Manipulating Host Signaling for Pathogenic Purposes: Hijacking of the Transcriptional Activity of the Host Protein Nrf2 by Kaposi’s Sarcoma-Associated Herpesvirus

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Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiological agent of two highly aggressive AIDS-related malignancies, endothelial Kaposi’s sarcoma (KS) and B cell primary effusion lymphoma (PEL). Although current antiretroviral therapy (ART) methods have decreased the overall morbidity and mortality in immunocompromised and AIDS patients, KS and PEL remain difficult to treat. Thus, novel therapeutic methods are essential to significantly improve the life expectancy and quality of life of PEL and KS patients, respectively. One of the signaling pathways that is highly modulated during KSHV infection is oxidative stress, which is a well-known inducer of the transcription factor Nrf2. Because Nrf2 plays an important role in the biology of several viral infections, and is an important agent in cancer progression and chemotherapeutic resistance, we recently undertook a study to investigate its role in KSHV infection of endothelial cells. Here, we provide important highlights of our recent findings, including the importance of oxidative stress and PKCζ in Nrf2 activation, a newly discovered feed-forward loop between Nrf2 and COX-2, and the impact of Nrf2 activation on host and KSHV gene expression and pathogenesis. Lastly, we provide insights on the potential therapeutic implications of Nrf2 modulation in KSHV and other virus-associated pathologies.

Keywords: Kaposi’s sarcoma-associated herpesvirus; KSHV; Nrf2; COX-2; PGE2; PKCζ; reactive oxygen species; ROS; oxidative stress; cancer

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The Oncogenic Kaposi’s Sarcoma-Associated Herpesvirus and Oxidative Stress

Kaposi’s sarcoma-associated herpesvirus (KSHV), discovered by Yuan Chang and Patrick S. Moore in 1994, is an oncogenic γ-herpesvirus [1]. KSHV infection of endothelial cells in immunocompromised patients often leads to the development of a highly proliferative, angiogenic tumor of polyclonal origin known as Kaposi’s sarcoma (KS) [1]. Infection of B cells in this patient population often leads to the development of primary effusion lymphoma (PEL), a highly aggressive malignancy with a poor prognosis [2].

In addition to endothelial and B cells, KSHV infects monocytes, fibroblasts and epithelial cells, although the significance of such infections in vivo remains elusive [3]. The wide tropism of KSHV is no surprise given that KSHV is one of the largest known human viruses, consisting of an ~145-kb genome that codes for over 90 open reading frames (ORF), several of which are viral envelope glycoproteins that mediate its wide tropism. These glycoproteins serve important functions during de novo KSHV infection, as their
interaction with cell-surface receptors induces signaling in the host that is critical for the subsequent virus entry, gene expression and, ultimately, establishment of latency [3].

A report by Bottero et al. (2013) showed that sustained induction of reactive oxygen species (ROS) during de novo KSHV infection was an imperative signal that aids KSHV infection [4]. Interestingly, the role of ROS in KSHV biology, albeit in different contexts, had been previously reported. Utilizing mouse endothelial cells (mECK36) overexpressing the KSHV lytic cycle-associated protein vGPCR and the KSHV Bac36 episome, Ma et al. (2013) demonstrated that inhibition of ROS through the free radical scavenger N-acetylcysteine (NAC) significantly abrogated the oncogenic effects of KSHV infection [5]. Moreover, Ye et al. (2011) showed that exogenous and endogenous induction of ROS in latently infected cells resulted in lytic reactivation of KSHV, and inhibition of such ROS prolonged the life span of mice modeled to develop KSHV-induced lymphoma [6]. Collectively, these studies further confirmed the significant findings by Mallery et al. (2004), who showed that increased oxidative stress was one of the underlying factors that enabled KSHV-positive patients, whether immunocompromised or not, to develop KS or PEL [7].

**Nrf2, the Master Gene Regulator**

The effects of ROS increase in a cell are numerous, including, but not limited to, induction of signaling, DNA damage and lipid oxidation. Few effects, however, are as significant as the activation of nuclear factor E2-related factor 2 (Nrf2), a Cap’n’Collar basic leucine zipper transcription factor. By its ability to bind to the anti-oxidant response element (ARE) on gene promoters, induced Nrf2 transcriptionally controls the activity of over 200 human genes [8]. Several Nrf2 target genes serve anti-oxidative functions that reduce the ROS levels that brought the original Nrf2 transcriptional activation of these genes, creating a negative feedback loop between ROS and Nrf2.

Transient Nrf2 activation in response to local ROS increase serves beneficial purposes to the cell as it protects it from the devastating effects of persistent oxidative stress. However, despite its well-characterized positive role as a master anti-oxidant, recent studies are unveiling a negative side of Nrf2. Contrary to the beneficial effects of transient Nrf2 activation, its constitutive activation has been blamed for the aggressiveness and chemotherapeutic resistance of certain cancers with poor prognosis, especially lung adenocarcinoma [9], rightfully bestowing Nrf2 the nickname “double-edged sword.” The mechanisms by which Nrf2 provides proliferative advantage to malignant cells are under intense scrutiny, are believed to be numerous, and some of them described below. First, Nrf2 induces the transcription of several drug resistance genes, which help secrete and attenuate the intracellular anti-cancerous effects of chemotherapeutic agents [10]. Second, Nrf2 inhibits the buildup of ROS, and while this is a desirable effect in healthy cells, ROS induction is also the mechanism of action of several chemotherapeutic drugs [11]. Third, Nrf2 induces the transcription of several proliferative and anti-apoptotic agents, including Bcl-2 and Bcl-xL [12, 13]. Fourth, Nrf2 plays an important role in the induction of the HIF-1α/VEGF pro-angiogenic axis during hypoxic and oxidative stress conditions, possibly providing an angiogenic milieu in the tumor microenvironment [14]. Fifth, Nrf2 inhibition decreases glioblastoma cell migration and invasion, likely by decreasing MMP9 expression [15].

**Nrf2 Regulation by ROS**

Probably due to Nrf2’s importance in promptly regulating oxidative stress while simultaneously avoiding excessive activation of Nrf2, both potentially detrimental to the cell, cells have developed an efficient system of Nrf2 control that can quickly lead to Nrf2 accumulation under stress conditions, while simultaneously ensuring its timely degradation once the original insult has been successfully attenuated. This system involves a dynamic E3 ubiquitin ligase complex consisting of a heterotrimeric interaction between Keap1 (scaffold) and Rbx-1 (E3 ligase) in a 2:1 ratio. Under basal conditions, the Keap1-Keap1 homodimer docks on Nrf2, bringing Rbx-1 close enough to mediate Nrf2 polyubiquitination and degradation through the 26S proteasome (Figure 1A) [16]. When ROS levels are elevated, conformational changes in Keap1 impair its ability to bind to Nrf2, preventing Rbx-1-mediated Nrf2 polyubiquitination (Figure 1B). Upon increased Nrf2 activity and the resulting attenuation of the original ROS insult, the conformational structure of Keap1 returns to its original state, once again binding to Nrf2 and mediating its degradation through Rbx-1-mediated polyubiquitination [16]. Phosphorylation of Nrf2 at Ser-40 has also been shown to disrupt, to some extent, its affinity for Keap1. More importantly, Ser-40 phosphorylation of Nrf2, hereon referred to as pNrf2, is crucial for its nuclear translocation and transactivation of several of its target genes [17].

**KSHV Induces Nrf2 and Its Target Genes**

Given the importance of ROS in KSHV biology and its well-known role in Nrf2 activation, we recently investigated the role of Nrf2 in KS pathogenesis. Our comprehensive studies demonstrate that KSHV induces Nrf2 during de novo infection of endothelial cells to create a microenvironment conducive to infection [18]. By immunostaining KS skin
lesions and healthy skin tissue with an anti-pNrf2 antibody, we observed that KS skin lesions had elevated levels of active Nrf2. Moreover, we determined that this Nrf2 activation was due to KSHV infection, as high pNrf2 levels in KS tissues correlated strongly with cells expressing the KSHV major latent ORF73 gene product known as LANA-1 (latency-associated nuclear antigen-1). We then used human dermal microvascular endothelial cells (HMVEC-d) to assess the effect of de novo KSHV infection on Nrf2 activation. Interestingly, as early as 30 minutes post-infection, KSHV induced both Nrf2 stability and Ser-40 phosphorylation of Nrf2. This resulted in the increase in transcriptionally active and nuclear pNrf2 that mediated the transcription of a significant number of host genes, including the de facto Nrf2 target genes NQO1, GCS and HO1 (Figure 1C). Moreover, KSHV infection also induced the anti-apoptotic protein Bcl-2, the pro-angiogenic protein VEGF, and the pentose phosphate pathway enzymes G6PD, TKT and TALDO (Figure 1C). Using a lentiviral transduction system to express short hairpin RNA against either a control vector or Nrf2, we successfully reduced Nrf2 expression levels in the target cells. Interestingly, infection of these Nrf2-deficient cells with KSHV failed to optimally induce the expression of any of the genes mentioned above, suggesting that Nrf2 is a key agent in mediating KSHV-induced early gene transcriptional modulation of infected cells that ultimately results in efficient KSHV infection and development of KS. It is interesting to note the involvement of Nrf2 in VEGF induction since we have previously shown that during de novo infection, KSHV-induced VEGF converts blood endothelial cells into lymphatic endothelial cells, a necessary transition for the establishment of latent infection in these cells [14].

**Hijacking the Nrf2 Control Mechanism**

When we pretreated the HMVEC-d with three powerful anti-oxidants, N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC) and diphenyleneiodonium (DPI), early de novo KSHV infection completely failed to induce Nrf2; latent KSHV infection, in contrast, still retained some of its ability to induce Nrf2 despite ROS inhibition. This combination of findings strongly suggests additional, ROS-independent mechanisms of Nrf2 activation during latency. Through co-immunoprecipitation analysis we determined that by inducing ROS, KSHV altered the ability of Keap1 to bind to newly synthesized Nrf2, allowing for its accumulation inside the cell untargeted by polyubiquitination (Figure 1C, Early Induction). KSHV also induced a signaling axis consisting of the Src, PI3-K and PKCζ kinases, which phosphorylated Nrf2, thereby further inducing its nuclear translocation and transcriptional activity (Figure 1C, Early Induction).

**The COX-2/PGE2/Nrf2 Loop**

KSHV infection of target cells induces a powerful induction of the proinflammatory enzyme cyclooxygenase-2 (COX-2), which, through its enzymatic product PGE2, unleashes a series of paracrine/autocrine signaling events that are important for KSHV infection [19-21]. To our surprise, infection of endothelial cells deficient in Nrf2 yielded a weak induction of COX-2 transcript and protein levels. Following up on these findings we identified two putative AREs (Nrf2 binding sites) on the COX-2 promoter, strongly suggesting that Nrf2 induction during de novo KSHV infection is important for transactivation of COX-2. More interestingly, when the COX-2 product PGE2 was added to uninfected

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**Figure 1 – Nrf2 regulation.** A) In a steady state, the Keap1/Rbx-1 E3 ubiquitin ligase complex mediates Nrf2 degradation. Red circles on Nrf2 indicate ubiquitin. B) Under oxidative stress, ROS induces conformational changes in Keap1, releasing Nrf2 from its inhibitory activity. C) KSHV induces ROS and PKCζ during the early stages of infection, and COX-2/PGE2 during latency via its latent protein vFLIP to induce Nrf2 activation, which is important for the burst of lytic gene expression observed during de novo infection. Nrf2 associates with LANA-1 and the KSHV genome, likely impacting viral and host gene modulation.
endothelial cells, it resulted in a robust, dose-dependent induction of pNrf2, an induction that required the activity of PKCζ (Figure 1C, Late Induction). This led us to the hypothesis that initial virus binding to host receptors induced COX-2 through Nrf2 activation, and the COX-2 product PGE2 subsequently induced further activation of Nrf2 at the later, post-entry stages of infection, potentially explaining the ROS-independent Nrf2 induction during latency. To test this hypothesis, we infected HMVEC-d cells in the presence or absence of Celecoxib, a COX-2-specific inhibitor. As expected, while infection of mock-treated cells yielded a robust and sustained Nrf2 activation, infection of COX-2-deficient cells induced Nrf2 only in the early stages of infection (1-2 hours post-infection), significantly abating by 8 hours. These experiments established an important feed-forward loop between Nrf2 and COX-2, with PGE2 and Nrf2 during infection to be used to their advantage.

Nrf2 and Virus Biology

Recent studies indicate that KSHV is not the only virus that induces Nrf2 during infection as herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV), hepatitis C virus (HCV), the influenza A virus, and recently the Marburg and Dengue viruses, have all been shown to induce Nrf2 activation during their infection of target cells through diverse mechanisms [23-28]. These findings strongly suggest that evolutionarily Nrf2 has become an important host response factor in determining the fate of virus infection. Pondering on whether Nrf2 plays pro- or anti-viral roles makes for an elusive answer because Nrf2 negatively affects the infection of certain viruses (HSV-1 and Influenza A) and positively the infection of others (HCV, HCMV, Marburg and Dengue). It is tempting to speculate that Nrf2 activation was initially employed by cells to fight virus infections, but given its tremendous transcriptional versatility, it was then later hijacked by certain viruses in the course of their infection to be used to their advantage.

Interestingly, although Nrf2 induction during de novo KSHV infection may reduce virus-induced stress, it also induces a cohort of cellular factors that are required for efficient establishment of infection. Importantly, when KSHV infected the Nrf2-deficient endothelial cells, it failed to express a set of lytic genes with important anti-apoptotic and immune-evasive functions, likely exposing the virus to host immune monitoring in vivo. From an oncogenic perspective, KSHV-mediated Nrf2 activation might provide some of the proliferative advantages mentioned above, such as anti-apoptotic signaling, drug resistance, angiogenesis and cell migration, all important features of both KS and PEL. Overall, these findings suggest that Nrf2 plays a potential pro-viral role during KSHV infection.

Therapeutic Implications

Despite a decreasing number of AIDS patients and AIDS-associated mortality and morbidity in Western countries, KS and PEL remain significant hurdles in the fight against AIDS in both the Western world and sub-Saharan Africa. For both malignancies, harsh chemotherapeutic treatment is often required following diagnosis, and with PEL the outcome is abysmal, with an average life expectancy of approximately six months post-diagnosis. Because of

vFLIP, LANA-1 and Nrf2: An Interesting Trio

It was interesting to note that KSHV infection induced Nrf2 during both early infection, when most of the events are driven by virus envelope glycoprotein and cell surface receptor interactions, and during latency, when most events are driven by a small set of KSHV latent genes. We speculated that the main viral culprit for Nrf2 induction during KSHV latency was the viral FLICE-inhibitory protein (vFLIP) encoded by the latency locus gene ORF71, which induces the COX-2/PGE2 axis during KSHV infection. This, in turn, could induce Nrf2 induction through both paracrine and autocrine mechanisms by PGE2, as explained in the previous section. In our study, we determined that vFLIP was indeed the viral agent that induced Nrf2 activity during latency. The exact mechanism of vFLIP-mediated Nrf2 induction was recently elucidated, showing that vFLIP-mediated inhibition of autophagy lead to Keap1 inhibition by the host protein p62 (SQSTM1) in endothelial cells carrying latent KSHV genome [22]. This finding further supports the model of a feed-forward amplification loop between Nrf2 and COX-2, with vFLIP expression during latency acting as a constitutive perpetuator of this loop. Using the novel imaging technique proximity ligation assay (PLA), which detects protein colocalizations fewer than 16 nm apart, we found that upon KSHV entry into the nucleus and Nrf2 induction by vFLIP, Nrf2 associated with LANA-1. More interestingly, Nrf2 and LANA-1 were observed to colocalize with the KSHV genome, suggesting that LANA-1 may hijack the transcriptional activity of Nrf2 to affect KSHV gene expression. Concomitantly, we observed that certain Nrf2/LANA-1 interactions did not colocalize with the KSHV genome, suggesting a similar effect on host gene expression. The extent to which the Nrf2/LANA-1 interaction affects KSHV and host gene expression during de novo infection as well as latency is of utmost importance and yet to be investigated. Similarly, host and viral partners of Nrf2 and their role in KSHV pathogenesis remain to be elucidated.
these reasons, finding alternative treatments or treatments that can be administered as adjuvants to current chemotherapeutic methods is very important, and Nrf2 modulation could be one such approach. Multiple recent studies have indicated that Nrf2 inhibition in several cancers, including lung and pancreatic adenocarcinomas, significantly reduced cell proliferation and resistance to chemotherapy [13,29]. Moreover, several Nrf2 inhibitors have been discovered in recent years, including, but not limited to, Brusatol, Tigroneilline, Luteolin and Apigenin [30].

It would be interesting to observe the combinatorial effects of COX-2 and Nrf2 modulators in the context of KSHV. As we have shown, COX-2 is crucial for proper Nrf2 activity during de novo KSHV infection, and considering the negative effects that COX-2 inhibition has on latent KSHV gene expression, it is tempting to speculate that some of these effects might be due to inhibition of Nrf2 activity mediated by the COX-2/PGE2 inhibitors. It is, therefore, worthwhile to further investigate the importance of Nrf2 in KSHV biology, gene expression and pathogenesis, and how its chemical manipulation affects the outcome of these pathologies both in in vitro and in vivo animal models.

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Conflicts of Interest:

The authors declare that they have no conflicting interests.

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