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Effects of NF κ B activation on KSHV latency and lytic reactivation are complex and context-dependent

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Abstract

Like all herpesviruses, Kaposi's sarcoma-associated herpesvirus (KSHV) can produce either latent or lytic infection. The latent v-FLIP gene is a strong activator of NF κ B, and in primary effusion lymphoma (PEL) cells, blockade of NF κ B activation is associated with enhanced lytic gene expression, while overexpression of p65 impairs expression of reporter genes driven by lytic promoters. This has led to the suggestion that NF κ B activation may promote latency by suppressing lytic reactivation. Here we examine in detail the effects of NF κ B activation on KSHV replication in several cell types. In accord with earlier work, we find that inhibition of NF κ B signaling in PEL cells is associated with enhanced lytic reactivation of KSHV. Similarly, in *de novo* KSHV infection of primary endothelial cells, inhibition of NF κ B signaling leads to an increase in lytic gene expression and enhanced virion production. By contrast, KSHV-infected human foreskin fibroblasts (HFF) show no increase in spontaneous lytic reactivation when NF κ B is inhibited. Moreover, if NF κ B activation is always inhibitory to lytic gene expression, one might expect its activation to be suppressed during the lytic cycle. However, we find that NF κ B signaling is strongly and consistently activated in lytically infected cells of all lineages. Together these data indicate that (i) the relationship of NF κ B activation to latency and lytic reactivation is not uniform, but is dependent on the cellular context; and (ii) even though NF κ B activation is inhibitory to lytic gene expression in some contexts, such inhibition is at least partially bypassed or overridden during lytic growth.

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Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV, also called human herpesvirus 8) is the etiologic agent of Kaposi's sarcoma (KS), an inflammatory and proliferative lesion affecting the microvascular endothelium. KSHV also targets B lymphocytes, and is linked to two rare lymphoproliferative syndromes, multicentric Castlemann's disease (MCD) and primary effusion lymphoma (PEL) (Arvanitakis et al., 1996; Moore and Chang, 1998; Soulier et al., 1995). Like all herpesviruses, KSHV can execute two different genetic programs, known as *latency* and *lytic growth*. In latency,

viral gene expression is strongly restricted, with only a handful of the virus' nearly 100 genes being expressed. The latent viral genome is maintained as a circular nuclear plasmid, and no viral progeny are produced. Latency is usually the default pathway following experimental infection in cultured cells (Vieira et al., 2001; Bechtel et al., 2003). However, if latently infected cells are exposed to certain stimuli (such as: phorbol esters or HDAC inhibitors) latency can be disrupted and lytic replication triggered. The lytic program proceeds via a temporally regulated cascade of gene expression, in which lytic cycle-specific genes are activated in at least 3 classes: immediate-early (IE), delayed-early (DE) and late (L). Viral DNA replication follows DE gene expression, and progeny genomes are encapsidated into virions and released.

KS tumors are primarily latently infected, but a small number of cells in the lesion are lytically infected (Staskus et al., 1997),

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and both latency and lytic replication have been postulated to play important roles in KS pathogenesis (Ganem, 2006). A single viral gene, RTA (Replication and Transcription Activator) controls this genetic switch; forced expression of RTA in latency triggers lytic reactivation, and mutational inactivation of RTA blocks lytic reactivation (Sun et al., 1998; Lukac et al., 1999). However, little is known of the physiologic stimuli that activate RTA expression to trigger lytic growth, and still less is known about how latency, once established, is stably maintained (that is to say, how the expression of RTA and other lytic genes is prevented).

Several years ago, Brown et al. (2003) demonstrated that treatment of PEL cells with Bay 11-7082, a known pharmacological inhibitor of NF κ B activation, triggered enhanced spontaneous lytic reactivation. Because KSHV latency is associated with expression of the v-FLIP gene, which tonically activates NF κ B by binding to NEMO and activating IKK (Field et al., 2003; Liu et al., 2002), latently infected cells have elevated NF κ B activity. A model has accordingly been proposed that NF κ B activation opposes lytic reactivation; in support of this notion, Brown et al. (2003) reported that overexpression of p65 inhibits expression of luciferase reporter genes driven by several KSHV lytic promoters. Here we have examined the role of NF κ B in lytic reactivation in several cell types other than PEL cells (which have been selected for stable latency *in vivo* and display a strong dependence on v-FLIP-mediated NF κ B activation (Guasparri et al., 2004)). We find that the dependence of latency on NF κ B activation is variable and dependent upon the cellular context — primary endothelial cells behave similarly to PEL cells, but equally permissive human fibroblasts show no enhancement of lytic reactivation when the NF κ B activation pathway is inhibited. Moreover, NF κ B is strongly activated during lytic replication in all cell types, indicating that if there is a block to lytic gene expression mediated by p65 it must be at least partially bypassed or overcome in the context of the lytic program.

Results

Inhibition of NF κ B in PEL cells leads to increased lytic gene expression and apoptosis

Although Brown et al. (2003) reported that NF κ B inhibition upregulated lytic gene expression, other investigators have not observed lytic derepression in this setting (Keller et al., 2000). We therefore re-examined this issue. In accord with previous work (Brown et al., 2003), we found that inhibition of NF κ B signaling in KSHV positive PEL cells leads to increased expression of an array of lytic genes. BCBL-1 cells, a line persistently infected with KSHV, were treated with a range of doses (1, 2, 4, 6 μ M) of the compound Bay 11-7082 (in DMSO), an irreversible inhibitor of I κ B α phosphorylation, or a corresponding amount of DMSO. Total RNA was isolated from the cells and expression of several lytic genes was assayed by Taqman real time RT-PCR. As shown in Fig. 1A, cells treated with Bay showed a marked increase in expression of an immediate-early lytic gene (ORF 50), a delayed-early gene (ORF 57) and a late lytic gene (gB).

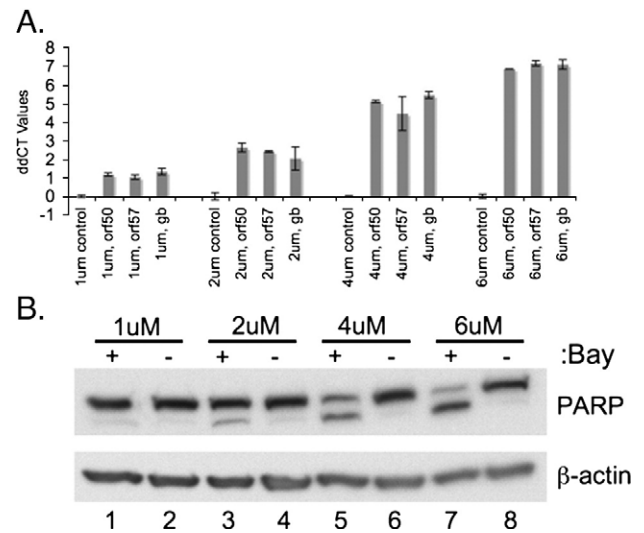


Fig. 1. Inhibition of NF κ B in BCBL-1 cells leads to increased lytic gene expression as well as increased apoptosis. BCBL-1 cells were treated with the indicated amounts of Bay 11-7082 or vehicle (DMSO) for 30 min at 37 $^{\circ}$ C. Cells were resuspended in fresh media and total RNA and total protein were harvested at 48 h post treatment. Taqman real time PCR was performed on cDNA made from total RNA. ddCT values (log based 2 indicator of fold change) for lytic genes Orf 50, Orf57 and gB are shown in panel (A). All values were normalized to GAPDH levels, and are expressed for each gene in BCBL-1 cells treated with Bay 11-7082 relative to those treated with the same amount of DMSO. Panel (B) shows a dose dependent increase in apoptosis as measured by PARP cleavage with increasing doses of the Bay 11-7082 compound. 20 μ g of total protein was blotted for PARP protein and β actin as a loading control.

This increase was seen at all assayed doses and followed a dose-dependent trend.

NF κ B inhibition in PEL cells has been reported to be associated with increased apoptosis of the PEL cells (Keller et al., 2006, 2000). Accordingly, we examined apoptosis levels by immunoblotting for PARP, a downstream cleavage target of caspases 3 and 7 in the apoptotic cascade (Fig. 1B). As expected, increased cleavage of PARP was seen in BCBL-1 cells treated with Bay 11-7082 (lanes 1,3,5,7) but not the corresponding vehicle, DMSO (lanes 2,4,6,8). Because apoptosis was assayed in the mass culture rather than at the single cell level, we do not know if the cell population experiencing lytic reactivation was the same or different from that undergoing apoptosis.

de novo KSHV infection of endothelial cells in the context of NF κ B inhibition leads to increased cytotoxicity, lytic reactivation and apoptosis

Previous experiments investigating the link between the NF κ B signaling pathway and the KSHV latent-lytic switch have been done almost exclusively in PEL lines (Brown et al., 2003; Guasparri et al., 2004; Keller et al., 2006, 2000; Sgarbanti et al., 2004). But PEL cells are very far removed from the initial latent infection, having been selected *in vivo* for stable episome maintenance despite rapid growth — a selection that we know requires epigenetic changes that are likely not present in KS-derived endothelial (spindle) cells or most latently infected cells

established in culture (Grundhoff and Ganem, 2004). Therefore, PEL cells may not be fully representative of all cells in which latent KSHV infection can be observed. Accordingly, we have examined several other cell types in which *de novo* KSHV infection can produce latency, and which are permissive for lytic reactivation. These include primary human umbilical vein endothelial cells (HUVEC) and secondary human foreskin fibroblasts (HFF).

To examine the role of NF κ B in HUVEC cells, we generated a HUVEC culture stably expressing a degradation-resistant mutant of I κ B α . This version of I κ B α (I κ B super-repressor, I κ BSR) contains two Ser/Ala mutations at positions 32 and 36, rendering it resistant to phosphorylation by the I κ B α kinase (IKK) and therefore refractory to subsequent degradation by the proteasome. This results in a stabilization of I κ B α -NF κ B complexes in the cytoplasm of the cell, inhibiting the ability of the NF κ B transcription factor to translocate into the nucleus. As previously described (Grossmann et al., 2006), HUVECs were transduced with retrovirus encoding I κ BSR and selected for a short time with puromycin. These cells were assayed for inhibition of NF κ B signaling by treatment with TNF α (10 ng/ml) for 2 h, isolation of nuclear extracts, followed by electrophoretic mobility shift assay (EMSA) to determine NF κ B DNA binding activity. Fig. 2A shows complete inhibition of inducible NF κ B DNA binding in I κ BSR HUVECs upon treatment with TNF α , in contrast to those expressing the empty vector, which display strong NF κ B induction.

Next, I κ BSR and control HUVECs were infected with KSHV at levels that were previously determined to give 100% LANA positive cells and low (<1%) spontaneous lytic reactivation; infected and mock-infected cells were then followed for 3 days. Fig. 2B shows representative pictures of the mock- or KSHV-infected HUVEC monolayers at 66 h post infection. Upon KSHV infection, I κ BSR expressing cells showed reproducible increases in cytotoxicity as compared to control cells. In some cases, this toxicity was visible at time points as early as 18 h post infection (data not shown), but was most pronounced at the 66 h time point.

To determine whether the observed increases in cell toxicity correlated with increased spontaneous lytic reactivation of the virus, I κ BSR and control HUVECs previously infected with KSHV were fixed and stained for the delayed-early protein ORF 59 (Fig. 2C). The lower panel of Fig. 2C shows representative fluorescence images of cells stained for ORF 59 expression (red) and DAPI (blue) at 66 h post infection (hpi). As is evident in the picture, and is quantified in the histogram in the upper panel, I κ BSR HUVECs showed a significant increase in ORF 59 positive cells. In order to better quantify the increase in lytic gene expression in I κ BSR HUVECs, total RNA was harvested from cells at 66 hpi. Real time quantitative RT-PCR was performed to determine the relative abundance of mRNAs that spanned the multiple phases of temporal regulation of the lytic cycle. As is shown in the upper panel of Fig. 2D, all three lytic genes assayed (ORF50, ORF57 and gB) showed increased expression in I κ BSR HUVECs over control cells. Values are normalized to levels of the endogenous GAPDH gene for each sample, and are displayed as ddCT values (log base 2) showing the relative

increase in expression of the genes in I κ BSR HUVECs over control cells.

To determine whether the observed increase in lytic gene expression in the I κ BSR HUVECs corresponded to an increased production of KSHV virions, supernatants from infected cells were collected at 42 and 66 hpi. Supernatants were cleared of dead cells and cellular debris and the remaining virions were concentrated by centrifugation. Exogenous DNA was removed by DNase digestion; following inactivation of the nuclease, virions were lysed and virion DNA was extracted as described in the Materials and methods section. Real time quantitative Taqman PCR was performed on the isolated virion DNA, and the abundance of viral genomes was quantified by measuring the relative levels of the PAN promoter sequence (Fig. 2D, lower panel), normalized to a spiked-in control. I κ BSR HUVECs showed increased production and release of virions into the supernatant at both the 42 and 66 hpi time points, reaching a maximum of 8-fold. In conjunction with the observed increases in staining for lytic protein and lytic gene expression, the increased production of KSHV virions supports the view that inhibition of the NF κ B pathway in primary human endothelial cells leads to increased spontaneous lytic reactivation of KSHV upon *de novo* infection.

Although much of the cytotoxicity observed in the infected I κ B-SR HUVECs could be attributed to cell necrosis from lytic infection, we also asked whether there was an increase in apoptosis in this setting. Lysates taken at 66 hpi were blotted with an anti-PARP antibody to assess the extent of PARP cleavage in the individual cultures (Fig. 2E). Increased PARP cleavage was observed in the KSHV-infected I κ BSR HUVECs (lane 4) as compared to the infected control cells (lane 2), suggesting enhanced levels of apoptosis in these cells. This is not entirely surprising, as many lytic herpesviral gene products can trigger apoptosis. Additionally, since NF κ B is known to upregulate an anti-apoptotic cascade in endothelial cells (Stehlik et al., 1998), inhibition of this pathway in infected cells might be expected to trigger enhanced apoptosis.

KSHV-infected human fibroblasts do not display enhanced lytic replication or cytotoxicity in the presence of NF κ B inhibition

To determine whether the cell death associated with KSHV infection in the context of NF κ B inhibition is common to all cell types, human foreskin fibroblasts (HFF) expressing the I κ BSR were constructed. HFFs have been previously shown to be fully permissive for both latent and lytic KSHV infection (Bechtel et al., 2003; Vieira and O'Hearn, 2004). As expected, I κ BSR HFFs display strong inhibition of NF κ B signaling in response to TNF α (Fig. 3A), relative to control HFFs transduced with an empty retroviral vector. These cells were then infected with KSHV under conditions promoting latent infection of nearly all cells. Despite robust latent infection, as evaluated by staining for LANA (green, Fig. 3C), no increase in cell death (Fig. 3B) or ORF 59 staining (red, Fig. 3C) was detected in I κ BSR HFFs as compared to control cells. Likewise, no increase in lytic mRNA expression or virion production was observed in these cells (Fig. 3D). (In fact, for unclear reasons the levels of lytic

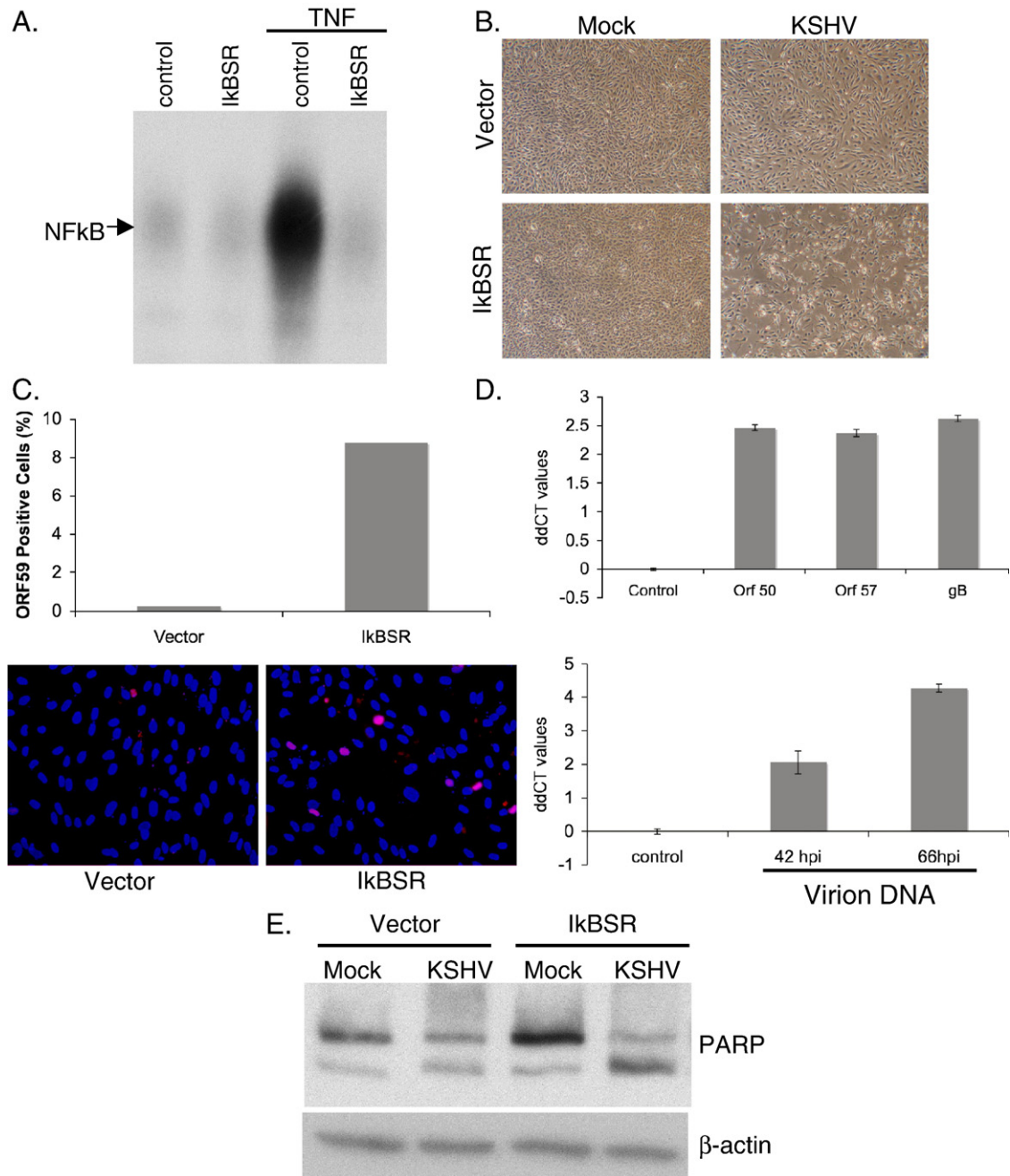


Fig. 2. Inhibition of NFκB in HUVEC cells leads to increased cellular toxicity, increased lytic gene expression, virion production and apoptosis upon *de novo* infection with KSHV. (A) HUVECs were transduced with retroviruses encoding a degradation-resistant mutant of IκBα (IκBSR). NFκB DNA binding activity in these cells was blocked upon treatment with 10 ng/ml TNFα for 2 h, as compared to those expressing vector alone. (B) HUVECs expressing IκBSR show increased cellular toxicity upon *de novo* latent KSHV infection. HUVECs expressing either vector or IκBSR were infected with KSHV for 6 h, pictures were taken at 66 h post infection. (C) KSHV-infected IκBSR HUVECs show increased staining for ORF 59 (red) as compared to HUVECs expressing vector alone. Cells were fixed and stained at 66 h post infection. (D) Taqman real time PCR ddCT values (log base 2) for ORF 50, ORF 57 and gB (upper panel) and PAN promoter values (lower panel) show increased lytic gene expression and enhanced virion production in IκBSR expressing HUVECs. All values were normalized to GAPDH or spike-in DNA levels, and are expressed for each gene in IκBSR HUVECs relative to vector HUVECs. (Please see Materials and methods section for details on virion DNA isolation.) (E) IκBSR HUVECs show increased apoptosis upon infection with KSHV. Total protein was isolated from all cells (floating and adherent to the dish) from vector and IκBSR HUVECs at 66 h post infection. 20 μg of total protein was blotted for PARP protein and β actin as a loading control.

transcripts modestly decreased in the presence of IκB-SR.) Thus, neither lytic reactivation nor cell injury is an ineluctable consequence of KSHV infection in the absence of NFκB activation. Clearly, the relationship between NFκB signaling and spontaneous lytic reactivation is dependent on the cellular context.

NFκB signaling is activated in lytically infected cells of all lineages

Data showing that inhibition of NFκB induces lytic gene expression in HUVEC and PEL cells suggest that NFκB

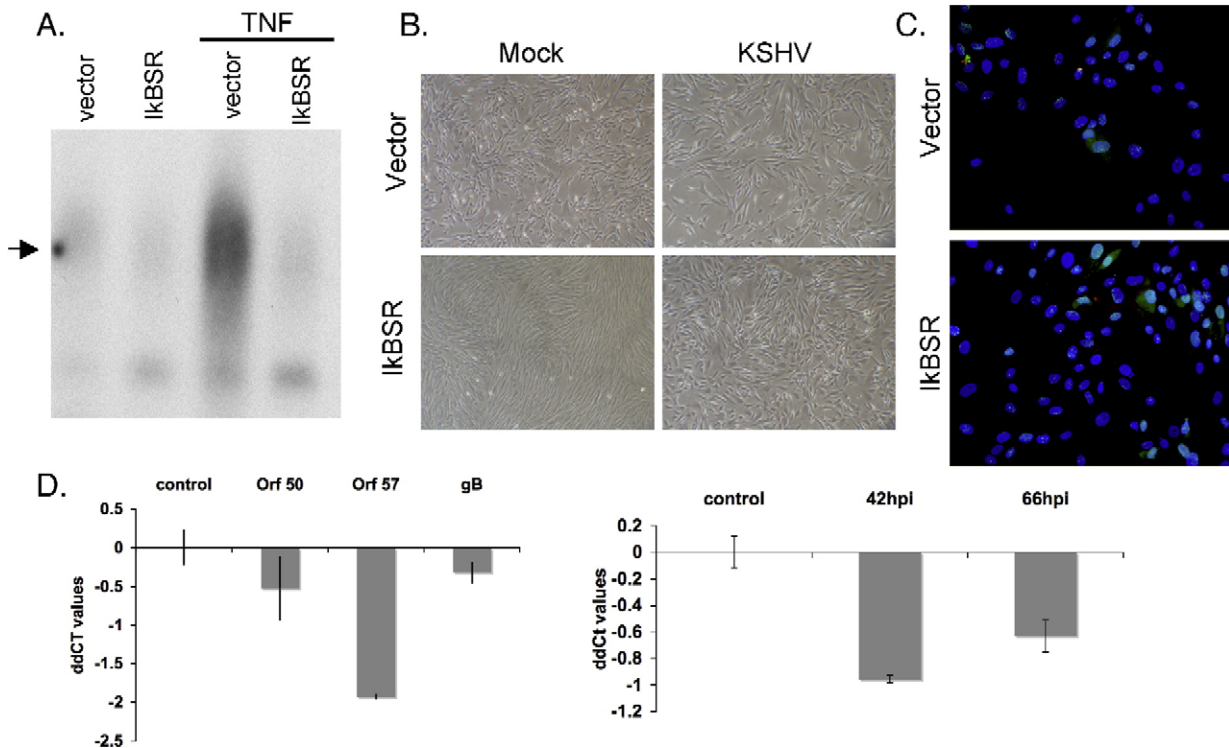


Fig. 3. Inhibition of NF κ B in human foreskin fibroblasts (HFF) does not lead to increased cellular toxicity, increased lytic gene expression and virion production upon *de novo* infection with KSHV. (A) HFFs were transduced with retrovirus encoding a degradation-resistant mutant of I κ B α (I κ BSR). NF κ B DNA binding activity in these cells was blocked upon treatment with 10 ng/ml TNF α for 2 h, as compared to those expressing vector alone. (B) HFFs expressing I κ BSR show no increase in cellular toxicity upon *de novo* latent KSHV infection. HFFs expressing either vector or I κ BSR were infected with KSHV for 6 h, pictures were taken at 66 h post infection. (C) No increase in ORF 59 staining was seen in I κ BSR HFFs as compared to vector despite extensive latent KSHV infection as determined by staining for LANA (green). Cells were fixed and stained at 66 h post infection. (D) Taqman real time PCR ddCt values for ORF 50, ORF 57 and gB (left panel) and PAN promoter values (right panel) show no increase in lytic gene expression and or virion production in I κ BSR expressing HFFs. All values were normalized to GAPDH or spike-in DNA levels, and are expressed for each gene in I κ BSR HFFs relative to vector HFFs.

activation is inhibitory to lytic gene expression. Consistent with this, [Brown et al. \(2003\)](#) reported that overexpression of recombinant p65 can extinguish gene expression from cotransfected reporter genes driven by lytic KSHV promoters. These data suggest that NF κ B activation may be incompatible with lytic gene expression and lead to the prediction that the lytic cycle might actively suppress NF κ B activation. To test this prediction, we examined the state of NF κ B activity at several time points during the progression of lytic KSHV infection. Contrary to the predictions of this model, we found robust NF κ B activation in several cell types including HUVECs, HFFs and 293T. The NF κ B activation state of HUVECs and HFFs was examined during both latent and lytic KSHV infections. Cells were infected with either KSHV alone or KSHV followed by an adenovirus encoding the lytic transactivator RTA (AdRTA). At 48 hpi nuclear extracts were prepared from the cells and NF κ B DNA binding was assessed by EMSA ([Fig. 4A](#)). Increased NF κ B DNA binding could be seen in HUVECs upon latent infection (upper panel, lane 3) and was further enhanced upon reactivation with AdRTA (upper panel, lane 4). HFFs showed similarly increased NF κ B DNA binding upon lytic KSHV infection ([Fig. 4A](#), lower panel). Moreover, this enhanced NF κ B was functionally active, as judged by its ability to upregulate expression of an NF κ B-dependent luciferase reporter. 293T cells were cotransfected with

plasmids encoding CD8, RTA, and an NF κ B-luciferase reporter. 36 h post transfection, cells expressing CD8 (and therefore RTA) were enriched for by binding to magnetic beads bearing anti-CD8. The next day, these cells were either infected with KSHV or mock-infected; at 48 h post infection, luciferase levels were assayed ([Fig. 3B](#)). KSHV-infected, RTA-expressing cells showed enhanced luciferase readings, indicating increased NF κ B-dependent transcriptional activation under these conditions. (We note that the extent of upregulation of the reporter observed here is surely an underestimate of the degree of NF κ B activation, owing to the global turnover of host mRNAs during lytic KSHV replication ([Glaunsinger and Ganem, 2004](#).)

As another indication of the activation state of the NF κ B pathway during lytic growth, we assayed for phosphorylation of I κ B α by the IKK complex in lytically infected HUVECs. Cells were infected with either KSHV alone or KSHV plus AdRTA; extracts were prepared at 48 and 72 hpi and assayed by immunoblotting for phospho-I κ B α and total levels of I κ B α . [Fig. 4C](#) shows increased phosphorylation of I κ B α in lytically infected cells by 48 hpi (top panel, lane 3, bottom band) and a corresponding decrease in steady state levels of total I κ B α protein in these cells (middle panel, lane 3). By 72 hpi the intracellular pool of I κ B α is virtually totally depleted, most likely as a result of proteasome action, and possibly exacerbated by KSHV-mediated

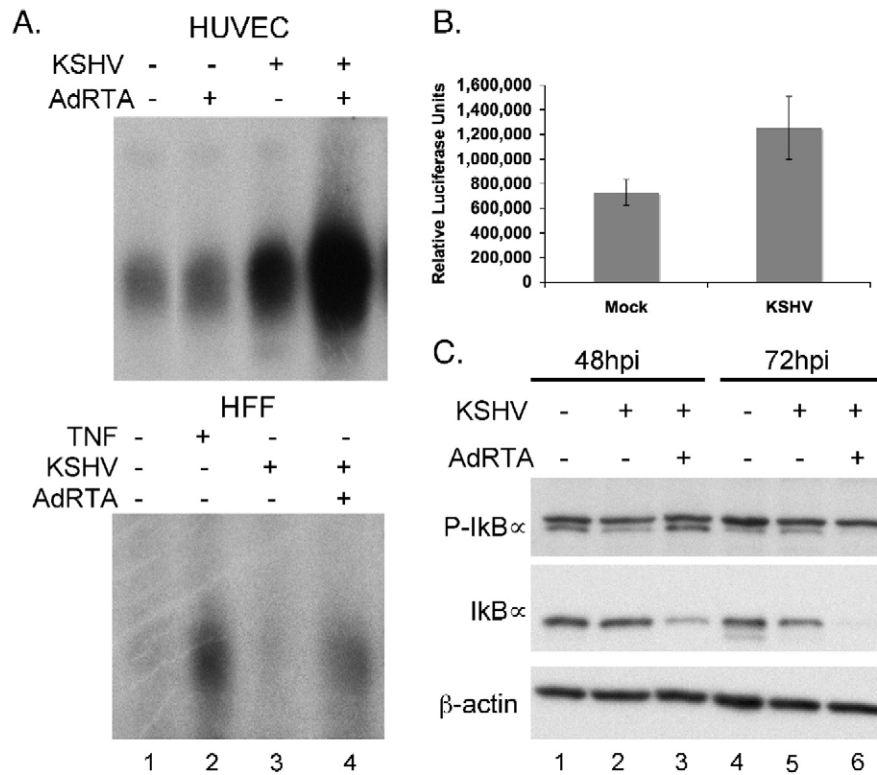


Fig. 4. NF κ B signaling is activated in lytically infected cells. (A) Increased NF κ B DNA binding activity is observed in lytically infected HUVECs (upper panel) and HFFs (lower panel), as compared to both uninfected, and latently infected cells. Nuclear fractions were harvested at 48 h post infection, 5 μ g of protein was used in each binding reaction. (B) Increased NF κ B transcription is shown in 293T cells cotransfected with RTA and NF κ B-luciferase reporter at 48 h post infection. Both mock and KSHV cells were transfected with CD8, RTA and luciferase plasmids for 36 h before infection, and enriched for CD8, and therefore RTA positive cells, using anti-CD8 coupled beads over magnetic columns. (C) Lytically infected HUVECs show increased phosphorylation of I κ B α (top blot, lower band) and corresponding decrease in levels of total I κ B α (middle blot) demonstrating active NF κ B signal transduction.

mRNA turnover, which is very pronounced late in infection (Glaunsinger and Ganem, 2004). These data affirm that the NF κ B pathway is upregulated during lytic replication, and that this induction is occurring at or above the level of IKK activation.

Discussion

Previous studies have described complicated interactions between the NF κ B signaling pathway and KSHV infection in the context of PEL cell lines (Brown et al., 2003; Guasparri et al., 2004; Keller et al., 2006, 2000; Sgarbanti et al., 2004). These studies have shown increased lytic gene induction upon chemical inhibition of NF κ B (Brown et al., 2003), enhanced apoptosis upon chemical inhibition of NF κ B or knockdown of v-FLIP, a potent NF κ B inducer (Guasparri et al., 2004; Keller et al., 2006), and even production of defective KSHV particles upon genetic inhibition of NF κ B in TPA-induced PEL cells (Sgarbanti et al., 2004). Additionally, inhibition of RTA-mediated transactivation of lytic gene promoters was shown in the context of overexpression of the p65 subunit of NF κ B (Brown et al., 2003). Taken together, these accounts have led some to propose a model of suppression of lytic gene expression and function by NF κ B activation in PEL cells.

In our hands, inhibition of NF κ B in BCBL-1 cells by treatment with Bay 11-7082 led to increases in both lytic gene expression, in agreement with the work of Brown et al. (2003), and enhanced apoptosis, in accord with the observations of Guasparri et al.

(2004). Results in HUVEC cells were largely congruent with these PEL observations; however, KSHV-infected human fibroblasts behaved completely differently — they displayed neither enhanced cytotoxicity nor increased spontaneous lytic gene reactivation. These results led us to conclude that the relationship between NF κ B and spontaneous reactivation of KSHV is complex, non-uniform and dependent on the cellular context. Cell specific differences in KSHV biology are not without precedent. For example, while many cell types can be latently infected *in vitro*, only a small subset of these can efficiently support full lytic replication following treatment with chemical inducers (Bechtel et al., 2003). Additionally, we and others have reported modifications in cellular morphology upon latent KSHV infection that occur only within the context of human primary endothelium (Ciuffo et al., 2001; Grossmann et al., 2006; Grundhoff and Ganem, 2004; Pan et al., 2004; Tang et al., 2003); differences in KSHV gene expression upon *de novo* infection and in stable latency have also been reported to differ between cell type (Krishnan et al., 2004; Rivas et al., 2001). Given these differences, which indicate intricate and complex interactions of the KSHV genome with the machinery of host gene expression, it is perhaps not surprising that the relationship of lytic induction to NF κ B activation is not invariant in different cellular environments.

Finally, despite the earlier finding (Brown et al., 2003) that p65-overexpression can impair reporter genes driven by KSHV lytic promoters (outside of the context of viral infection), we find

that lytic replication is associated with *increased* NF κ B activity in endothelial, epithelial and fibroblast cell lines upon lytic infection. Increased NF κ B activity has also been observed in PEL cell lines during lytic reactivation (Sgarbanti et al., 2004), though those studies were complicated by the use of TPA as an inducer (and the fact that isogenic KSHV-negative PEL cells are not available as controls). Therefore, NF κ B activation and lytic gene expression cannot be strictly incompatible under all circumstances. Whatever inhibitory influence p65 activation may have on lytic promoters, it clearly can be at least partially bypassed or overcome during full lytic infection. (That this bypass is not complete is indicated by our recent observation that when TPA is used to induce lytic reactivation in infected HUVECs, titers of released virions are 2–3-fold higher in the presence of I κ B-SR expression; C.G., unpublished data.) Exactly how this bypass is accomplished will require further investigation.

Materials and methods

Cells and KSHV infection

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics and cultured in EGM-2 media supplemented with the endothelial supplement pack (Clonetics). Human foreskin fibroblasts (HFFs) were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine. BCBL-1 cells were carried in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamine and β -mercaptoethanol. KSHV was concentrated from supernatants of induced BCBL-1 cells as previously described (Bechtel et al., 2003; Lagunoff et al., 2002). KSHV infections were done in media containing 2 μ g/ml polybrene and incubated with cells for 6 h, after which cells were rinsed and media was added back. In the case of lytic infection, Adeno-RTA (1×10^9 particles/ml) was incubated in media plus 0.5 μ g/ml polylysine (Sigma) for 2 h before being applied to cells after the removal of the KSHV containing media.

Retrovirus production and infection

Retroviruses were produced using the amphotropic Phoenix packaging cell line transfected with the Moloney Murine Leukemia Virus based vector pMXpie (a kind gift from Lewis Lanier). Phoenix cells were transfected using FuGENE 6 (Roche) according to the manufacturer's specifications. 36 h after transfection supernatants were collected and concentrated at 5000 rpm for 16 h. Concentrated retroviruses were resuspended in EGM-2 media with 6 μ g/ml polybrene and filtered through a 0.2 μ m filter. Concentrated retrovirus was diluted in EGM-2 media with 6 μ g/ml polybrene and applied to cells. These cultures were spun at 2000 rpm for 1.5 h after which virus-containing media was removed and regular culture media was added back. In cases where selection was employed, media containing the selective agent was added to cells at the stated concentration 24 h after transduction.

Immunofluorescence and Western blotting

Immunofluorescence assays were performed as previously described using anti-ORF59 (ABI) and an anti-mouse rhodamine (Santa Cruz Biotechnology) secondary antibody was used at 1:300.

Lysates for Western blots were obtained by rinsing cells twice with ice cold PBS, scraping adherent cells from the plate, spinning cells at 1000 rpm for 5 min and resuspending pellets in 2–3 \times the pellet volume of RIPA lysis buffer. Lysates were quantified using Biorad Protein Assay. 20 μ g of protein were run on 4–15% Tris–HCl gels and protein was transferred to PVDF at 100 mV for 1 h at 4 $^{\circ}$ C. Blots were blocked for 1 h at room temperature in 5% milk in TBS plus 0.1% Tween. Primary antibodies were incubated overnight at 4 $^{\circ}$ C at 1:1000 anti-PARP (Cell Signaling Technology), 1:5000 anti- β -actin (A1978, Sigma); secondary antibodies, anti-mouse-HRP and anti-rabbit-HRP (Santa Cruz) were incubated at 1:5000 for 1 h at room temperature. Signal was detected using ECL Detection Reagent (Amersham).

Bay 11-7082 treatment of BCBL-1 cells

Bay 11-7082 was purchased from Calbiochem, diluted in DMSO and used at the concentrations stated. For reactivation assays, BCBL-1 cells were resuspended in media containing Bay 11-7082, incubated for 30 min, then spun down and resuspended in fresh media. RNA and protein lysates were harvested at 48 h post Bay 11-7082 treatment.

KSHV virion DNA isolation

Media was removed from virus-producing cells, filtered through a 0.45 μ m filter and stored at 4 $^{\circ}$ C. 20 μ /ml DNase was added to filtered supernatants and incubated at 37 $^{\circ}$ C for 1 h. Supernatants were then chilled on ice and spun at 15,000 rpm for 2 h at 4 $^{\circ}$ C. Media was then removed and pelleted virus was resuspended in 600 μ l lysis buffer (20 mM Tris–HCl at pH 8, 10 mM EDTA, 100 mM NaCl, 0.5% SDS) and incubated at room temperature for 10 min. 0.7 mg/ml Proteinase K and linearized plasmid encoding an unrelated malaria gene were diluted in 100 μ l lysis buffer and added to the 600 μ l resuspended virions. The 700 μ l total volume was incubated at 55 $^{\circ}$ C for 2 h; this was then extracted once with 700 μ l phenol/chloroform/isoamyl. 600 μ l of the aqueous phase was removed and to it was added 3 μ l glycogen, 100 μ l 3 M sodium acetate and 700 μ l isopropanol; this was then spun at 15,000 rpm for 15 min at 4 $^{\circ}$ C. The resulting pellet was rinsed once with 70% ethanol, spun and air dried before being resuspended in 85 μ l of water. 2 μ l of this was used in the subsequent Taqman and Sybergreen assays for PAN promoter and spiked-in malaria gene as a normalizing factor.

Taqman and Sybergreen real time PCR assays

For all Taqman assays, 200 ng of RNA was reverse transcribed using SuperScript III and oligo d(T) primer (Invitrogen) according to the manufacturer's instructions. 2 μ l of this reaction was used in all subsequent real time PCR Taqman assays. Primer and

probe sequences used for lytic gene expression ORF50 are previously described (Bechtel et al., 2005). Other primers used were ORF 57 forward: TGGACATTATGAAGGGCATCC, reverse: CGGGTTCGGACAATTGCT; gB forward: TCGCCGCACCAATACCATA, reverse: CCTGCGATCTACGTCGGG; PAN promoter forward: GCCAGCTTGAGTCAGTTTAGCA, reverse: CGAGCACAAAATCCATAGGTG; and Malaria DNA forward: AGGACCCGATCAACAACAT, reverse: AAGCTGAACAA-GAACGCGAT. Taqman reactions (all but malaria DNA) were performed using Taqman Universal Master Mix (Applied Biosystems) and Sybergreen reactions (malaria DNA) were performed using SybergreenER mastermix (Invitrogen) as per the manufacturer's specifications.

NfκB Emsa

Nuclear enriched lysates were made from cells by incubation in hypotonic buffer (20 mM HEPES at pH 7.8, 5 mM KCl, 1.5 mM MgCl₂, 1 mM DTT and protease inhibitors), followed by pelleting and disruption of nuclei by incubation in high salt buffer (0.4 M KCl, 50 mM HEPES at pH 7.9, 0.1% NP40, 0.5 μM EDTA, 10% glycerol and protease inhibitors). 5 μg of lysate was incubated with ³²P-labelled oligonucleotide encoding the NFκB consensus sequence (Santa Cruz Biotechnology), and dI:dC DNA (Sigma) in binding buffer (20 μM HEPES at pH 7.9, 50 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM MgCl₂, 1 mM DTT). Reactions were incubated at room temperature for 30 min without the labeled probe and an additional 30 min after addition of the probe. Complexes were resolved in a 1× TBE, 4% acrylamide gel.

NFκB-luciferase assay

293T cells were transfected using Fugene 6 (Roche) as per the manufacturer's specifications with increasing amounts for the following constructs: pNFκB-luc (BD Biosciences), pCDNA3.1-RTA, pCD8 (Mylteni Biotech). 36 h post transfection cells were passed over anti-CD8 MACS columns (Mylteni Biotech) to enrich for cells expressing CD8 and RTA. The separated cells were plated and allowed to recover overnight. The next morning cells were either mock-infected or infected with KSHV in triplicate. 48 h post infection luciferase levels were assayed as per the manufacturer's specifications (Promega).

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