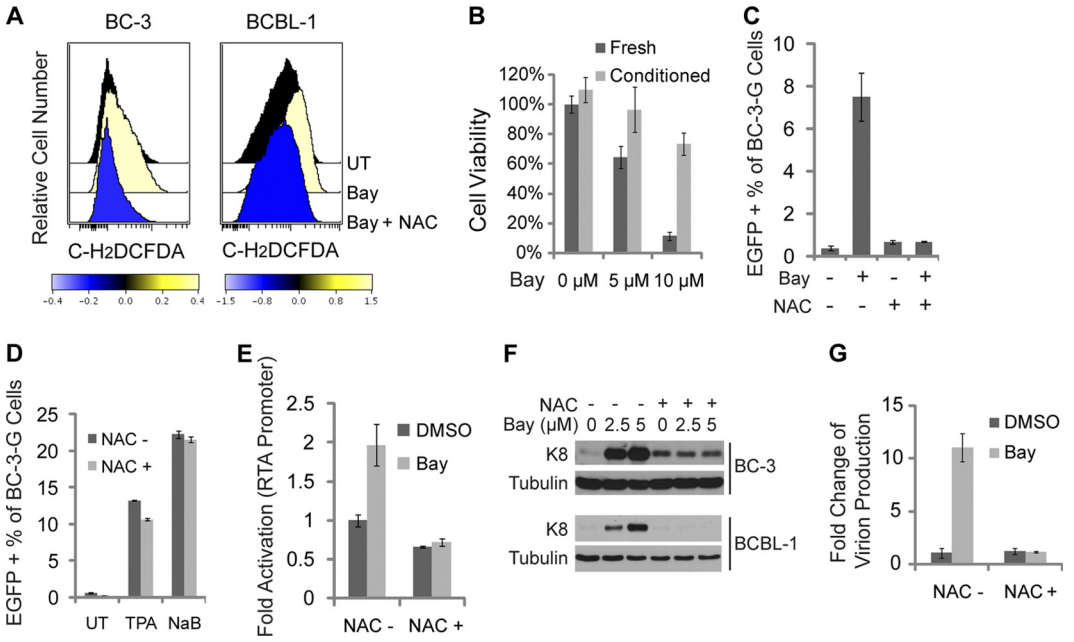

FIG. 1. Inhibition of NF-κB induces ROS-dependent KSHV reactivation. (A) Fluorescence-activated cell sorter (FACS) analysis of c-H2DCFDA staining, showing ROS levels in BC-3 and BCBL-1 cells untreated (UT) or treated with 5 μM Bay 11-7082 (Bay) for 2 h in the presence or absence of 10 mM NAC added 1 h prior to Bay treatment. (B) Cell viability measured by trypan blue exclusion assay of BC-3 cells treated with 0, 5, or 10 μM Bay for 24 h in the presence of conditioned medium or fresh medium. (C) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 5 μM Bay for 48 h in the presence or absence of 10 mM NAC added 1 h prior to Bay treatment. (D) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 5 ng/ml of TPA or 0.2 mM NaB for 48 h in the presence or absence of 10 mM NAC added 1 h prior to Bay treatment. (E) KSHV RTA promoter activity measured by a luciferase reporter assay in BC-3 cells treated with 5 μM Bay or its vehicle dimethyl sulfoxide (DMSO) for 48 h in the presence or absence of 10 mM NAC added 1 h prior to Bay treatment. (F) Western blotting with anti-K8 and anti-α-tubulin antibodies of cell lysates of BC-3 and BCBL-1 cells treated with 5 μM Bay or its vehicle DMSO for 48 h in the presence or absence of 10 mM NAC added 1 h prior to Bay treatment. (G) Relative virion production by BCBL-1 cells treated with 5 μM Bay or its vehicle DMSO for 72 h in the presence or absence of 10 mM NAC added 1 h prior to Bay treatment. For panels A and F, data are representative of three independent experiments. For panels B, C, D, E, and G, data are the means ± standard deviations from three independent experiments.

(36), suggesting that a greater degree and/or a longer duration of NF-κB inhibition favors cell death, whereas a lesser degree and/or a shorter duration of NF-κB inhibition favors KSHV reactivation. During our optimization for conditions that limit PEL cell death and promote KSHV reactivation, we found that culturing PEL cells in conditioned medium during prolonged treatment with Bay promoted cell survive (Fig. 1B). We therefore cultured PEL cells in conditioned medium when assaying KSHV reactivation. After 48 h of treatment with 5 μM Bay in conditioned medium, significantly higher percentages of BC-3-G cells than of untreated cells were EGFP positive (Fig. 1C). Pretreatment of cells with the antioxidant NAC was able to abolish the increase of the percentage of EGFP-positive cells induced by Bay treatment (Fig. 1C), indicating that ROS are required for KSHV reactivation induced by NF-κB inhibition. In contrast, NAC minimally affected KSHV reactivation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (NaB) (Fig. 1D), two well-known inducers of KSHV reactivation that reactivate KSHV through different pathways, suggesting that NAC does not affect the reactivation assay *per se* and ROS are specifically required for reactivation induced by NF-κB inhibition.

Previous studies showed that NF-κB inhibition induces KSHV reactivation through activating the RTA promoter (5). Therefore, we examined whether ROS are required for the activation of the RTA promoter by NF-κB inhibition. Treatment of BC-3 cells with 5 μM Bay for 48 h activated the RTA promoter in a luciferase reporter assay and NAC abolished the activation of the RTA promoter by Bay (Fig. 1E), indicating that NF-κB inhibition activates the RTA promoter in a ROS-dependent manner.

Next, we examined the expression of viral lytic protein K8 in BC-3 and BCBL-1 cells treated with 2.5 or 5 μM Bay in the presence or absence of NAC pretreatment. After 48 h of treatment with Bay, significantly higher levels of K8 protein were observed in both BC-3 and BCBL-1 cells than in untreated cells (Fig. 1F). Importantly, NAC pretreatment was able to largely prevent the increase of K8 expression induced by Bay (Fig. 1F), suggesting that NF-κB inhibition enhances K8 expression in a ROS-dependent manner. Consistent with these results, Bay treatment enhanced the production of viral particles in a ROS-dependent manner (Fig. 1G).

Depletion of GSH induces KSHV reactivation. NAC can increase intracellular glutathione (GSH) levels by functioning as a precursor of GSH (74). GSH plays a major role in cellular defense against oxidative stress (50). Because NAC was able to inhibit KSHV reactivation induced by NF-κB inhibition (Fig. 1), we asked whether intracellular GSH plays a role in maintaining KSHV latency in PEL cells. Treatment of BC-3-G cells with 0.1 or 0.2 mM diethyl maleate (DEM), which can deplete

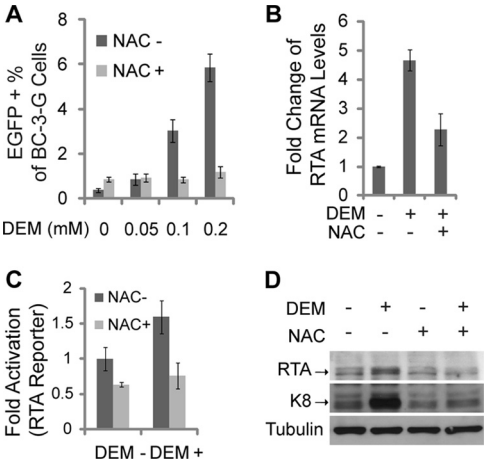

FIG. 2. Glutathione depletion induces ROS-dependent reactivation of KSHV. (A) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 0.05, 0.1, or 0.2 mM DEM for 48 h in the presence or absence of 10 mM NAC added 1 h prior to DEM treatment. (B) RT-Q-PCR analysis of RTA transcripts in BC-3 cells untreated or treated with 0.1 mM DEM for 48 h in the presence or absence of 10 mM NAC pretreatment. (C) KSHV RTA promoter activity measured by a luciferase reporter assay in BC-3 cells untreated or treated with 0.1 mM DEM for 48 h in the presence or absence of 10 mM NAC pretreatment. (D) Western blotting with anti-RTA, anti-K8, and anti-α-tubulin antibodies of cell lysates of BCBL-1 cells untreated or treated 0.1 mM DEM for 48 h in the presence or absence of 10 mM NAC pretreatment. For panel D, data are representative of three independent experiments. For panels A to C, data are the mean ± standard deviations from three independent experiments.

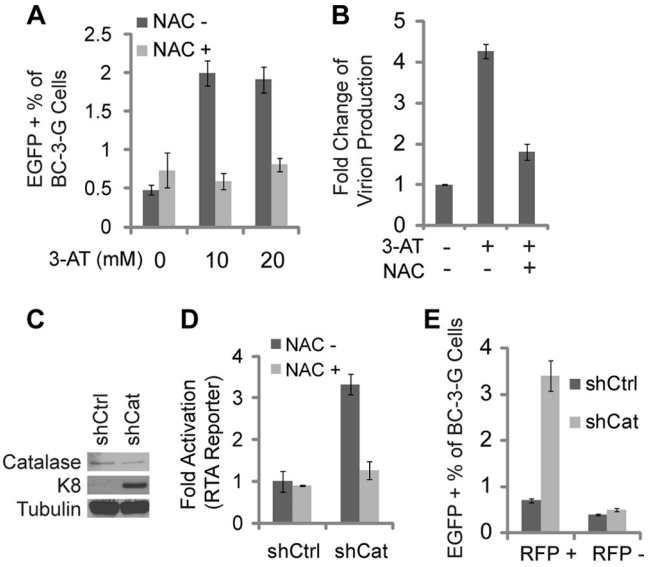

FIG. 3. Inhibition or depletion of catalase induces ROS-dependent reactivation of KSHV. (A) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 10 or 20 mM 3-AT for 48 h in the presence or absence of 10 mM NAC pretreatment. (B) Relative virion production by BC-3 cells untreated or treated with 10 mM 3-AT for 72 h in the presence or absence of 10 mM NAC pretreatment. (C) Western blotting with anti-catalase, anti-K8, and anti-α-tubulin antibodies of cell lysates of BC-3 cells transfected with the shRNA plasmid targeting catalase (shCat) or the control shRNA plasmid (shCtrl). Transfected cells were selected with 3 μg/ml of puromycin at 24 h posttransfection and collected for analysis at 72 h posttransfection. (D) KSHV RTA promoter activity measured by a luciferase reporter assay in BC-3 cells transfected with shCat or shCtrl. Cells were untreated or treated with 10 mM NAC at 12 h posttransfection and collected for analysis at 60 h posttransfection. (E) KSHV reactivation measured by FACS analysis of EGFP expression in RFP-positive and RFP-negative BC-3-G cells cotransfected with an RFP expression plasmid and shCat or shCtrl at 72 h posttransfection. For panel C, data are representative of three independent experiments. For panels A, B, D, and E, data are the means ± standard deviations from three independent experiments.

intracellular GSH (69), for 48 h increased the percentages of EGFP-positive BC-3-G cells, which is indicative of enhanced reactivation of KSHV (Fig. 2A). Importantly, pretreatment of cells with NAC was able to inhibit reactivation induced by DEM (Fig. 2A), suggesting that DEM induced KSHV reactivation specifically through depleting intracellular GSH. We noticed that further increasing the concentration of DEM to 0.4 mM and the duration of DEM treatment from 48 h to 72 h caused a significant decrease of the percentage of EGFP-positive cells and a large increase in cell death (data not shown), suggesting that severe depletion of GSH favors cell death at the expense of KSHV reactivation.

Consistent with the ability of DEM to induce KSHV reactivation, treatment of BC-3 cells with DEM for 48 h significantly increased the mRNA levels of RTA and NAC pretreatment inhibited the enhancement of RTA mRNA levels by DEM (Fig. 2B). Furthermore, treatment of BC-3 cells with DEM activated the RTA promoter in a luciferase reporter assay (Fig. 2C), suggesting that depletion of GSH induces KSHV reactivation through activating the RTA promoter. We also observed increased protein levels of RTA and K8 in BCBL-1 cells treated with DEM (Fig. 2D), consistent with virus reactivation.

Inhibition of catalase induces KSHV reactivation in a ROS-dependent manner. An important mechanism by which GSH protects cells against oxidative stress is through functioning as a cofactor of glutathione peroxidase (GPx) to detoxify hydrogen peroxide (H_2O_2), a major type of ROS (64). Therefore, the reactivation of KSHV after depletion of GSH suggests that intracellular hydrogen peroxide may be an inducer of KSHV reactivation. Another major antioxidant enzyme that protects cells from hydrogen peroxide is catalase. To investigate the role of hydrogen peroxide in KSHV reactivation, we examined the effects of catalase inhibition on KSHV reactivation. Treatment of BC-3-G cells with a 10 mM or 20 mM concentration of a catalase inhibitor, 3-amino-1,2,4-triazole (3-AT), for 48 h increased the percentage of EGFP-positive cells (Fig. 3A). Pretreatment of cells with NAC inhibited KSHV reactivation induced by 3-AT (Fig. 3A), suggesting that catalase inhibition induced KSHV reactivation in a ROS-dependent manner. Consistent with the ability of 3-AT to induce virus reactivation, treatment of BC-3 cells with 10 mM 3-AT also enhanced the production of viral particles in a ROS-dependent manner (Fig. 3B).

Knockdown of catalase induces KSHV reactivation. To further confirm the role of catalase in KSHV reactivation, we also used shCat, a plasmid that expresses short hairpin RNA (shRNA) targeting catalase, to examine the effects of catalase depletion on KSHV reactivation. We transfected BC-3 cells

with shCat or shCtrl, a control shRNA plasmid that does not target any cellular genes. We also cotransfected cells with shCtrl and a plasmid that expresses RTA as a positive control for inducing KSHV reactivation. Because of the low transfection efficiency of BC-3 cells, with fewer than 30% of cells transfected, we selected transfected cells with puromycin since shCtrl and shCat carry a puromycin resistance gene. Western blotting showed that at 72 h posttransfection, shCat was able to moderately knock down catalase and increase the protein levels of K8 (Fig. 3C), suggesting that catalase knockdown can induce KSHV reactivation. The degree of catalase knockdown was moderate but consistent in three independent experiments. We also found that enforced expression of KSHV RTA increases the protein level of catalase in BC-3 cells (data not shown). The upregulation of catalase by RTA expression may be a reason why we observed only moderate levels of catalase knockdown. In addition, transfection of BC-3 cells with shCat activated the RTA promoter in a luciferase reporter assay (Fig. 3D), suggesting that knockdown of catalase induces KSHV reactivation through activating the RTA promoter.

Catalase protects cells from oxidative stress through decomposing hydrogen peroxide into water (64), and hydrogen peroxide can diffuse through membranes (66). Therefore, it is possible that hydrogen peroxide released by cells in which catalase expression has been inhibited can diffuse to neighboring cells and induce KSHV reactivation. To investigate whether knockdown of catalase induces KSHV reactivation in a cell-autonomous manner, we cotransfected BC-3-G cells with a plasmid expressing red fluorescent protein (RFP) and shCat or shCtrl. At 72 h posttransfection, we examined the percentages of EGFP-positive cells in RFP-positive and RFP-negative cell populations. shCat transfection was able to significantly increase the percentage of EGFP-positive cells only in the RFP-positive cell population and not in the RFP-negative cell population (Fig. 3E), suggesting that knockdown of catalase induces reactivation of KSHV in a cell-autonomous manner.

p38 signaling is required for KSHV reactivation induced by ROS. Having established a role for ROS in KSHV reactivation, we asked what downstream signaling events mediate virus reactivation induced by ROS. ROS can activate mitogen-activated protein kinase (MAPK) pathways (30) that have been shown to play a role in KSHV reactivation (72, 76). Therefore, we used chemical inhibitors of MAPK pathways to examine whether MAPK pathways are required for KSHV reactivation induced by ROS. SB 203580 (SB), a specific inhibitor of p38 MAPK, inhibited KSHV reactivation in BC-3-G cells treated with Bay, whereas PD 98059 (PD), a specific inhibitor of MEK1/2, did not (Fig. 4A), suggesting that p38 signaling is required for KSHV reactivation induced by Bay.

We next asked whether NF-κB inhibition can activate p38. Treatment of BC-3 and BCBL-1 cells with 5 μM Bay significantly activated p38 at 2 and 8 h posttreatment, as shown by increased activating phosphorylation of p38 at these time points (Fig. 4B). Importantly, pretreatment of cells with NAC inhibited the activation of p38 by Bay (Fig. 4B), suggesting that NF-κB inhibition activates p38 in a ROS-dependent manner.

We also examined whether p38 signaling is required for KSHV reactivation induced by GSH depletion or catalase inhibition. Inhibition of p38 with p38 inhibitor III (p38 I-III) or p38 inhibitor VIII (p38 I-VIII), two highly specific inhibitors of p38, prevented KSHV reactivation induced by either DEM or 3-AT (Fig. 4C), suggesting that KSHV reactivation induced by GSH depletion or catalase inhibition also requires p38 signaling. In addition, activation of the RTA promoter by DEM or 3-AT was also inhibited by p38 inhibition (Fig. 4D). Treatment of KSHV-negative 293T cells with 3-AT also increased RTA promoter activity (Fig. 4E), suggesting that ROS can activate the RTA promoter independent of the autoactivation of the RTA promoter by RTA itself. Furthermore, overexpression of

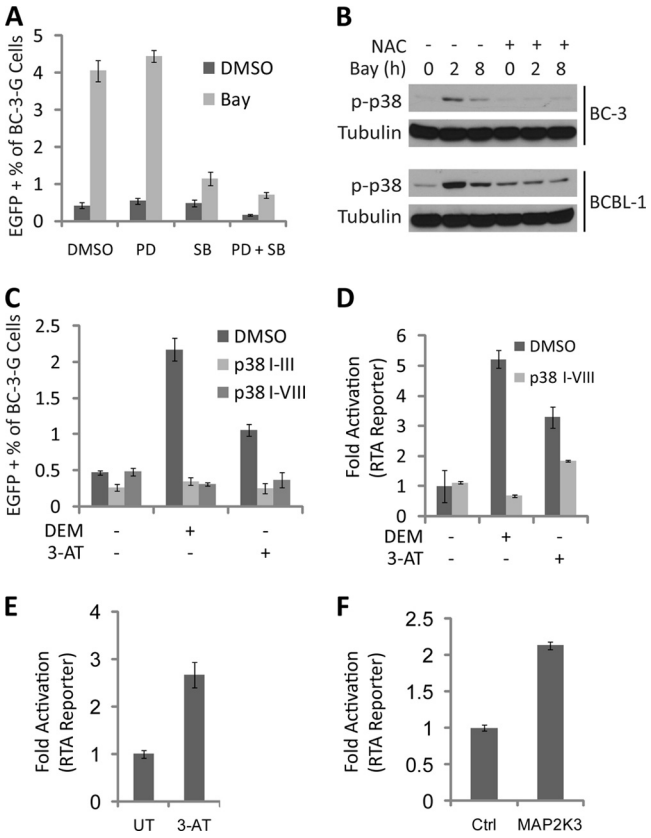

FIG. 4. Requirement of p38 signaling for KSHV reactivation induced by ROS. (A) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells treated with 5 μM Bay or DMSO vehicle control for 48 h in the presence or absence of pretreatment with 20 μM MEK inhibitor PD 98059 (PD), 20 μM p38 inhibitor SB 203580 (SB), PD and SB, or DMSO vehicle control. (B) Western blotting with anti-phospho-p38 (Thr180/Tyr182) and anti-α-tubulin antibodies of cell lysates of BC-3 and BCBL-1 cells untreated or treated with 5 μM Bay for 2 or 8 h in the presence or absence of 10 mM NAC pretreatment. (C) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 0.1 mM DEM or 10 mM 3-AT for 48 h in the presence or absence of pretreatment with 20 μM p38 inhibitor III (p38 I-III), 40 μM p38 inhibitor VIII (p38 I-VIII), or DMSO vehicle control. (D) KSHV RTA promoter activity measured by a luciferase reporter assay in BC-3 cells untreated or treated with 0.1 mM DEM or 10 mM 3-AT for 48 h in the presence of 40 μM p38 I-VIII or DMSO pretreatment. (E) KSHV RTA promoter activity measured by a luciferase reporter assay in 293T cells untreated or treated with 20 mM 3-AT for 24 h. (F) KSHV RTA promoter activity measured by a luciferase reporter assay in 293T cells transfected with a MAP2K3 expression plasmid or a control plasmid. For panel B, data are representative of two independent experiments. For panels A, C, D, E, and F, data are the means ± standard deviations from three independent experiments.

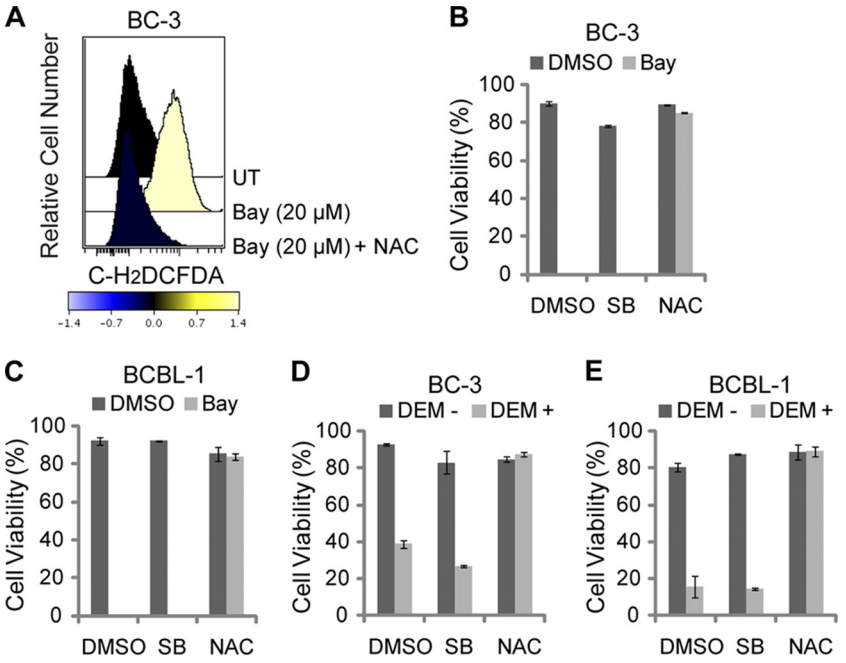

FIG. 5. ROS induce PEL cell death. (A) FACS analysis of c-H2DCFDA staining, showing ROS levels in BC-3 cells untreated (UT) or treated with 20 μM Bay for 2 h in the presence or absence of 10 mM NAC added 1 h prior to Bay treatment. Data are representative of three independent experiments. (B and C) Cell viability measured by trypan blue exclusion assay of BC-3 cells (B) and BCBL-1 cells (C) treated with 20 μM Bay or DMSO vehicle control for 48 h in the presence of pretreatment with 10 mM NAC, 20 μM SB, or DMSO vehicle control. (D and E) Cell viability measured by trypan blue exclusion assay of BC-3 cells (D) and BCBL-1 cells (E) untreated or treated with 0.25 mM DEM for 72 h in the presence of pretreatment with 10 mM NAC, 20 μM SB, or DMSO vehicle control. For panels B to E, data are the means ± standard deviations from three independent experiments.

MAP2K3, which can activate p38 (18), activated the RTA promoter (Fig. 4F), suggesting that p38 activation is sufficient for RTA promoter activation. Thus, p38 signaling mediates KSHV reactivation induced by ROS through facilitating the activation of the RTA promoter.

ROS are required for PEL cell death induced by NF-κB inhibition and GSH depletion. ROS are known to play important roles in cell death (23, 63). Previous studies showed that inhibition of NF-κB induces apoptosis in PEL cells (28, 36). NF-κB inhibition can induce ROS-dependent death of fibroblast and T-cell lymphoma cells (37, 62). Therefore, we asked whether ROS play a role in PEL cell death induced by NF-κB inhibition. Treatment of BC-3 cells with 5 μM Bay for 24 h (Fig. 1B) induced certain degrees of cell death, whereas treatment of BC-3 and BCBL-1 cells with 20 μM Bay reduced cell viability to zero at 48 h posttreatment as determined by trypan blue exclusion assay (Fig. 5B and C), consistent with the notion that a greater degree of NF-κB inhibition shifts the balance from KSHV reactivation toward cell death. We also observed a greater degree of ROS upregulation inhibited by NAC pretreatment in cells treated with 20 μM Bay (Fig. 5A) than in cells treated with 5 μM Bay (Fig. 1A), suggesting that higher levels of ROS favor cell death. Importantly, pretreatment of BC-3 and BCBL-1 cells with the antioxidant NAC completely prevented cell death induced by Bay, whereas inhibition of p38 with SB did not (Fig. 5B and C), suggesting that ROS, but not downstream p38 signaling, are required for cell death induced by NF-κB inhibition.

We also measured the viability of BC-3 and BCBL-1 cells treated with the GSH-depleting reagent DEM. We found that increasing the concentrations of DEM and the duration of DEM treatment increased cell death (data not shown). At 72 h posttreatment with 0.25 mM DEM, a concentration comparable to the highest concentration of 0.2 mM (Fig. 2A) used for inducing KSHV reactivation, cell viability was reduced to below 40% in BC-3 cells and to below 20% in BCBL-1 cells (Fig. 5D and E). Pretreatment of cells with NAC completely prevented increased cell death induced by DEM, whereas pretreatment of cells with SB did not (Fig. 5D and E), consistent with the results observed in cells treated with Bay (Fig. 5B and C).

The anticancer drugs cisplatin and arsenic trioxide induce KSHV reactivation and PEL cell death in a ROS-dependent manner. Certain anticancer drugs are known to induce ROS generation (63). For example, the widely used chemotherapeutic drug cisplatin has been shown to induce ROS through disrupting the function of mitochondria (38). We therefore asked whether cisplatin can induce KSHV reactivation in a ROS-dependent manner. Treatment of BC-3-G cells with 4.1 μg/ml of cisplatin for 48 h induced KSHV reactivation, which was inhibited by pretreatment with NAC (Fig. 6A), suggesting that cisplatin can indeed induce KSHV reactivation in a ROS-dependent manner. Consistent with the requirement of p38 signaling in KSHV reactivation induced by ROS, reactivation induced by cisplatin also required p38 signaling (Fig. 6B).

Another anticancer drug that has been shown to induce ROS is arsenic trioxide (As_2O_3). Arsenic trioxide has been used successfully to treat acute promyelocytic leukemia (58).

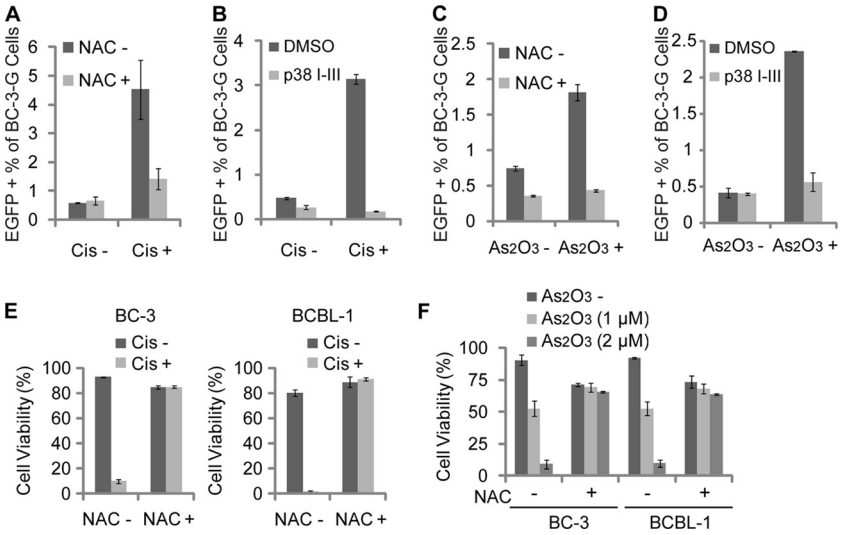

FIG. 6. Cisplatin and arsenic trioxide induce KSHV reactivation and PEL cell death in a ROS-dependent manner. (A) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 4.1 μg/ml of cisplatin for 48 h in the presence or absence of 10 mM NAC pretreatment. (B) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 4.1 μg/ml of cisplatin for 48 h in the presence or absence of pretreatment with 20 μM p38 inhibitor III or DMSO vehicle control. (C) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 1 μM arsenic trioxide for 48 h in the presence or absence of 10 mM NAC pretreatment. (D) KSHV reactivation measured by FACS analysis of EGFP expression in BC-3-G cells untreated or treated with 0.5 μM arsenic trioxide for 48 h in the presence or absence of pretreatment with 20 μM p38 inhibitor III or DMSO vehicle control. (E) Cell viability measured by trypan blue exclusion assay of BC-3 and BCBL-1 cells untreated or treated with 4.1 μg/ml of cisplatin for 72 h in the presence or absence of pretreatment with 10 mM NAC. (F) Cell viability measured by trypan blue exclusion assay of BC-3 and BCBL-1 cells untreated or treated with 1 μM or 2 μM arsenic trioxide for 48 h in the presence or absence of pretreatment with 10 mM NAC. Data are the means ± standard deviations from three independent experiments.

Treatment of BC-3-G cells with 0.5 μM and 1 μM arsenic trioxide, concentrations easily achievable *in vivo*, induced KSHV reactivation (Fig. 6C). Inhibition of p38 signaling with p38 inhibitor III inhibited reactivation induced by arsenic trioxide (Fig. 6D). Therefore, arsenic trioxide can also induce KSHV reactivation through the ROS-p38 signaling pathway.

We then asked whether cisplatin and arsenic trioxide can also induce PEL cell death in a ROS-dependent manner. Treatment of BC-3 and BCBL-1 cells with 4.1 μg/ml of cisplatin for 72 h induced cell death, which was prevented by pretreatment with NAC (Fig. 6E). Similarly, treatment of BC-3 and BCBL-1 cells with 1 μM or 2 μM arsenic trioxide for 48 h induced significant cell death, which was also prevented by pretreatment with NAC (Fig. 6F). Therefore, both cisplatin and arsenic trioxide can induce KSHV reactivation and PEL cell death in a ROS-dependent manner.

DISCUSSION

Latency is a strategy for a virus to establish persistent infection in an immunocompetent host. It presents a major hurdle to the destruction of KSHV-associated tumor cells by facilitating immune evasion of KSHV-infected tumor cells and promoting tumor cell survival and proliferation through the expression of oncogenic latent genes. In this study, we investigated the role of ROS in the regulation of viral latency and cell survival. An overview of the intracellular antioxidant system (reviewed in references 23 and 64) and a working model of the regulation of KSHV reactivation and PEL cell death by ROS are summarized in Fig. 7. Cellular antioxidant systems

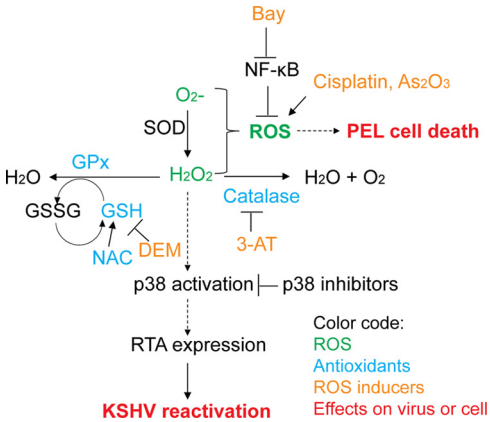

FIG. 7. Model of the regulation of KSHV reactivation and PEL cell death by oxidative stress. Cellular antioxidant systems protect cells from oxidative stress by converting reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) into H_2O and O_2. These antioxidant systems include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). SOD converts O_2^- into H_2O_2, which can be further converted into H_2O and O_2 by catalase or into H_2O by GPx. Glutathione (GSH) provides the reducing potential for GPx to reduce H_2O_2 to H_2O, while being oxidized into an oxidized form (GSSG). NAC functions as an antioxidant by increasing intracellular concentrations of GSH. Oxidative stress resulting from inhibition of NF-κB or inhibition of various other intracellular antioxidant systems leads to p38 activation, which enhances RTA expression and ultimately reactivation of KSHV. Oxidative stress can also lead to death of primary effusion lymphoma cells.

protect cells from oxidative stress. These antioxidant systems include enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). SOD catalyzes the conversion of superoxide (O_2^-) into hydrogen peroxide (H_2O_2), which can be further converted into H_2O and O_2 by catalase or into H_2O by GPx. Glutathione (GSH) is essential for the function of GPx by providing the reducing potential for GPx to reduce H_2O_2 to H_2O, while being oxidized to an oxidized form (GSSG). NAC functions as an antioxidant by increasing the intracellular concentration of GSH. Inhibition of NF-κB has been shown to result in virus reactivation, and we show here that ROS play a critical role. We show that ROS are required for KSHV reactivation induced by GSH depletion, inhibition or depletion of catalase, and anticancer drugs such as cisplatin and As_2O_3. Furthermore, we demonstrate that ROS are required for PEL cell death induced by NF-κB inhibition, GSH depletion, cisplatin, and As_2O_3. Therefore, our findings establish a critical role for ROS and cellular antioxidant systems in regulating KSHV reactivation and the survival of KSHV-infected tumor cells.

Previous studies showed that NF-κB inhibition can lead to KSHV reactivation (5, 42) and the death of PEL cells (28, 35, 36), two seemingly contradictory phenotypes. Our data indicate that ROS are required for both KSHV reactivation (Fig. 1) and PEL cell death (Fig. 5B and C) induced by NF-κB inhibition, providing a mechanistic link between these two important consequences of NF-κB inhibition. Furthermore, our data suggest that the degree of NF-κB inhibition and the levels of ROS affect the balance between KSHV reactivation and PEL cell death, since a lower (5 μM) concentration of Bay induced lower levels of ROS and KSHV reactivation (Fig. 1), whereas a higher (20 μM) concentration of Bay led to higher levels of ROS (Fig. 5A) and mostly cell death (Fig. 5B and C). We did observe that treatment of cells with a moderate (5 μM) concentration of Bay resulted in both KSHV reactivation (Fig. 1C and F) and moderate levels of cell death (Fig. 1B) in a population of cells. A possible explanation for the coexistence of KSHV reactivation and cell death could be that individual cells within the whole population behave differently due to stochastic cell-to-cell differences such as variation in antioxidant levels, resulting in virus reactivation in some of the cells and cell death in other cells. The coexistence of cells in which KSHV was reactivated and cells that died due to oxidative stress in the same cell population may be one of the reasons that KSHV is not reactivated in all cells under oxidative stress. Nonetheless, ROS levels seem to be a critical factor in regulating the balance between KSHV reactivation and PEL cell death.

Despite the requirement of ROS for both KSHV reactivation and PEL cell death induced by NF-κB inhibition or GSH depletion (Fig. 1 and 5B to E), p38 signaling is required only for virus reactivation and not for tumor cell death (Fig. 5B to E), suggesting that signaling events bifurcate downstream of ROS to mediate two processes, i.e., virus reactivation and cell death. We showed that ROS moderately activate the RTA promoter (Fig. 1E, 2C, 3D, and 4E), likely via p38 (Fig. 4F). RTA can activate its own promoter, constituting a positive feedback loop (17). Thus, a moderate increase of the activity of the RTA promoter may lead to a greater degree of KSHV reactivation, making RTA a sensitive switch between latency and the lytic cycle. ROS may also upregulate the expression of other KSHV lytic genes independent of its ability to increase RTA expression.

Previous studies have demonstrated that NF-κB can reduce ROS through upregulating antioxidant proteins, such as manganese superoxide dismutase (MnSOD) (62) and ferritin heavy chain (52). A recent study showed that in endothelial cells, the viral latent protein vFLIP/K13 can upregulate MnSOD in an NF-κB-dependent manner (62a), suggesting that MnSOD may also play an important role in ROS suppression in PEL cells. The authors also showed that vFLJP/Kl3 can protect endothelial cells from superoxide-induced cell death through upregulating MnSOD (62a), consistent with our results demonstrating the essential role of ROS in PEL cell death induced by NF-κB inhibition.

Oxidative stress caused by excessive amounts of ROS can damage various cellular components and jeopardize cell survival (23). Because the survival of KSHV in its latent state depends entirely on the survival of infected cells, in order to avoid elimination, it is logical and critical for KSHV to sense oxidative stress and initiate lytic reactivation either to escape from those cells that are destined to die or possibly to counter oxidative stress through regulating viral and cellular gene expression. In fact, both *de novo* infection by KSHV and reactivation of KSHV can activate NF-κB (27, 54), suggesting that once lytic replication is initiated, upregulated NF-κB activity may help KSHV control the intracellular redox balance to facilitate lytic gene expression and the completion of the viral lytic cycle. Thus, cells under the assault of oxidative stress may not provide the optimal cellular environment for efficient lytic replication of KSHV, in accordance with a previous study showing that inhibition of NF-κB in endothelial cells reduces lytic gene expression upon *de novo* infection (54). Nonetheless, reactivation of KSHV in cells under oxidative stress is probably an appropriate response that is essential for the survival and propagation of KSHV.

Although the extent of reactivation induced by ROS is less than the extent of reactivation induced by the strongest inducers, such as TPA or butyrate (Fig. 1C and D), it is comparable to or greater than the extent of reactivation induced by some other known inducers of KSHV reactivation, such as Toll-like receptor ligands (26), hypoxia (16), and KSHV-encoded microRNAs (2). More importantly, in contrast to TPA or butyrate, ROS are natural products of cellular metabolism and play critical roles in a number of physiological and pathological conditions. For example, cells of the immune system can produce high levels of ROS during inflammatory processes (64). Because inflammation is one of the key features of KS (24), ROS may play an important role in regulating the balance between latency and lytic replication in KS. Thus, our finding in this study can provide one explanation for the pathogenesis associated with KSHV infection and reactivation.

ROS might also play a role in virus reactivation induced by hypoxia. Hypoxia induces KSHV reactivation (16). The transcription factors hypoxia-inducible factor 1 alpha (HIF-1α) (6, 15) and X-box binding protein 1 (XBP-1) (15, 71, 75) have been shown to be involved in hypoxia-induced reactivation of KSHV. ROS generated by mitochondria signal hypoxia and are required for HIF-1α stabilization during hypoxia (9, 10),

suggesting that ROS may play a role in KSHV reactivation induced by hypoxia.

Our results also show that anticancer drugs such as cisplatin and arsenic trioxide can induce KSHV reactivation and PEL cell death in a ROS-dependent manner (Fig. 6). Notably, virus reactivation and cell death were induced by arsenic trioxide at concentrations readily achievable *in vivo* (Fig. 6), suggesting that arsenic trioxide may be useful for treating KSHV-associated malignancies. Taken together, our findings establish a key role for ROS in regulating KSHV latency and the survival of KSHV-infected lymphoma cells, providing the framework for us to understand and exploit viral and cellular regulation of the redox balance in order to design novel approaches for treating KSHV-associated malignancies.

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X.L. and R.S. conceived and designed the experiments, X.L. and J.F. performed flow cytometry experiments, X.L. performed other experiments, and X.L. and R.S. analyzed the data and wrote the paper.

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