Reactive Oxygen Species Hydrogen Peroxide Mediates Kaposi’s Sarcoma-Associated Herpesvirus Reactivation from Latency

Fengchun Ye1,2, Fuchun Zhou1,2, Roble G. Bedolla1,2, Tiffany Jones1,3, Xiufen Lei1,2, Tao Kang1, Moraima Guadalupe1,2, Shou-Jiang Gao1,2,4*

1 Tumor Virology Program, Greehey Children’s Cancer Research Institute, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States of America, 2 Department of Pediatrics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States of America, 3 Department of Molecular Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States of America, 4 Cancer Therapy and Research Center, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States of America.

Abstract

Kaposi’s sarcoma-associated herpesvirus (KSHV) establishes a latent infection in the host following an acute infection. Reactivation from latency contributes to the development of KSHV-induced malignancies, which include Kaposi’s sarcoma (KS), the most common cancer in untreated AIDS patients, primary effusion lymphoma and multicentric Castleman’s disease. However, the physiological cues that trigger KSHV reactivation remain unclear. Here, we show that the reactive oxygen species (ROS) hydrogen peroxide (H$_2$O$_2$) induces KSHV reactivation from latency through both autocrine and paracrine signaling. Furthermore, KSHV spontaneous lytic replication, and KSHV reactivation from latency induced by oxidative stress, hypoxia, and proinflammatory and proangiogenic cytokines are mediated by H$_2$O$_2$. Mechanistically, H$_2$O$_2$ induction of KSHV reactivation depends on the activation of mitogen-activated protein kinase (ERK)1/2, JNK, and p38 pathways. Significantly, H$_2$O$_2$ scavengers N-acetyl-L-cysteine (NAC), catalase and glutathione inhibit KSHV lytic replication in culture. In a mouse model of KSHV-induced lymphoma, NAC effectively inhibits KSHV lytic replication and significantly prolongs the lifespan of the mice. These results directly relate KSHV reactivation to oxidative stress and inflammation, which are physiological hallmarks of KS patients. The discovery of this novel mechanism of KSHV reactivation indicates that antioxidants and anti-inflammation drugs could be promising preventive and therapeutic agents for effectively targeting KSHV replication and KSHV-related malignancies.

Citation: Ye F, Zhou F, Bedolla RG, Jones T, Lei X, et al. (2011) Reactive Oxygen Species Hydrogen Peroxide Mediates Kaposi’s Sarcoma-Associated Herpesvirus Reactivation from Latency. PloS Pathog 7(5): e1002054. doi:10.1371/journal.ppat.1002054

Editor: Klaus Früh, Oregon Health & Science University, United States of America

Received September 30, 2010; Accepted March 16, 2011; Published May 19, 2011

Copyright: © 2011 Ye et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by a Pilot Grants Program from the Nathan Shock Center of Excellence in the Biology of Aging from the Barshop Institute for Longevity and Aging Studies at San Antonio, Texas to F.Y., and grants from National Institute of Health (DE017333, CA096512, CA124332, and CA119889) to S.-J.G. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: gao@uthscsa.edu

Introduction

A hallmark of herpesviral infections is the establishment of latency in the hosts following acute infections [1]. Reactivation of herpesviruses from latency results in production of infectious virions and often development of their associated diseases. KSHV is a gammaherpesvirus associated with KS, a vascular malignancy of endothelial cells commonly seen in AIDS patients [2]. KSHV is also linked to other lymphoproliferative diseases including primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD) [2–4]. Similar to other herpesviruses, KSHV establishes a lifelong persistent infection in the host [1]. In KS tumors, most tumor cells are latently infected by KSHV, indicating an essential role of viral latency in tumor development [5]. However, KSHV lytic replication also contributes to KS pathogenesis [6]. Both viral lytic products and de novo infection promote cell proliferation, invasion, angiogenesis, inflammation and vascular permeability [6]. In fact, higher KSHV lytic antibody titers and peripheral blood viral loads are correlated with high incidence and advanced stage of KS [7–13], and KS regressed following anti-herpesviral treatments that inhibit lytic replication [14,15].

While several cellular pathways such as mitogen-activated protein kinase (MAPK) pathways and protein kinase C delta regulate KSHV lytic replication [16–20], the common physiological trigger that reactivates KSHV from latency in patients remains unclear. A number of factors including proinflammatory and proangiogenic cytokines [21,22], hypoxia [23], HIV and its product Tat [24–26], coinfection with human cytomegalovirus and human herpesvirus 6 [27,28], and the activation of toll-like receptors [29] can cause KSHV reactivation in cultures. However, none of them is likely the trigger in all the clinical scenarios, which include different forms of KS, PEL and MCD. The mechanisms by which these factors reactivate KSHV from latency also remain unclear.

There are four clinical forms of KS. Patients with all forms of KS are characterized by high levels of inflammation and oxidative stress [30,31]. Classical KS, mostly seen in elderly men in the Mediterranean and Eastern European regions, is ubiquitously associated with high level of inflammation and oxidative stress...
Author Summary
Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiologic agent of all clinical forms of Kaposi’s sarcoma (KS) and several other malignancies. The life cycle of KSHV consists of latent and lytic phases. While establishment of viral latency is essential for KSHV to evade host immune surveillances, viral lytic replication promotes KSHV-induced malignancies. In this study, we show that the reactive oxygen species (ROS) hydrogen peroxide (H2O2) induces KSHV reactivation from latency. Furthermore, induction of KSHV reactivation by oxidative stress, hypoxia, and proinflammatory and proangiogenic cytokines, which are physiological hallmarks in all clinical forms of KS patients, is mediated by H2O2. Significantly, antioxidants inhibit H2O2-induced KSHV lytic replication in culture and in a mouse model of KSHV-induced lymphoma. These results show that ROS is likely an important physiological cue that triggers KSHV replication. The discovery of this novel mechanism of KSHV reactivation indicates that antioxidants and anti-inflammation drugs might be promising preventive and therapeutic agents for effectively targeting KSHV replication and KSHV-related malignancies.

because of its close link with aging [32]. In African endemic KS, excessive iron exposure due to high content of iron in the local soils coupled with bare foot walking is a possible cofactor that can induce inflammation and oxidative stress [33,34]. In transplantation KS, inflammation and oxidative stress are common because of immunosuppression and organ rejection [35]. Patients with AIDS-related KS (AIDS-KS) have high levels of inflammation and oxidative stress as a result of host responses to HIV infection and chronic inflammation [36]. In all clinical forms of KS, inflammation and oxidative stress are also the hallmarks in the tumors [37]. Both PEL and MCD often coexist with KS, and are commonly seen in HIV-infected patients [6]. PEL is often found in elderly men, particularly in HIV-negative cases. Thus, similar to KS patients, these patients often have high levels of inflammation and oxidative stress. Since inflammation and oxidative stress induce ROS, and high level of ROS activates the MAPK pathways [38], we postulated that ROS, as a result of inflammation and oxidative stress, might mediate KSHV reactivation from latency.

The most common ROS molecule in non-immune cells is H2O2, which is mainly produced by mitochondria as a byproduct of oxidative metabolism [38]. Because high level of H2O2 is cytotoxic, cells express multiple antioxidant enzymes such as catalase and glutathione peroxidase to remove H2O2 so that it is below the detrimental threshold in normal condition. During oxidative stress, cells produce and release a large amount of H2O2 as a consequence of lost balance between its production and its scavenging [38]. During infections and inflammatory responses, host phagocytes such as macrophages and neutrophils produce and release excessive amounts of H2O2 [39–41]. Thus, KS patients are deemed to have high levels of H2O2. In this study, we investigated the physiological role of H2O2 on KSHV reactivation.

Results
H2O2 induces KSHV reactivation through both paracrine and autocrine mechanisms
To examine the relationship of H2O2 with KSHV lytic replication, we stably expressed an H2O2-specific yellow fluorescent protein (pYFP) sensor from the HyPer-cyto cassette in KSHV-infected BCBL1 cells [42]. As previously reported [20], the majority of BCBL1 cells were latently infected by KSHV but a small percentage of them underwent spontaneous lytic replication, which was detected by staining for viral late lytic protein ORF65 (Figure 1A). Notably, these ORF65-positive cells were strongly positive for cpYFP (Figure 1A). In contrast, ORF65-negative cells were either weakly positive or negative for cpYFP. Treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), a common chemical inducer for KSHV lytic replication, increased the number of lytic cells, all of which also expressed high level of cpYFP while ORF65-negative cells remained weakly positive for cpYFP (Figure 1A). Extended exposure of the images or adjustment of the contrast showed that almost all the TPA-treated cells were positive for cpYFP albeit with diverse intensities (data not shown).

These diverse levels of H2O2 among the individual cells could be due to their different sizes of intracellular antioxidant enzyme pools. Together, these results showed a close correlation between high level of intracellular H2O2 and KSHV lytic replication.

To investigate the role of H2O2 in KSHV lytic replication, we examined whether exogenous H2O2, which uses water channels (aquaporins) to cross the cell membrane [43], is sufficient to induce KSHV reactivation. We observed a dose-dependent induction, at both mRNA and protein levels, of KSHV replication and KSHV-related malignancies. In this study, we show that the reactive oxygen species (ROS) hydrogen peroxide (H2O2) induces KSHV reactivation by oxidative stress, hypoxia, and proinflammatory and proangiogenic cytokines, which are physiological hallmarks in all clinical forms of KS patients, is mediated by H2O2. Significantly, antioxidants inhibit H2O2-induced KSHV lytic replication in culture and in a mouse model of KSHV-induced lymphoma. These results show that ROS is likely an important physiological cue that triggers KSHV replication. The discovery of this novel mechanism of KSHV reactivation indicates that antioxidants and anti-inflammation drugs might be promising preventive and therapeutic agents for effectively targeting KSHV replication and KSHV-related malignancies.

To confirm that the effect of ATZ on KSHV reactivation was due to an increase in intracellular H2O2 level, we stably expressed a siRNA specific to catalase in BCBL1 cells harboring a recombinant KSHV BAC36 [45]. Compared to cells stably expressing a scrambled siRNA, those expressing the catalase-specific siRNA had significantly lower expression levels of catalase transcript and protein (Figure 2F–G). Similar to treatment with ATZ, knockdown of catalase increased the expression of KSHV lytic transcripts of RTA, ORF57, ORF59, kbZIP and ORF65, genes, the expression of lytic protein ORF65, and production of infectious virions (Figure 2C–E). Interestingly, we observed an additive effect when either H2O2 or ATZ was used together with TPA to induce KSHV lytic replication (Figure 2C–E).

To confirm that the effect of ATZ on KSHV reactivation was due to an increase in intracellular H2O2 level, we stably expressed a siRNA specific to catalase in BCBL1 cells harboring a recombinant KSHV BAC36 [45]. Compared to cells stably expressing a scrambled siRNA, those expressing the catalase-specific siRNA had significantly lower expression levels of catalase transcript and protein (Figure 2F–G). Similar to treatment with ATZ, knockdown of catalase increased the expression of KSHV lytic transcripts of RTA, ORF57, ORF59, kbZIP and ORF65, lytic protein ORF65, and production of infectious virions (Figure 2F–H).

Taken together, our results so far have shown that an increase in intracellular or exogenous H2O2 level induces KSHV reactivation, indicating that H2O2 produced during inflammation and oxidative stress, might mediate KSHV reactivation from latency. Furthermore, induction of ROS, as a result of inflammation and oxidative stress as a consequence of lost balance between its production and its scavenging [38]. During infections and inflammatory responses, host phagocytes such as macrophages and neutrophils produce and release excessive amounts of H2O2 [39–41]. Thus, KS patients are deemed to have high levels of H2O2. In this study, we investigated the physiological role of H2O2 on KSHV reactivation.

Results
H2O2 induces KSHV reactivation through both paracrine and autocrine mechanisms
To examine the relationship of H2O2 with KSHV lytic replication, we stably expressed an H2O2-specific yellow fluorescent protein (pYFP) sensor from the HyPer-cyto cassette in KSHV-infected BCBL1 cells [42]. As previously reported [20], the majority of BCBL1 cells were latently infected by KSHV but a small percentage of them underwent spontaneous lytic replication, which was detected by staining for viral late lytic protein ORF65 (Figure 1A). Notably, these ORF65-positive cells were strongly positive for cpYFP (Figure 1A). In contrast, ORF65-negative cells were either weakly positive or negative for cpYFP. Treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), a common chemical inducer for KSHV lytic replication, increased the number of lytic cells, all of which also expressed high level of cpYFP while ORF65-negative cells remained weakly positive for cpYFP (Figure 1A). Extended exposure of the images or adjustment of the contrast showed that almost all the TPA-treated cells were positive for cpYFP albeit with diverse intensities (data not shown).

These diverse levels of H2O2 among the individual cells could be due to their different sizes of intracellular antioxidant enzyme pools. Together, these results showed a close correlation between high level of intracellular H2O2 and KSHV lytic replication.

To investigate the role of H2O2 in KSHV lytic replication, we examined whether exogenous H2O2, which uses water channels (aquaporins) to cross the cell membrane [43], is sufficient to induce KSHV reactivation. We observed a dose-dependent induction, at both mRNA and protein levels, of KSHV replication and KSHV-related malignancies. In this study, we show that the reactive oxygen species (ROS) hydrogen peroxide (H2O2) induces KSHV reactivation by oxidative stress, hypoxia, and proinflammatory and proangiogenic cytokines, which are physiological hallmarks in all clinical forms of KS patients, is mediated by H2O2. Significantly, antioxidants inhibit H2O2-induced KSHV lytic replication in culture and in a mouse model of KSHV-induced lymphoma. These results show that ROS is likely an important physiological cue that triggers KSHV replication. The discovery of this novel mechanism of KSHV reactivation indicates that antioxidants and anti-inflammation drugs might be promising preventive and therapeutic agents for effectively targeting KSHV replication and KSHV-related malignancies.

ROS Induction of KSHV Reactivation
stress in KS patients can be the physiological trigger that reactivates KSHV from latency through both autocrine and paracrine mechanisms.

**H₂O₂ scavengers inhibit H₂O₂-induced KSHV lytic replication**

To determine whether H₂O₂ is required for KSHV lytic replication, we used H₂O₂ scavengers to reduce the intracellular H₂O₂ level. As shown in Figure 1A, TPA not only induced KSHV reactivation but also increased the intracellular H₂O₂ level. At 12 h, H₂O₂ scavengers including catalase, reduced glutathione and NAC inhibited TPA induction of intracellular H₂O₂ as shown by the reduced median fluorescent levels in the cpYFP-expressing BCBL1 cells (Figure 3C). None of these treatments affected the viability and growth rate of the cells (data not shown). As expected, H₂O₂ scavengers inhibited TPA induction of RTA transcript and protein (Figure 3D–E). Consistent with these results, RTA promoter activities were induced 2.2- and 4.3-fold by H₂O₂ and TPA, respectively, and these induction effects were inhibited by NAC (Figure 3F). In contrast, a latent LANa promoter was not induced by H₂O₂ and only marginally induced by TPA for 1.4-fold (Figure 3G). NAC also abolished TPA induction of the LANa promoter activity. Furthermore, TPA induction of ORF65 protein and production of infectious virions were inhibited by H₂O₂ scavengers in a dose-dependent manner (Figure 3H). To examine whether H₂O₂ scavengers also inhibit KSHV spontaneous lytic replication, we measured the expression of ORF65 protein in BCBL1 cells treated with the scavengers. As shown in Figure 3J, both catalase and NAC inhibited the expression of ORF65 protein after 6 days but not 1 day of treatment, which is consistent with the late expression kinetics of this viral capsid protein. These results indicate that H₂O₂ is required for KSHV spontaneous lytic replication and TPA-induced KSHV reactivation, and antioxidants such as reduced glutathione and NAC can suppress KSHV lytic replication.

**H₂O₂ scavengers inhibit KSHV lytic replication induced by hypoxia, and proinflammatory and proangiogenic cytokines**

Because high levels of hypoxia, and proinflammatory and proangiogenic cytokines are features of KS tumors, and previous studies have shown that these conditions can induce KSHV reactivation [21–23], we determined whether KSHV reactivation induced by these conditions is mediated by H₂O₂. Short-time treatment with sodium azide (NaN₃), which induces hypoxia [46], increased intracellular H₂O₂ level as shown by cpYFP fluorescent level in BCBL1 cells (Figure 4A–B). As a result, the expression of RTA transcript was increased 12.2-fold by NaN₃, which was inhibited by both NAC and catalase (Figure 4C). Similarly, the expression of RTA and ORF65 proteins were induced by NaN₃, which was also inhibited by NAC and catalase (Figure 4D). As expected, HIF-1α was induced by NaN₃, which was also inhibited by NAC and catalase, suggesting that H₂O₂ mediates hypoxia induction of HIF-1α. These results are consistent with previous observations that H₂O₂ can directly induce HIF-1α [47].

Next, we determined the role of H₂O₂ in KSHV reactivation induced by proinflammatory and proangiogenic cytokines. Treatment of BCBL1 cells with vascular endothelial growth factor (VEGF), fibroblast growth factor-B (bFGF), interleukin-6 (IL-6) or tumor necrosis factor-alpha (TNF-α) alone minimally induced the expression of ORF65 protein by 1.5-, 1.2-, 1.4- and 1.2-fold, respectively, and these induction effects were reversed by NAC and catalase (Figure 4E). In contrast, insulin-like growth factor-1 (IGF-1), epithelial growth factor (EGF) and interferon gamma (IFN-γ) were more potent inducers, which increased the expression of ORF65 protein by 2.6-, 2.3- and 3.2-fold, respectively. Similarly, NAC and catalase inhibited the induction of ORF65 proteins by these cytokines. Since KS tumors contain abundant infiltration of proinflammatory immune cells such as monocytes, we further examined the effects of proinflammatory and proangiogenic cytokines on KSHV reactivation in the presence of monocytic cells U937 (Figure 4E). Co-culture of BCBL1 cells with U937 cells alone increased the expression of ORF65 protein by 2.4-fold. In the presence of U937 cells, weak inducers VEGF, bFGF, IL-6 and TNF-α more effectively increased the expression of ORF65 protein by 3.5-, 2.8-, 4.2- and 7-fold, respectively, suggesting a synergistic effect of these cytokines with U937 cells. These synergistic effects were also observed with strong inducers EGF and IFN-γ, which increased the expression of ORF65 protein by 5.2- and 10-fold in the presence of U937 cells. In contrast, IGF-1 did not further increase the expression of ORF65 protein in the presence of U937 cells. Both NAC and catalase inhibited the induction of ORF65 protein by proinflammatory and proangiogenic cytokines in the presence of U937 cells (Figure 4E). Together, these results indicate that H₂O₂ mediates KSHV reactivation induced by proinflammatory and proangiogenic cytokines with and without co-culture with the monocytic cells.

**H₂O₂ scavenger NAC inhibits KSHV lytic replication and tumor progression in vivo**

We next sought to inhibit KSHV lytic replication in vivo with H₂O₂ scavengers. To monitor KSHV lytic activity in vivo, we generated a recombinant KSHV Δ65Luc by replacing ORF65 with a firefly luciferase gene (Figure S1A–C). Because ORF65 is a late viral lytic gene, detection of its expression would imply nearly complete KSHV lytic replication cycle. We reconstituted Δ65Luc
in BCBL1 cells and generated a cell line harboring both wild type KSHV and Δ65Luc. As expected, BAC36-Δ65Luc cells expressed low level of ORF65 protein and luciferase, reflecting the spontaneous viral lytic replication in a small number of cells (Figure S1D). Treatment with TPA increased the expression of ORF65 and luciferase proteins. The corresponding luciferase activity was also increased by 6.2-fold (Figure S1E). These results indicate that the luciferase activity closely mimicked the expression of ORF65 protein, and thus can be used to monitor KSHV lytic activity. As expected, addition of NAC inhibited the luciferase activity in a dose-dependent manner (Figure 5A).

To examine KSHV lytic replication and determine the inhibitory effect of antioxidant NAC in vivo, we intraperitoneally inoculated NOD/SCID mice with BCBL1 cells harboring Δ65Luc. The mice were then fed daily with drinking water containing 5 mM NAC. All mice developed PEL at about five weeks (Figure 5B).
Figure 3. TPA-induced KSHV reactivation is mediated by H₂O₂. (A) Intracellular H₂O₂ levels in untreated BCBL1 cells and BCBL1 cells treated with TPA for 6 h. (B) Catalase protein levels in untreated BCBL1 cells and BCBL1 cells treated with TPA for 6 and 12 h. Catalase protein was detected by Western-blotting and calibrated with β-tubulin for sample loading. (C) H₂O₂ scavengers reduced TPA-induction of intracellular H₂O₂ levels measured by flow cytometry analysis of fluorescence intensity in BCBL1 cells stably expressing a H₂O₂ sensor protein cpYFP. Cells were treated with 20 ng/ml of TPA without any scavengers, or with scavenger catalase at 400 U/ml, reduced glutathione at 400 μM or NAC at 400 μM for 6 h. (D–E) H₂O₂ scavengers reduced TPA-induction of RTA transcript and protein in BCBL1 cells. Treatments of the cells were the same as in (C). RTA transcript was detected by RT-qPCR (D). RTA protein was detected by Western-blotting and calibrated with β-tubulin for sample loading (E). (F–G) H₂O₂ mediated the activation of lytic RTA but not latent LANA promoter. RTA promoter was induced by H₂O₂ and TPA, and this induction effect was inhibited by NAC (F). Latent LANA promoter was marginally activated by TPA but not by H₂O₂ (G). Luciferase activities were measured for 293 cells.
transfected with promoter reporter plasmids for 24 h, and treated with H2O2 (150 μM) or TPA (20 ng/ml) with or without NAC (400 μM) for 12 h. (I–H) H2O2 scavengers reduced TPA-induction of ORF65 protein and production of infectious virions in BCBL1 cells in a dose-dependent manner. TPA was used at 20 ng/ml. Relative ORF65 protein levels shown in numbers were detected following 72 h of treatment by Western-blotting and calibrated with β-tubulin for sample loading (H). Relative virus titers were determined by using supernatants collected at 5 days of treatment to infect endothelial cells and calculating the numbers of GFP-positive cells at 48 hpi (I). (J) H2O2 scavengers inhibited KSHV spontaneous lytic replication. ORF65 protein in BCBL1 cells treated with catalase (400 U/ml) and NAC (400 μM) for 1 and 6 day was determined by Western-blotting.

doi:10.1371/journal.ppat.1002054.g003

Discussion

We have shown that ROS H2O2 induces KSHV lytic replication through both paracrine and autocrine mechanisms, and in both PEL and endothelial cells. Because oxidative stress and chronic inflammation are characteristic features in patients of all clinical forms of KS, as well as PEL and MCD [6,30,31], H2O2 could be an important physiological factor that triggers KSHV reactivation in these patients. Several other factors that induce KSHV lytic replication [21–29] also induce oxidative stress and inflammation [36,32–36]. Thus, it is likely that H2O2 mediates KSHV lytic replication induced by these factors. Indeed, our data show that KSHV reactivation induced by oxidative stress, hypoxia, and proinflammatory and proangiogenic cytokines depends on the induction of H2O2. Importantly, co-culture of BCBL1 cells with monocytic U937 cells enhances KSHV reactivation induced by proinflammatory and proangiogenic cytokines, particularly IL-6, TNF-α and IFN-γ, which are highly expressed in KS tumors [6]. Because of the abundance of proinflammatory cells, and proinflammatory and proangiogenic cytokines in KS tumors, these synergistic effects could further boost KSHV lytic replication. Thus, tumor microenvironments consisting of proinflammatory cells, proinflammatory and proangiogenic cytokines, and possibly extracellular matrix and other stromal cells are likely to have essential roles in inducing and mediating KSHV lytic replication in KS tumors, which should be further examined in more details.

Mechanistically, we have shown that H2O2 induction of KSHV reactivation is mediated by ERK, JNK, and p38 MAPK pathways. Previous studies have shown that these pathways are required for KSHV infection and lytic replication [17–20]. Consistent with these observations, oxidative stress, hypoxia, and a number of proinflammatory and proangiogenic cytokines are known to induce MAPK pathways [57–65].

Based on our results, we propose a model in which KS tumors are initiated by either the homing of KSHV-infected cells, most likely progenitor endothelial cells or B-cells, from virus reservoirs to the affected sites or de novo infection by the newly produced virions, both of which are promoted by inflammation and oxidative stress [6]. Since KSHV de novo infection and lytic replication further promote inflammation and oxidative stress [6], one can expect the establishment of a positive feedback loop once the cycle is initiated. If these inflammatory conditions are not appropriately contained, they can lead to the rapid progression of KS as in the case of untreated AIDS-KS. Therefore, both active inflammation and host control of viral replication are likely to determine the course of KS.

It is interesting that only a subset of KSHV-infected cells undergo lytic replication in KS tumors or in cell culture induced for KSHV lytic replication (Figure 1A and F). KSHV has evolved a complex mechanism consisting of multiple blocks to regulate its replication [66]. Whether a cell undergoes lytic replication is likely to depend on the extent of release of these blocks. While KSHV lytic replication induces inflammation and weeks post-inoculation as previously reported [48]. However, mice fed with NAC had an average 15.6-fold lower luciferase activities than those fed with drinking water alone (Figure 5B–C). We also detected lower expression levels of ORF65 protein in lymphoma cells isolated from the NAC-treated mice than those from the control mice by Western-blotting (Figure 5D). Immunohistochemical staining showed that the majority of the lymphoma cells from both groups were positive for LANA; however, cells from NAC-treated mice had significantly lower number of ORF65-positive cells than those from the control group (Figure S2A–B). To determine the production of infectious virions by the lymphoma cells, we used cell-free supernatants from the pleural fluids of the mice to infect HUVEC and examined the presence of infectious virions by staining for ORF65 protein [49]. We observed abundant virus particles in many of the cells infected with supernatants from NAC-treated mice had almost no detectable virus particles (Figure S2G). Consistent with these results, mice fed with NAC had an average 2.7-fold lower virus loads in the blood than the control group (Figure 5E). As many as 50% of the mice from both groups also developed solid tumors. ORF65 protein was detected in over 10% of the tumor cells from the control solid tumors but was almost not detectable in the solid tumors from the NAC-treated mice (Figure S2D). By examining the survival curves, we found that NAC-treated mice had an extended lifespan compared to the control group (Figure 5F). At 12-week post-inoculation, 72.2% of the NAC-treated mice survived compared to only 45.4% in the untreated group (P = 0.016). Collectively, results from these in vivo experiments indicated that PEL induced in mice had active KSHV lytic replication, and antioxidant NAC effectively inhibited KSHV lytic replication, and extended the lifespan of the mice.

**H2O2 induces KSHV reactivation by activating the ERK1/2, JNK and p38 MAPK pathways**

H2O2 is known to activate multiple MAPK pathways [50], which are required for KSHV lytic replication [17,20]. Similar to TPA, both exogenous and ATZ-induced endogenous H2O2 activated ERK1/2, JNK, and p38 MAPK pathways, and increased the total and phosphorylated forms of their downstream target c-Jun in a dose-dependent manner in BCBL1 cells in addition to induction of RTA protein expression (Figure 6A). Treatment with specific inhibitors of all three MAPK pathways effectively inhibited TPA activation of their respective MAPKs and c-Jun, as well as the induction of RTA protein (Figure 6A). Importantly, these inhibitors also strongly inhibited H2O2 and ATZ induction of RTA expression and production of infectious virions (Figure 6B–C).

To further confirm the essential roles of MAPK pathways in H2O2-induced KSHV reactivation, we used dominant negative (DN) constructs to block these pathways. In 293T cells harboring BAC36, treatment with TPA induced KSHV reactivation [51]. Treatment with H2O2 and ATZ induced the expression of RTA transcript (Figure 6D). As expected, DN constructs of all three MAPK pathways and c-Jun effectively inhibited the induction of RTA by TPA and H2O2 (Figure 6E). Together, these results indicate that H2O2 induction of KSHV reactivation is mediated by all three MAPK pathways.
Figure 4. H$_2$O$_2$ mediates KSHV reactivation induced by hypoxia, and proinflammatory and proangiogenic cytokines. (A–B) Sodium azide (NaN$_3$) and TPA increased intracellular H$_2$O$_2$ levels measured by flow cytometry analysis of fluorescence intensity in BCBL1 cells stably expressing a H$_2$O$_2$ sensor protein cpYFP. Cells were treated with NaN$_3$ (10 mM) for 90 min or 20 ng/ml of TPA for 12 h, and their fluorescence levels measured by flow cytometry shown in histogram (A) and median fluorescence intensity (B). (C) H$_2$O$_2$ scavengers inhibited NaN$_3$ induction of RTA transcript. BCBL1 cells were treated with NaN$_3$ (10 mM) for 90 min with and without NAC (400 mM) or catalase (400 U/ml). RTA transcript was detected by RT-qPCR following elimination of NaN$_3$, and continuous culture with NAC or catalase for another 24 h. (D) H$_2$O$_2$ scavengers inhibited NaN$_3$ induction of HIF-1α, RTA and ORF65 proteins. BCBL1 cells were treated with NaN$_3$ (10 mM) for 90 min with and without NAC (400 mM) or catalase (400 U/ml). Following elimination of NaN$_3$, cells were cultured with NAC or catalase, and Western-blotting was performed to measure the expression of HIF-1α protein at 6 h, RTA protein at 24 h, and ORF65 protein at 72 h. (E) H$_2$O$_2$ scavengers inhibited the expression of ORF65 protein in BCBL1 cells induced by proinflammatory and angiogenic cytokines. ORF65 protein was determined by Western-blotting in BCBL1 cells with or without co-culture with U973 cells treated with cytokines with or without NAC (400 mM) or catalase (400 U/ml) for 72 h. The concentrations of the cytokines were stated in the Materials and Methods. Relative ORF65 protein level was calculated using untreated BCBL1 cells as a reference (1-fold) and after calibration for protein loading with β-tubulin.

doi:10.1371/journal.ppat.1002054.g004
promotes the overall tumor growth through a paracrine mechanism, it is also detrimental to the lytic cells [6]. Thus, a fine balance of latent and lytic programs in KS tumors combined with active inflammation and oxidative stress in the tumor microenvironment is likely required for the development of advanced stage of KS.

We have shown that H$_2$O$_2$ scavengers such as NAC can inhibit KSHV lytic replication in vitro and in a KSHV lymphoma animal
**Figure 6. H₂O₂ induction of KSHV reactivation is mediated by ERK1/2, JNK, and p38 MAPK pathways.** (A) Treatment with H₂O₂, ATZ or TPA activated ERK1/2, JNK, and p38 pathways and their downstream transcriptional factor c-Jun, and induced the expression of RTA protein in BCBL1 cells harboring BAC36. Cells were treated with different concentrations of H₂O₂ and ATZ, or TPA at 20 ng/ml with DMSO control or inhibitors of MAPK pathways including 10 μM U0126 (ERK inhibitor), 50 μM JNK inhibitor II, and 50 μM SB203580 (p38 inhibitor) for 12 h. Total ERK1/2, JNK, p38 and c-Jun, and their phosphorylated forms, RTA protein, and β-tubulin were detected by Western-blotting. (B–C) Inhibitors of MAPK pathways inhibited the induction of RTA and production of infectious virions by H₂O₂, ATZ, and TPA in BCBL1 cells harboring BAC36. H₂O₂, ATZ, and TPA were used at concentrations of 300 μM, 5 mM, and 20 ng/ml, respectively. Inhibitors of MAPK pathways were used at concentrations described in (A). Relative RTA...
transcript level at 24 h of treatment was detected by RT-qPCR with untreated cells set as “1” (B). Relative virus titers were determined by using supernatants collected at 5 days of treatment to infect endothelial cells and calculating the numbers of GFP-positive cells at 48 hpi (C). Virus titers from untreated cells were set as “1”. (D) $H_2O_2$ and ATZ, and TPA at concentrations of 300 µM, 5 mM and 20 ng/ml, respectively, induced the expression of RTA transcript in 293T cells harboring BAC36. RTA transcript was detected by RT-qPCR following 24 h of treatment. (E) Dominant negative (DN) constructs of MAPK pathways inhibited the induction of RTA transcript by $H_2O_2$ and TPA in 293T cells. 293T cells harboring BAC36 were transiently transfected with the control plasmid and DN constructs of ERK, JNK, p38, and c-Jun for 24 h, and treated with $H_2O_2$ at 300 µM and TPA at 20 ng/ml for an additional 12 h.

doi:10.1371/journal.ppat.1002054.g006

model. Significantly, NAC extends the lifespan of the lymphoma-bearing mice. These results indicate that antioxidants and anti-inflammation drugs might be effective for inhibiting KSHV lytic replication, and thus could be promising preventive and therapeutic agents for KSHV-induced malignancies. Because many of these agents are affordable, their use is attractive, particularly in the African settings.

Materials and Methods

Cell culture and chemical reagents

BCBL1 cells and BCBL1 cells carrying pHycr-cyto or BAC36, a recombinant KSHV [45], were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Human embryonic kidney 293T cells, 293 cells and 293T cells carrying BAC36 were cultured in DMEM plus 10% FBS.

$H_2O_2$, ATZ, Na$\text{N}_3$, and antioxidants NAC, reduced L-glutathione, and bovine liver catalase were purchased from Sigma Life Science (St. Louis, MO). The ERK inhibitor U0126, p38 inhibitor SB203580, and JNK inhibitor II were all purchased from Calbiochem (Gibbstown, NJ). TPA was from Sigma.

Plasmids and lentiviruses

The p38 DN plasmid pDNA3-p38/AF was provided by Jiahua Han at The Scripps Institute [67]. The JNK DN (HA-JNK1 [APF]) plasmid was from Lin Mantell at the University of Texas Southwestern Medical Center [69]. The c-Jun DN plasmid pCMV-TAM67 was from Bradford W. Ozanne at Beatson Institute [70]. The pHycr-cyto plasmid was purchased from Biocompare (South San Francisco, CA). Transfection of 293T and BCBL1 cells was carried out using the Lipofectamine LTX Reagent from Invitrogen (Carlsbad, CA). The lentiviruses expressing specific siRNA to human catalase and scrambled control were purchased from Santa Cruz (Santa Cruz, CA). BCBL1-BAC36 cells stably expressing catalase-specific or scrambled siRNAs were obtained following lentiviral infection and puromycin selection according to the instructions of the manufacturer.

Measurement of cellular catalase activity and intracellular level of $H_2O_2$

A total of $5 \times 10^6$ BCBL1 cells cultured with or without ATZ or TPA for 12 h were harvested by brief centrifugation. The cell pellets were homogenized in 0.5 ml ice cold phosphate saline buffer at pH 7.4 containing 1 mM EDTA. After centrifugation at 10,000 g for 15 min at 4°C, the supernatants were collected and used for measuring catalase activity or intracellular $H_2O_2$. Cellular catalase activity was determined with the OxiSelect Catalase Activity Assay Kit (Cell Biolabs, San Diego, CA), and intracellular $H_2O_2$ determined with the Fluorescent Hydrogen Peroxide/Peroxidase Detection Kit from Cell Technology (Columbia, MD). Alternatively, we tracked the intracellular $H_2O_2$ level with a $H_2O_2$ sensor by stable transfection of BCBL1 cells with a HyPer-cyto cassette consisting of a circularly permuted yellow fluorescent protein (cYFP) under the control of the regulatory domain of the prokaryotic $H_2O_2$-sensing protein, OxyR [42].

Induction of viral lytic replication and titration of infectious virions

To induce viral lytic replication, $2 \times 10^6$ BCBL1 cells were treated with $H_2O_2$, ATZ or TPA alone, or in combination, in 10 ml RPMI 1640 medium containing 10% FBS for 48 h. The cells were then harvested, washed 1 time by centrifugation to eliminate the chemicals, and cultured in 5 ml fresh RPMI 1640 media with 10% FBS for three additional days. The supernatants were collected following centrifugation to eliminate cells and cell debris at 5,000 g for 15 min, and used for titration as previously described [44]. Relative virus titers were calculated based on the numbers of GFP-positive cells. Detection of virus particles in lymphoma supernatants from mice was carried out by staining for ORF65 with a monoclonal antibody following infection of HUVEC for 4 h as previously described [49].

To examine the effects of antioxidants on KSHV spontaneous lytic replication, BCBL1 cells were cultured in the presence NAC (400 µM) or catalase (400 U/ml) for 1 or 6 day, and cells were collected for Western-blotting analysis of ORF65 protein.

For induction of lytic replication by hypoxia, BCBL1 cells at $1.5 \times 10^7$ cells/ml were treated with 10 mM Na$\text{N}_3$ with or without antioxidants NAC (400 µM) and catalase (400 U/ml) for 90 min. Following washing by centrifugation, the cells were cultured for the specified lengths of time with or without NAC and catalase.

For induction of lytic replication by cytokines, BCBL1 cells at $1.5 \times 10^7$ cells/ml cultured in fresh medium with cytokines with or without antioxidants NAC (400 µM) and catalase (400 U/ml) for 72 h, and collected for Western-blotting. In parallel induction experiments, BCBL1 cells at $1.5 \times 10^7$ cells/ml in fresh medium were induced with cytokines with or without antioxidants by co-culture with U937 cells at $5 \times 10^7$ cells/ml pretreated with cytokines with or without antioxidants for 4 h. The following cytokines and concentrations were used: recombinant human VEGF at 200 ng/ml (Lonza, Walkersville, MD), recombinant human long R IGF-1 at 200 ng/ml (Lonza), recombinant human bFGF at 200 ng/ml (Lonza), recombinant human EGF at 200 ng/ml (Lonza), human IL-6 at 1 µg/ml (R&D Systems, Minneapolis, MN), human TNF-α at 1 µg/ml (R&D Systems) and human IFN-γ at 4000 U/ml (Sigma).

To induce KSHV lytic replication in endothelial cells, latent KSHV-infected HUVEC obtained as previously described [44] were treated with $H_2O_2$ (150 µM), ATZ (10 µM) for 24 h or 72 h, and cells were collected for RNA analysis or immunostaining for ORF65 protein, respectively.

RNA extraction and quantitative reverse transcription real-time PCR (RT-qPCR)

RNA was purified using a Total RNA Purification Kit (Promega, Madison, WI). Total RNA (10 µg) was reversely transcribed into first-strand cDNAs by using a Superscript III
First-Strand cDNA Synthesis Kit (Invitrogen). RT-qPCR was carried out in a DNA Engine Opticon 2 Continuous Fluorescence Detector (Bio-Rad, Hercules, CA). Each sample was measured in triplicate. The expression level of each transcript (mRNA) was first normalized to β-actin mRNA as previously described [71]. The relative expression level of a transcript in the treated cells was compared to the untreated cells, and calculated as fold changes. Specific primers for all KSHV genes were previously described [71]. Primers for human catalase were: 5'-aggactaccctctctcatcccagttg-3' (forward) and 5'-gggtccagggaggggtgcag-3' (reverse).

**Immunofluorescence antibody assay (IFA), immunohistochemistry and Western-blotting**

KSHV lytic proteins ORF59 or ORF65 in BCBL1 cells were detected by IFA as previously described [51]. Expression of KSHV lytic proteins in lymphomas and solid tumors in mice were detected by immunohistochemistry. Briefly, cells from PEL lytic proteins in lymphomas and solid tumors in mice were detected by immunohistochemistry. Briefly, cells from PEL induced in NOD/SCID mice were collected by centrifugation at 1,000 g for 5 min, fixed with formalin, and embedded in paraffin. Sections at 5 nm cut from the paraffin blocks were deparaffinized at 60°C, cleared, and rehydrated in xylene and graded alcohols. Antigen retrieval was done with citrate buffer at pH 6 for 20 min at 121°C in a pressure chamber. Sections were blocked successively with 3% H2O2 and bovine serum albumin buffer. Sections were then incubated with a monoclonal antibody to ORF65 or a mouse immunoglobulin fraction (DAKO, Carpinteria, CA) as a negative control for 1 h at 25°C. After three washes with PBS, the slides were further incubated with a secondary antibody conjugated to horseradish peroxidase (DAKO) for 15 min. The slides were then incubated with the diaminobenzidine substrate (DAKO), counterstained with hematoxylin, and mounted for observation.

Western-blotting was carried out as previously described [51]. The rabbit polyclonal antibodies to ERK1, p-ERK (Tyr 204), JNK1, p-JNK (Thr 183/Tyr 185), p38, p-p38 (Tyr 182), c-Jun, and catalase were from Santa Cruz; a polyclonal antibody to p-c-Jun (Ser73) was from Calbiochem; and a monoclonal antibody to β-tubulin was from Sigma. A rabbit polyclonal antibody to KSHV lytic protein RTA was a generous gift from Dr. Charles Wood at the University of Nebraska, Lincoln.

**Reporter assay**

293 cells transfected with either the RTA promoter luciferase reporter plasmid or the latent LANA promoter (LTd) luciferase reporter plasmid using Lipofectamine-2000 Transfection Reagent (Invitrogen) for 24 h were treated with H2O2 (300 μM) or TPA (20 ng/ml) with or without antioxidants NAC (400 μM) or catalase (400 U/ml) for 12 h. Cells were collected and their luciferase activity determined as previously described [51]. Transfection efficiency was calibrated by co-transfection with the pSV-β-galactosidase construct (Promega).

**Detection of blood viral loads in mice**

Cell-free DNA was isolated from two drops of blood from each mouse collected by tail bleeding using the QiAamp DNA Blood Mini Kit (Qiagen). KSHV DNA was detected by real-time PCR using v Cyclin (ORF72) primers and purified BAC36 as copy number control as previously described [71]. KSHV viral loads expressed as genome copies per ml of blood were calculated. PCR assay for human β-actin gene was also carried out for these DNA samples to monitor the absence of any contamination of human cells [71]. None of the samples had any detectable signal for human β-actin gene.

**Generation of a recombinant KSHV expressing firefly luciferase under the control of the late lytic ORF65 promoter**

A recombinant KSHV genome with the entire ORF65-coding frame deleted and replaced with the firefly luciferase gene was constructed using a “two-step” homologous recombination strategy as previously described (Figure S1A) [51]. Firstly, the firefly luciferase gene was amplified from the luciferase reporter plasmid pGL3-Basic Luciferase Reporter Vector (Promega) using primers 5'-tttgttttaatgtgtggagttg-3' (luciferase forward) and 5'-tttgattttaaatgtaggttctg-3' (luciferase reverse). The Kanamycin resistance cassette (KanR) flanked by two LoxP sites was amplified from the transposon EZ-Tn5TM <Kan-2> (Epicenter, Madison, WI) using primers 5'-tttttaaatgtaggttccg-3' (KanR forward) and 5'-ttttttaaatgtaggttccg-3' (KanR reverse). The two PCR products were ligated using a T4 DNA ligase (New England Biolabs, Ipswich, MA). The resulting fragment was then used as a template to generate the KanR-luc cassette by PCR amplification using primers 5'-ctttttaggttccg-3' (KanR-5') and 5'-ttttttaatagtgtaggttccg-3' (KanR-3'). This PCR product, flanked by a 50 bp sequence from the immediate downstream region of ORF65 at its 5’ end and a 50 bp sequence from the start codon (ATG) region of ORF65 at its 3’ end, was electroporated into Escherichia coli strain DH10B containing recombinant KSHV BAC36 [45]. Upon homologous recombination, the KanR-luc cassette was integrated into KSHV genome. The Kanamycin-resistant colonies, containing the mutant KSHV genome, named Δ65Kan-luc, with ORF65 replaced with KanR-luc, were selected. To eliminate the KanR cassette, a Cre-expression plasmid pCre carrying a tetracycline resistant marker and a temperature (37°C)-sensitive replication origin was electroporated into the selected bacteria. The expression of Cre protein led to the removal of KanR cassette by LoxP-mediated recombination. The resulting colonies containing the mutant KSHV genome, named Δ65Luc, with ORF65 replaced with firefly luciferase gene, which was Tetacycline resistant but Kanamycin sensitive, were selected. The pCre plasmid was removed by culturing the bacteria at 37°C.

The mutant KSHV genomes were purified using the Large Construct DNA Purification Kit (Qiagen, Valencia, CA), verified for integrity by restriction digestion and PCR amplification of specific genes (Figure S1B-C), and electroporated into BCBL1 cells as previously described [45]. Following Hygromycin selection, a cell line harboring both the wild type KSHV genome and Δ65Luc was established. Expression of luciferase by this cell line was confirmed by Western-blotting using a luciferase-specific antibody (Figure S1D), and by measuring luciferase activity using the firefly luciferase substrate (Promega) and a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA) (Figure S1E).

**Examination of the effect of antioxidant NAC on KSHV lytic replication in a mouse lymphoma model**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Health Science Center at San Antonio (Animal Welfare Assurance Number: 450144).
A3345-01). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Male NOD/SCID mice at 6 weeks from Jackson Laboratories (Bar Harbor, ME) were intraperitoneally inoculated with BCBL1 cells carrying Δ65Luc at 5 × 10^6 per mouse. One week after inoculation, mice (n = 36) were given drinking water supplemented with 5 mM of NAC while the control mice (n = 44) were given drinking water without the antioxidant. Mice were monitored daily for “PEL-like” symptoms. Luciferase activity and GFP intensity were measured five weeks after injection using a Xenogen IVIS 200 Imaging System (Xenogen, Alameda, CA). Mice were monitored daily, and terminated when they became inmobile. Lymphoma cells, supernatants and solid tumors were collected and analyzed as indicated.

Supporting Information

Figure S1 Construction of a recombinant KSHV with ORF65 replaced with firefly luciferase gene. (A) Schematic illustration of the “two-steps” recombination strategy for generating the mutant KSHV genome. (B) Genetic analysis of wild type BAC36, intermediate mutant Δ65Kan-Luc and recombinant virus Δ65Luc genomes by restriction digestion with Hind III. (C) Confirmation of the replacement of ORF65 by luciferase gene in intermediate mutant Δ65Kan-Luc and recombinant virus Δ65Luc genomes by PCR amplification. (D) Detection of luciferase and ORF65 proteins in uninduced and TPA-induced BCBL1 cells harboring Δ65Luc by Western-blotting. $b_{D65Luc}$ at 5

References

1. Speck SH, Ganem D (2010) Viral latency and its regulation: lessons from the gamma-herpesviruses. Cell Host Microbe 8: 109–115.
2. Chang Y, Cesaran E, Posin MS, Lee F, Collepeper J, et al. (1994) Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266: 1865–1869.
3. Cesaran E, Chang Y, Moore PS, Said JW, Knowles DM (1995) Kaposi's sarcoma-associated herpesvirus DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med 332: 1196–1191.
4. Soulier J, Grolet I, Oksenenden E, Cacoub P, Cazals-Hatem D, et al. (1995) Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood 86: 1276–1280.
5. Staskus KA, Zhong W, Gehlhards K, Herndier B, Wang H, et al. (1997) Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol 71: 715–719.
6. Greene W, Kuhne K, Ye F, Chen J, Zhou F, et al. (2007) Molecular biology of KSHV in relation to ADN-associated oncogenesis. Cancer Treat Res 133: 69–127.
7. Wherry DT, Howard MR, Tenant-Flowers M, Brink NS, Copas A, et al. (1995) Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet 346: 799–802.
8. Moore PS, Kingsey LA, Holmberg SD, Spira T, Gupta P, et al. (1996) Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol 70: 715–719.
9. Cattelan AM, Calabro ML, Aversa SM, Zanchetta M, Meneghetti F, et al. (2003) Human herpesvirus (KSHV) reactivation. Int J Cancer 97: 795–799.
10. Campbell TB, Fitzpatrick L, MaWhinney S, Zhang X, Schooley RT (1999) Regression of AIDS-related Kaposi's sarcoma following antiretroviral therapy with protease inhibitors: biological correlates of clinical outcome. J Virol 73: 17–25.
11. Blackbourn DJ, Fujimura S, Kutzkey T, Levy JA (2000) Induction of human herpesvirus latency. J Virol 74: 5371–5382.
12. Chang J, Renne R, Dittmer D, Ganem D (2000) Inflammatory cytokines and the reactivation of Kaposi's sarcoma-associated herpesvirus lytic replication. Virology 266: 17–25.
13. Blackkloerl DJ, Fujinuma S, Kutayek T, Levy JA (2000) Induction of human herpesvirus gene expression by recombinant interferon gamma. AIDS 14: 98–99.
14. Jones JL, Hanson DL, Chu SY, Ward JW, Jaffe HW (1995) AIDS-associated Kaposi’s sarcoma. Science 267: 1078–1079.
15. Morrell AO, Yosse M, Gazzard B, Moreinck J, Hakai R, et al. (1996) Antiherpervirus treatment and risk of Kaposi's sarcoma in HIV infection. Royal Free/Chelsea and Westminster Hospital Collaborative Group. AIDS 10: 1101–1105.
16. Denitch E, Cohen A, Kazimirsly G, Dovset S, Rubinfeld H, et al. (2004) Role of protein kinase C delta in reactivation of Kaposi's sarcoma-associated herpesvirus. J Virol 78: 10187–10192.
17. Pan H, Xie J, Ye F, Gao SJ (2006) Modulation of Kaposi's sarcoma-associated herpesvirus infection and replication by MEK/ERK, JNK, and p38 multiple mitogen-activated protein kinase pathways during primary infection. J Virol 80: 537–5382.
18. Cohen A, Brodie C, Sasid R (2006) An essential role of ERK signalling in TPA-induced reactivation of Kaposi’s sarcoma-associated herpesvirus. J Gen Virol 87: 795–802.
19. Ford BW, Bryan BA, Dyson OF, Weidner DA, Chintalgattu V, et al. (2006) Raf/MEK/ERK signalling triggers reactivation of Kaposi's sarcoma-associated herpesvirus latency. J Gen Virol 87: 1139–1144.
20. Xie J, Ajihade AO, Ye F, Kuhne K, Gao SJ (2006) Reactivation of Kaposi's sarcoma-associated herpesvirus from latency requires MEK/ERK, JNK and p38 multiple mitogen-activated protein kinase pathways. Virology 371: 139–154.
21. Chang J, Renne R, Dittmer D, Ganem D (2000) Inflammatory cytokines and the reactivation of Kaposi's sarcoma-associated herpesvirus lytic replication. Virology 266: 17–25.
22. Blackkloerl DJ, Fujinuma S, Kutayek T, Levy JA (2000) Induction of human herpesvirus gene expression by recombinant interferon gamma. AIDS 14: 98–99.
23. Davis DA, Rinderknecht AS, Zoeteweij JP, Aoki Y, Read-Connole EL, et al. (1995) Lytic replication of Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. J Virol 73: 1101–1105.
24. Harrington W, Jr., Sieczkowski L, Sosa C, Chan-a-Sue S, Cai JP, et al. (1997) Human herpesvirus-8 gene expression by recombinant interferon gamma. AIDS 11: 175–180.
25. Varthakavi V, Smith RM, Deng H, Sun R, Spearman P (2002) Human immunodeficiency virus type-1 activates lytic cycle replication of Kaposi's sarcoma-associated herpesvirus through induction of KSHV Rta. Virology 297: 270–280.
26. Merat R, Amara A, Lebbe C, de The H, Morel P, et al. (2002) HIV-1 infection of primary effusion lymphoma cell line triggers Kaposi's sarcoma-associated herpesvirus (KSHV) reactivation. Int J Cancer 97: 791–795.
27. Vieira J, O’Hearn P, Kimball L, Chandran B, Corey L (2003) Activation of Kaposi’s sarcoma-associated herpesvirus (human herpesvirus 8) lytic replication by human cytomegalovirus. J Virol 75: 1378–1386.

28. Liu C, Zeng Y, Huang Z, Huang L, Qian C, et al. (2003) Human herpesvirus 6 activates lytic cycle replication of Kaposi’s sarcoma-associated herpesvirus. Am J Pathol 160: 175–183.

29. Gregory SM, West JA, Dillon PJ, Hitchen C, Ditmer DP, et al. (2009) Toll-like receptor signaling controls reactivation of KSHV from latency. Proc Natl Acad Sci USA 106: 11725–11730.

30. Douglas JL, Gustin JK, Dezuze B, Pantanowitz JL, Moses AV (2007) Kaposi’s sarcoma: a model of both malignancy and chronic inflammation. Panminerva Med 49: 119–138.

31. Ganem D (2010) KSHV and the pathogenesis of Kaposi’s sarcoma: learning to human biology and medicine. J Clin Invest 120: 939–949.

32. Khatami M (2009) Inflammation, aging, and cancer: tumouricidal versus tumourigenic of immunity: a common denominator mapping chronic diseases. Cell Biochem Biophys 55: 53–79.

33. Simonart T, Noël JC, Andrei G, Parent D, Van Vooren JP, et al. (2000) Iron as a potential co-factor in the pathogenesis of Kaposi’s sarcoma? Int J Cancer 76: 720–726.

34. Salahudeen AA, Bruick RK (2009) Maintaining Mammalian iron and oxygen homeostasis: sensors, regulation, and cross-talk. Ann N Y Acad Sci 1177: 30–38.

35. Laubach VE, Kron IL (2009) Pulmonary inflammation after lung transplantation. Surgery 146: 1–4.

36. Gil L, Martinez-Gonzalez I, Tarinas A, Alvarez A, et al. (2003) Contribution to characterization of oxidative stress in HIV/AIDS patients. Pharmacol Res 47: 217–224.

37. Malley SR, Pei P, Landwehr DJ, Clark CM, Bradbury JE, et al. (2004) Implications for oxidative and nitrative stress in the pathogenesis of AIDS-related Kaposi’s sarcoma. Carcinogenesis 25: 597–603.

38. Pan JS, Hong MZ, Ren JL (2009) Reactive oxygen species: a double-edged sword in oncogenesis. World J Gastroenterol 15: 1702–1707.

39. Chochola J, Strosberg AD, Stanislawski M (1995) Release of hydrogen peroxide from human T cell lines and normal lymphocytes co-infected with HIV-1 and mycoplasma. Free Radic Res 23: 197–212.

40. Elbim C, Pillet S, Prevost MH, Preira A, Girard PM, et al. (2001) The role of NADPH oxidase 2 in generating reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation. J Biol Chem 276: 23435–23439.

41. Babbar N, Casero RA, Jr. (2006) Tumor necrosis factor-alpha increases reactive oxygen species by inducing spermine oxidase in human lung epithelial cells: a potential mechanism for inflammation-induced carcinogenesis. Cancer Res 66: 11125–11130.

42. Yang D, Elner SG, Baim ZM, Till GO, Petty HR, et al. (2007) Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells. Exp Eye Res 85: 462–472.

43. Soderberg-Naucler C (2006) Does cytochrome oxidase play a causative role in the development of various inflammatory diseases and cancer? J Intern Med 259: 219–246.

44. Gill R, Tsung A, Billiar T (2010) Linking oxidative stress to inflammation: Toll-like receptors. Free Radic Biol Med 48: 1121–1132.

45. Wang X, Martindale JL, Liu Y, Holbrook NJ (1998) The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J 333: 291–300.

46. Muller JM, Krauss B, Kelshmidt C, Barurela PA, Rupe R (1997) Hypoxia induces c-fos transcription via a mitogen-activated protein kinase-dependent pathway. J Biol Chem 272: 23145–23149.

47. Matsuda N, Morita N, Matsuda K, Watanabe M (1998) Proliferation and differentiation of human osteoblast cells associated with differential activation of MAP kinases in response to epidermal growth factor, hypoxia, and mechanical stress in vitro. Biochem Biophys Res Commun 249: 350–354.

48. D’Angelo G, Struman I, Martial J, Weiner RI (1995) Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. Proc Natl Acad Sci USA 92: 6374–6378.

49. Giorgietti S, Pellici PG, Pellici G, Van Obberghen E (1996) Involvement of Src-homology/collagen (SHC) proteins in signaling through the insulin receptor and the insulin-like-growth-factor-Receptor. Eur J Biochem 239: 195–202.

50. Williams R, Sanghera J, Wu F, Carbonaro-Hall D, Campbell DL, et al. (1993) Identification of a human epidermal growth factor receptor-associated protein kinase as a new member of the mitogen-activated protein kinase/extracellular signal-regulated protein kinase family. J Biol Chem 268: 11899–11905.

51. Lobao RJ, Treter AV, Cerione RA, Gitlin J (2004) Activation of the mitogen-activated protein kinase cascade by leukemia signal-regulated protein kinase family. J Biol Chem 279: 235–246.

52. D’Angelo G, Struman I, Martial J, Weiner RI (1995) Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. Biochem Biophys Res Commun 238: 256–260.

53. Li Q, Zhou F, Ye F, Gao SJ (2008) Genetic disruption of KSHV major latent gene vFLIP inhibits viral lytic replication through human fibroblast. J Biol Chem 268: 7219–7226.

54. Derijard B, Hibi M, Wu IH, Barrett T, Su B, et al. (1994) JNK1: a protein kinase related to c-Jun amino-terminal kinase with specificity for the c-Jun activation domain. Cell 76: 1025–1037.

55. Schiemann WP, Nathanson NM (1994) Involvement of protein kinase C during activation of the mitogen-activated protein kinase cascade by leukemia inhibitory factor. Evidence for participation of multiple signalling pathways. J Biol Chem 269: 6376–6382.

56. Boshoff C, Gao SJ, Ye F, Crane KD, Slack F, et al. (1997) Signal-regulated protein kinase family. J Biol Chem 268: 18213–18217.

57. Schiemann WP, Nathanson NM (1994) Involvement of protein kinase C during activation of the mitogen-activated protein kinase cascade by leukemia inhibitory factor. Evidence for participation of multiple signalling pathways. J Biol Chem 269: 6376–6382.

58. Williams R, Sanghera J, Wu F, Carbonaro-Hall D, Campbell DL, et al. (1993) Identification of a human epidermal growth factor receptor-associated protein kinase as a new member of the mitogen-activated protein kinase/extracellular signal-regulated protein kinase family. J Biol Chem 268: 11899–11905.

59. D’Angelo G, Struman I, Martial J, Weiner RI (1995) Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. Biochem Biophys Res Commun 238: 256–260.

60. Li Q, Zhou F, Ye F, Gao SJ (2008) Genetic disruption of KSHV major latent nuclear antigen LANA enhances viral lytic transcriptional program. Virology 379: 234–244.

61. Reigner G, Eaton RH, Chen TD, Winslow BM, Stanisch M, Henson PM (1997) Interferon-gamma and transforming growth factor-beta modulate the activation of mitogen-activated protein kinases in tumor necrosis factor-alpha production induced by Fr gamma- and Fr alpha-receptor stimulation in murine macrophages. Biochem Biophys Res Commun 238: 256–260.

62. Han J, Richter B, Li Z, Kravchenko V, Ulewicz R (1995) Molecular cloning of human p38 MAP kinase. Biochem Biophys Acta 1263: 224–227.

63. Derijard B, Hibi M, Wu IH, Barrett T, Su B, et al. (1994) JNK1: a protein kinase related to c-Jun amino-terminal kinase with specificity for the c-Jun activation domain. Cell 76: 1025–1037.

64. Froehlich T, Gobbert TD, Cobb MH, Feramisco JR (1994) A requirement for extracellular signal-regulated kinase (ERK) function in the activation of AP1 by Ha-Ras, phosphol 12-myristate 13-acetate, and serum. Proc Natl Acad Sci USA 91: 3844–3848.

65. Brown PH, Chen TK, Birrer MJ (1994) Mechanism of action of a dominant-negative mutant of c-Jun. Oncogene 9: 781–789.

66. Yoo SM, Zhou FC, Ye FC, Pan HY, Gao SJ (2005) Early and sustained expression of latent and host modulating genes in coordinated transcriptional program of KSHV productive primary infection of human primary endothelial cells. Virology 343: 47–64.