STAT3 Regulates Lytic Activation of Kaposi’s Sarcoma-associated Herpesvirus

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Abstract

Lytic activation of Kaposi’s Sarcoma-associated Herpesvirus (KSHV) from latency is a critical contributor to pathogenesis and progression of KSHV-mediated disease. Development of targeted treatment strategies and improvement of lytic phase-directed oncolytic therapies therefore hinge on gaining a better understanding of latency-to-lytic transition. A key observation in that regard, also common to other herpesviruses, is the partial permissiveness of latently-infected cells to lytic cycle inducing agents. Here, we address the molecular basis for why only some KSHV-infected cells respond to lytic stimuli. Since cellular Signal Transducer and Activator of Transcription 3 (STAT3) is overactive in KSHV-associated cancers, KSHV activates STAT3, and STAT3 has been found to regulate lytic activation of Epstein-Barr virus (EBV)-infected cells, we asked if STAT3 contributes similarly to the life cycle of KSHV. We found that high levels of STAT3 correlate with the refractory state at the single cell level under conditions of both spontaneous and induced lytic activation; importantly, STAT3 also regulates lytic susceptibility. Further, knockdown of STAT3 suppresses the cellular transcriptional co-repressor Krüppel-associated box domain-associated protein 1 (KAP1), and suppression of KAP1 activates lytic genes including the viral lytic switch RTA, thereby linking STAT3 via KAP1 to regulation of the balance between lytic and latent cells. These findings, taken together with those from EBV-infected, and more recently HSV-1-infected cells, cement the contribution of host STAT3 to persistence of herpesviruses, and simultaneously reveal an important lead to devise strategies to
improve lytic phase-directed therapies for herpesviruses.

Importance

Lytic activation of the cancer-causing Kaposi’s Sarcoma-associated Herpesvirus (KSHV) is vital to its life cycle and causation of disease. Like other herpesviruses, however, a substantial fraction of latently-infected cells are resistant to lytic-inducing stimuli. Investigating the molecular basis for this refractory state is essential for understanding how the virus persists, how it causes disease, and to guide efforts to improve treatment of KSHV-mediated diseases. We find that like two other herpesviruses, EBV and HSV-1, KSHV exploits the cellular transcription factor STAT3 to regulate susceptibility of latently-infected cells to lytic triggers. These findings highlight a common STAT3-centered strategy used by herpesviruses to maintain persistence in their host, while also revealing a key molecule to pursue while devising methods to improve herpesvirus lytic phase-directed therapies.
Introduction

The oncogenic human gammaherpesvirus human herpesvirus 8 (HHV8), widely known as Kaposi’s sarcoma-associated herpesvirus (KSHV), is the etiologic agent of three human malignancies: Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD) (1, 2). Like other herpesviruses, KSHV exhibits a dual-phase life cycle that includes latency and lytic infection (3). During latency, the KSHV episome expresses a limited number of viral genes which mediate multiple functions that include maintaining viral genomes, repressing viral lytic gene expression, promoting proliferation of infected cells, and perturbing host immune surveillance (4-6). Periodic switching from latency to the lytic phase results in an orderly expression of a large number of viral genes to produce infectious virions.

Clinico-epidemiologic studies indicate that KSHV lytic activation is a critical contributor to pathogenesis of KS, PEL, and MCD (7-13). Lytic activation also correlates with disease progression and prognosis (14-16). Indeed treatment with antiviral agents such as Ganciclovir and Foscarnet that target the lytic phase of KSHV life cycle reduces the risk of development of KS as well as progression of KS and MCD (9, 14-16). A few studies have also reported remission of PEL following treatment with the antiviral drug Cidofovir (17-19). These studies argue for a better understanding of the molecular mechanisms underlying latency to lytic switch with the ultimate goal of “therapeutically” increasing the number of latently infected cancer cells that switch to the lytic phase and thereby become susceptible to antiviral
agents. In fact, a major hurdle to such oncolytic therapy is the presence of large
fractions of cells among a population of latently infected cells that are resistant to lytic
cycle inducing agents (20).

The goals of this study were to detect lytically-infected cells using a monoclonal
antibody and begin investigating the contribution of cellular factors towards
susceptibility to lytic cycle inducing signals at the single cell level. The downstream
goal is to use our findings to increase the number of lytic cells in response to lytic
cycle activating agents. Our studies on Epstein-Barr virus (EBV)-infected cells
revealed that high levels of Signal Transducer and Activator of Transcription 3
(STAT3), a cellular transcription factor important for oncogenesis and inflammation,
correlate with resistance to lytic phase inducing signals. Furthermore, STAT3
regulates susceptibility of latently infected EBV+ cells to lytic cycle activating
triggers (21, 22). Partly because of the newly-discovered contribution of STAT3 to
lytic susceptibility in another human gammaherpesvirus (i.e. EBV) and partly because
JAK2/STAT3 signaling is constitutively active in KSHV+ PEL cells (23), we focused
our efforts on STAT3. Using KSHV+ PEL cells that are derived from B cells and
provide an excellent model to study KSHV latency-to-lytic switch, we show here that
high levels of STAT3 correlate with and promote the lytic cycle refractory state of
KSHV-infected cells. We also show that STAT3 regulates the cellular transcriptional
corepressor KAP1 which represses lytic genes, thus functionally linking STAT3 via
KAP1 to lytic susceptibility of KSHV+ latent cells.
Materials and Methods

Cell culture and chemical treatment
The body-cavity-based KSHV+ lymphoma cell line BCBL-1 (a kind gift from Dr. Shane McAllister at University of Minnesota Medical School) were maintained in RMPI-1640 supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco). BCBL-1 cells were sub-cultured at 3×10^5 cells/ml and treated after 24 hours with 20ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA; 79346, Sigma), 0.6μM of valproic acid (VPA; 152064, MP Biomedicals), or 5μM of WP1066 (573097, EMD). Primary Human Umbilical Vein Endothelial cells (HUVECs; Lonza) were cultured in EGM-2 medium (Lonza) in 6-well tissue culture plates coated with 0.1% (w/v) gelatin (in PBS) and used at passage 6 for experiments.

Nucleofection of BCBL-1 cells
BCBL-1 cells were sub-cultured at 5×10^5 cells/ml 24 hours prior to transfection. 2×10^6 of BCBL-1 cells were transfected with either 20μg of plasmid [pEGFPN1 or pEGFPN1-STAT3, from Dr. Nancy Reich, Stony Brook University (24, 25)] or 130 pmol of siRNA (30 pmol FITC+ scrambled siRNA plus 100 pmol scrambled siRNA or 100pmol siRNA targeting human STAT3; Santa Cruz) in Cell line Nucleofection solution V (VCA-1003, Lonza) using program T-001. For experiments in Figures 3A, 3B, 3D, 3E and 5, 150 pmol of siRNA (scrambled siRNA, siRNAs targeting human STAT3 or KAP1; all from Santa Cruz) were used.
Infection of HUVEC cells with KSHV

HUVECs were plated on gelatin-coated 6-well plates with glass coverslips at $1 \times 10^5$ cells/well. The next day, monolayers were inoculated for 2 hours at 37°C with 1.8 ml of cell-free supernatants derived from BCBL-1 cells left un-treated or treated for 48 hours with 0.6μM VPA or 5μM WP1066. This was followed by spinoculation at 2000 rpm for 15min at 25 °C, after which inoculum was removed and cells were incubated at 37°C and 5% CO2 in fresh EGM-2 medium for 48 hours.

Immunoblotting and immunofluorescence

Total cell extracts were electrophoresed in 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes and reacted with antibodies as described. Mouse monoclonal antibodies (Ab) were used to detect K8.1 (1:50; a kind gift from Dr. Bala Chandran, Rosalind Franklin University) and β-actin (AC-15, 1:10,000 dilution; Sigma), a rabbit polyclonal Ab was used to detect STAT3 (sc-482, 1:500 dilution; Santa Cruz) and a rabbit polyclonal Ab was used to detect KAP1 (A300-274A, 1:3000 dilution; Bethyl Laboratories). Signals were detected using enhanced chemiluminescence.

Immunofluorescence for KSHV LANA-1 was carried out in HUVECs treated with cell-free supernatants from treated or un-treated BCBL-1 cells. At 48 hours post-treatment, HUVECs were washed in PBS, fixed in 4% paraformaldehyde for 10 minutes at room temperature, washed and permeabilized in 0.1% Triton X-100. Coverslips were blocked in 1% human AB serum in 5% BSA/PBS for 30 mins and
then stained using rabbit anti-LANA Ab (1:500, a kind gift from Dr. Craig McCormick at Dalhousie University). After primary antibody binding, cell monolayers were washed and incubated for 45 min with Alexa Fluor 488 anti-rabbit Ab (1:2000, Molecular Probes) and mounted using Vectashield with Dapi (Molecular Probes).

**Flow cytometry**

To stain for K8.1 alone, BCBL-1 cells were washed with 1× PBS and stained with mouse anti-K8.1 Ab diluted at 1:10 in FACS buffer (1× PBS with 0.5% FBS) followed by staining with allophycocyanin (APC)-conjugated anti-mouse IgG (sc-3818, Santa Cruz) diluted at 1:200 in FACS buffer. For double staining of K8.1 and STAT3, BCBL-1 cells were washed with 1× PBS and stained with mouse anti-K8.1 Ab followed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (F0257, Sigma) diluted at 1:200. Cells were then fixed with BD Cytofix/Cytoperm solution and stained with rabbit anti-STAT3 Ab diluted at 1:50 in 1× BD Perm/Wash solution followed by staining with Alexa Fluor 647-conjugated anti-rabbit IgG (A21245, Life Technologies). Events were acquired using FACSCalibur (BD), and data were analyzed using FlowJo software (Tree Star). Gating was performed by comparing with similarly-treated cells that were stained with isotype control antibodies or secondary antibodies alone.

**Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)**
Total RNA was isolated from BCBL-1 cells using an RNeasy kit (Qiagen) followed by DNase digestion (Promega). RNA was quantitated by NanoDrop (Thermo Scientific). RNA (1μg) was converted to cDNA using qScript cDNA SuperMix (Quanta BioSciences). Relative transcript levels of selected cellular and viral genes were determined with gene-specific primers using Fast SYBR Green Master Mix on a StepOne Plus thermocycler (Applied Biosystems). Sequences of primers used are as follows: forward primer GTAACCCGTTGAACCCATT and reverse primer TTGATTTTGGAGGATCTCG for 18S rRNA; forward primer GAGTCAACGGATTTGGTGTG and reverse primer GGCGTCGTGATTAGTGATGAT for GAPDH; forward primer GGGCTACAATGTGATGGCCT for HPRT1; forward primer GAGTATGCCTGCGTGTG and reverse primer AATCCAAATGCGGCATCT for β2 microglobulin; forward primer GCCTCTGTGTGAGACCTGTG and reverse primer AGTACGTTACCACCCCGAG for KAP1; forward primer CCCTGAGCCAGTTTGTCATT and reverse primer ATGGGTTAAAGGGGATGATG for ORF50; forward primer TGGTCGGCGGTTCAGTCATCAA and reverse primer GCGGCCGCTAAGAAAATCGA for ORFK8.1; forward primer TAGGCGCTTCGTGCTGG and reverse primer CCGGATTGCTGCACTCGTA for ORF59.
Relative expression levels were calculated using the \( \Delta \Delta^{'Ct} \) method after normalization to 18S rRNA, HPRT1, or GAPDH. Individual samples were assayed in triplicate.

**KSHV viral load assay**

One million BCBL-1 cells transfected with siRNAs or plasmids and treated with VPA (or untreated) were resuspended in 100 µL of 0.2× PBS, heated to 95°C for 1h, and then treated with 10 mg/mL of proteinase K (19131, Qiagen) at 56°C overnight. The enzyme was inactivated by heating at 95°C for 1h. Aliquots of lysate (10 µL from siRNA-transfected and 0.01 µL from plasmid-transfected cells) were used to amplify the KSHV \textit{ORFK9} gene by real-time PCR using the following primers:

\begin{align*}
\text{ORFK9-F, 5'}-\text{GTCTCTGCGCCATTCAAAAC-3'}; \\
\text{ORFK9-R, 5'}-\text{CCGGACACGACAACTAAGAA-3'}.
\end{align*}

Relative KSHV load was quantified based on a standard curve PCR generated using VPA-treated BCBL-1 cells; standard qPCR curve gave linear detection over 5 logs of target dilutions.

**Statistical analysis**

P values were calculated by comparing the means of two groups of interest using unpaired Student t test.
Results

High levels of STAT3 mark KSHV+ cells that are refractory to lytic activation.

To determine the relationship between cellular STAT3 and KSHV lytic activation, we used a well-validated monoclonal antibody to the KSHV glycoprotein K8.1 (a late lytic protein) (26) in flow cytometric analysis of single cells. To establish the optimal time for detection of K8.1+ lytic cells, we treated BCBL-1 cells with lytic cycle inducing agents TPA (12-O-tetradecanoylphorbol-13-acetate) or VPA (Valproic acid). We found that ORFK8.1 message and protein levels were increased at 24 hours and even more so at 48 hours post-treatment with both agents compared to un-treated cells (Figs. 1A and B). Further, VPA treatment showed a more pronounced increase in steady state ORFK8.1 RNA and protein levels compared to TPA treatment after 48 hours (Figs. 1A and B). We therefore expected to be able to detect K8.1+ lytic cells as early as 24 hours. However, to ensure maximal detection of lytic cells by flow cytometry, we treated cells with TPA or VPA for 48 hours. Figure 1C shows that after TPA- and VPA-treatment, 6.5% and 10.5% cells expressed high levels of K8.1, respectively compared to 0.6% cells in the un-treated population. Notably, in this experiment, higher levels of K8.1 protein in 48-hour VPA-treated cells (Fig.1B) corresponded to an increase in the number of K8.1+ lytic cells compared to that in TPA-treated cells (Fig.1C).

To correlate the levels of STAT3 with lytic activation, we divided the cells into STAT3hi (high), STAT3int (intermediate) and STAT3lo (low) based on the relative
levels of expression of endogenous STAT3 and then examined them for K8.1 expression (Fig.1C). We found that STAT3hi cells were predominantly refractory with only 0.3-1.3% cells expressing high levels of K8.1 whether under spontaneous lytic conditions (i.e. un-treated) or treated with lytic cycle inducing agents. In contrast, lytic cells, spontaneous or induced by chemicals, arose primarily from STAT3int and STAT3lo cells. Of note, the majority (81.1% to 86.9%) of cells expressing low levels of K8.1, and likely to be early lytic cells, also expressed low levels of STAT3. Thus, high levels of STAT3 correlate with the refractory state in KSHV-infected cells.

Chemical inhibition of STAT3 results in KSHV lytic activation.

STAT3 is a convergent point of multiple signaling pathways, especially cytokine and growth factor signaling pathways (27). Receptor engagement typically results in activation of STAT3 via members of the Janus kinase (JAK) family. Activated STAT3 then functions as a transcription activator of many genes including itself (28). Changes in STAT3 protein levels would be expected to occur under a variety of physiologic and pathologic conditions, leading us to investigate the effect of a STAT3-inhibitor on KSHV lytic activation. We found that exposing BCBL-1 cells to WP1066, an agent that inhibits JAK2-mediated activation and nuclear localization of STAT3 (29), resulted in substantial increases in steady state levels of messages from KSHV lytic genes ORF50 (immediate early lytic gene), ORF9 and ORF59 (early lytic genes) and ORFK8.1 (late lytic gene) (Fig.2A). Compared to the kinetics in VPA-treated cells, increase in ORF50 and ORF59 RNA levels showed a slight lag.
following treatment with WP1066 alone; this was not the case for ORFK8.1 message.

Also by 18 hours, exposure to VPA and WP1066 resulted in substantial and synergistic increases in steady state levels of lytic messages beyond that caused by VPA alone. Thus, chemical inhibition of STAT3 resulted in increase in levels of messages from KSHV lytic genes belonging to all 3 kinetic classes.

**Inhibition of STAT3 results in lytic activation.**

We next asked if increase in lytic gene expression actually resulted in increase in the number of lytically infected cells. Figure 2B shows that there were 3.2% K8.1+ lytic cells after 24 hour of treatment with WP1066 compared to 1.5% in VPA-treated cells.

As expected, treatment of cells with WP1066 resulted in lower levels of STAT3 in the majority of cells (Fig.2B, right-hand dotplot compared to the 2 dotplots on the left). Also, nearly all lytic cells arose from cells expressing low levels of STAT3 whereas cells with higher levels of STAT3 remained strictly refractory. To further investigate if lytic activation secondary to STAT3 impairment results in the production and release of infectious particles, we harvested cell culture supernatants 48 hours after treatment of cells with VPA or WP1066. Exposure of HUVECs to culture supernatants from VPA- and WP1066-treated cells resulted in greater numbers of Latency Associated Nuclear Antigen (LANA)-1 positive HUVECs compared to exposure to supernatants from un-treated cells (Fig.2C). These data indicate that functionally inhibiting STAT3 enhances productive lytic activation in KSHV-infected B cells.
STAT3 regulates KSHV lytic activation. Since chemical inhibitors are often associated with off-target effects, we used previously validated siRNAs (22, 30) to specifically inhibit STAT3. Figure 3A shows that compared to scrambled siRNA-treated cells, introduction of 2 different siRNAs targeting STAT3 into latently infected cells resulted in significant increases in mRNA levels from ORF50, ORF59, ORF9 and ORFK8.1. Furthermore, these increases in lytic gene expression corresponded with increases in cell-associated KSHV load (Fig.3B), the percentage of K8.1+ lytically infected cells (Fig.3C; 15.8% in siSTAT3 compared to 8.3% in scrambled), and infectious virions in the cell-free supernatant (Fig.3D); treatment of siRNA-transfected cells with VPA did not cause an additional increase in lytic cells (data not shown), likely due to the combined stresses of lowering STAT3 levels and exposure to an HDAC inhibitor. Figure 3E shows that, as expected, siRNAs to STAT3 suppressed the level of STAT3 compared to scrambled siRNA treatment. Thus, suppressing the levels of STAT3 is sufficient to induce the KSHV lytic cycle.

To determine if increasing the levels of STAT3 had an opposite effect on lytic activation, i.e., restrain KSHV lytic cycle, we introduced a GFP-tagged STAT3 plasmid into latently infected cells and then induced the lytic cycle with VPA. Figure 4A shows that compared to empty vector (GFP)-transfected cells, cells transfected with STAT3-overexpression plasmid demonstrated statistically significant reductions in message levels from ORF50, ORF59, ORF9 and ORFK8.1. Overexpression of
STAT3 also suppressed KSHV load in VPA-treated cells (Fig. 4B) and the amount of infectious virions in the cell-free supernatant (Fig. 4D). To determine if STAT3 overexpression caused fewer cells to support the KSHV lytic cycle, we gated on GFP+ (i.e. transfected) cells and then enumerated K8.1-expressing cells. As shown in Figure 4C, there was a nearly 90% reduction (6.9% for GFP versus 0.7% for STAT3-GFP) in the percentage of lytic cells among those that had been successfully transfected. Also as expected, introduction of STAT3 plasmid resulted in an increase in the levels of STAT3 (Fig. 4E). Taken together, our data demonstrate that levels of cellular STAT3 regulate susceptibility of latently infected cells to KSHV lytic cycle activation.

**Cellular KAP1 links STAT3 to KSHV lytic activation.**

STAT3 is generally considered a transcription activator; yet, it represses lytic genes. When we examined a publically available STAT3 ChIP-seq dataset (22, 31), we identified cellular KAP1 (Krüppel-associated box domain-associated protein 1), a transcriptional corepressor, as a potential STAT3-transcriptional target. Importantly, KAP1 has been previously found to regulate lytic susceptibility in CMV and KSHV-infected cells (32-35); however, how KAP1 itself is regulated in virus-infected cells is not entirely clear. Introduction of the 2 siRNAs targeting STAT3 in BCBL-1 cells resulted in suppression of KAP1 transcript and protein levels (Figs. 5A and 5B), indicating that STAT3 regulates KAP1 in BCBL-1 cells. Furthermore, transfection of siRNA to KAPI resulted in increases in levels of transcripts from lytic genes belonging to all 3 kinetic classes (Fig. 5C). As expected, siRNA to KAPI caused
suppression of KAP1 protein level (Fig.5D). These results collectively implicate cellular KAP1 in STAT3-mediated regulation of KSHV-lytic susceptibility.

**Discussion**

The lytic phase of KSHV is critical to its pathogenesis; in this study, we provide evidence for STAT3 as a mechanistic link between cells that are susceptible versus those that are refractory to lytic stimuli. We make this connection between a host transcription factor and a key event in the life cycle of the virus at the single cell level. Not only do high levels of STAT3 correlate with the refractory state regardless of whether cells respond to spontaneous lytic triggers in culture or exogenously provided chemical triggers, but suppression of STAT3 makes KSHV-infected cells more permissive to signals that trigger lytic activation; this culminates in production of infectious virus. In contrast, experimental overexpression of STAT3 causes maintenance of the refractory/latency state. Importantly, we find that STAT3 functions via KAP1, a cellular transcriptional corepressor, to regulate lytic susceptibility. These findings implicate STAT3, an important cellular pro-survival and pro-proliferative protein overactive in KSHV+ tumors, in a regulatory role that limits exit of KSHV from latency.

Although KSHV lytic activation is linked to pathogenesis and often progression of KS, hemophagocytic lymphohistiocytosis, MCD and PEL (7-13), a host of disparate diseases arising from a multitude of cell types, KSHV+ B cell lines such as
BCBL-1 cells that were originally derived from clinical specimens of PEL provide the most robust tissue culture models for understanding molecular events underlying viral latency and lytic activation. Another important reason for selecting BCBL-1 cells is that they are not co-infected with EBV, thereby avoiding the confounding presence of another gammaherpesvirus while examining this mechanistic question. Indeed while most PEL are co-infected with EBV (36), the existence of EBV-negative PEL indicates that KSHV infection is sufficient for disease. Nonetheless, future studies need to address the contribution of STAT3 and KAP1 to regulation of KSHV lytic cycle in other cell lines and more importantly, in KSHV-infected tissues.

STAT3 is an important molecular node for many cellular signaling pathways and can potentially regulate expression of thousands of genes (22, 31). This study and our earlier studies demonstrate that in addition to phosphorylation and dimerization of STAT3, modulation of the protein level of STAT3 also regulates its function (22, 30, 37-39). Our data also indicate that STAT3 functions at least partly by repressing the immediate early gene product RTA, the key latency-to-lytic viral switch. While generally thought to be a transactivator, STAT3 can suppress gene expression in some instances either directly (40) or indirectly. Indeed, we find that STAT3 exploits a transcriptional corepressor to repress lytic genes. Whether the effects on transcript levels of early and late lytic genes are secondary to increase in RTA levels or due to direct repression of these genes by KAP1 remain to be determined.
KSHV LANA and cellular Nrf2 have been found to repress ORF50 by recruiting KAP1 during the early stage of KSHV primary infection and in latently-infected KSHV+ cell lines, respectively (33, 35). Also, phosphorylation of KAP1 at S824 has been linked to de-repression of lytic genes in CMV and KSHV-infected cells (32, 34).

Our results now reveal modulation of KAP1 levels by STAT3 as a mechanism to regulate KSHV lytic susceptibility. However, whether KAP1 is recruited to ORF50 (and potentially the other 3 lytic genes) by LANA, cellular Nrf2 or some other means is presently unclear.

Dysregulation of STAT3 is linked to most human cancers including KSHV+ PEL and KS (23, 41, 42). Notably, infection of endothelial cells with KSHV results in phosphorylation of STAT3 at S727 and phosphorylation of KAP1 at S473; the latter relieves STAT3 from KAP1-mediated repression (37). Furthermore, KSHV-encoded viral IL6 is able to activate STAT3 via binding to gp130, the common IL6 receptor (43); though a lytic gene product, viral IL6 could contribute to STAT3 activation and therefore increased STAT3 levels in a paracrine manner in latent cells. Our findings now indicate that apart from contributing to the inflammatory milieu and subsequent growth transformation (37, 44), this increased STAT3 also contributes to viral persistence. This exploitation of cellular STAT3 by both human oncogenic herpesviruses EBV and KSHV, to ensure maintenance of a persistent pool of latently infected cells despite presence of lytic activation triggers, appears to extend beyond the gammaherpesvirus subfamily. A recent report showed that STAT3 restricts HSV-1...
lytic activation (45). Thus, STAT3-mediated regulation of viral lytic activation may be a common theme among human herpesviruses. This knowledge could guide novel strategies to prevent lytic activation of HSV-1 and therefore prevent reactivation-associated alphaherpesvirus disease. On the other hand, increasing the number of latently infected cells undergoing lytic activation could greatly enhance the efficacy of oncolytic therapies directed against KSHV+ and EBV+ cancers.

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Authorship contributions: C.K., X.L. and S.B.-M. designed the study, C.K., A.B.-G., and X.L. carried out the experiments, C.K., X.L., and S.B.-M. analyzed data and interpreted the findings, and X.L. and S.B.-M. wrote the manuscript.

Conflict of interest disclosures: None
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Figure Legends

Figure 1. Cells expressing high levels of STAT3 protein are refractory to spontaneous and induced KSHV lytic activation. BCBL-1 cells were treated with TPA, VPA, or left untreated (U). Cells were harvested at 1, 24, and 48 hours post-treatment in A and B, and at 48 hours in C. A. RNA was isolated and subjected to qRT-PCR to determine the relative levels of lytic ORFK8.1 transcripts. Fold changes were calculated by the $\Delta \Delta C_T$ method, normalized to three housekeeping genes, GAPDH, HPRT1 and B2M ($\beta$2 microglobulin). Data are presented as mean ± SEM, and are representative of two experiments with 3 technical replicates. B. Cell lysates were immunoblotted using antibodies to K8.1 and $\beta$-actin. C. Cells were immunostained for K8.1 and STAT3, and subjected to flow cytometry. Numbers within dotplots on the left-hand side indicate percent K8.1$^{hi}$ cells under un-treated, TPA-, or VPA-treated conditions, after comparison with similarly-treated cells stained with isotype control antibody; oval gates indicate K8.1$^{lo}$ cells. Numbers within dotplots in the three middle columns indicate percent K8.1$^{lo}$ cells of sub-populations expressing different levels of STAT3, i.e., STAT3$^{hi}$ (high), STAT3$^{int}$ (intermediate) and STAT3$^{lo}$ (low) subpopulations. Numbers in dotplots on the right-hand side indicate
percent K8.1lo cells (from oval gates in left-hand side dotplots) expressing low, intermediate and high levels of STAT3.

Figure 2. Chemical inhibition of STAT3 results in KSHV lytic activation, increase in the number of lytic cells, and increase in production of infectious virions. BCBL-1 cells were treated with VPA, WP1066, VPA + WP1066 or left un-treated. Cells were harvested at 1, 6, 12 and 18 hours post-treatment in A, at 24 hours post-treatment in B, and 48 hours post-treatment in C. A. RNA was analyzed for relative transcript levels of 4 lytic genes, ORF50, ORF59, ORF9 and ORFK8.1 at the different times compared to un-treated cells, by qRT-PCR. KSHV-specific transcript levels were normalized to GAPDH, HRPT1, and B2M (β2 microglobulin) and fold changes determined by the ΔΔCT method. Data are presented as mean ± SEM and are representative of two separate experiments with 3 technical replicates. B. Cells were immunostained for K8.1 and STAT3, and subjected to flow cytometry. Numbers indicate percent K8.1+ (lytic) cells; these percentages were determined after comparison with similarly-treated cells stained with isotype control antibody. C. Cell-free supernatants were used to inoculate primary HUVECs. After 48 hours, HUVECs were fixed, permeabilized, stained with anti-LANA antibody and DAPI, and visualized at 40X magnification.

Figure 3. Knock-down of endogenous STAT3 results in increased spontaneous KSHV lytic activation. A. BCBL-1 cells were transfected with scrambled siRNA or
two different siRNAs to STAT3 [siSTAT3(1), siSTAT3(2)] and harvested 18 hours and 48 hours later for determination of relative amounts of transcripts from KSHV lytic genes ORF50, ORF59, ORF9 and ORFK8.1 by qRT-PCR after normalization to 18S rRNA using the \( \Delta \Delta C_T \) method. Error bars: SEM of 3 technical replicates from each of 2 transfection experiments. B. BCBL-1 cells were transfected with scrambled siRNA (Sc) or siRNAs to STAT3 (si-1, si-2) and harvested 48 hours later for determination of relative amounts of cell-associated KSHV DNA by qPCR. Error bars: SEM of 3 technical replicates from each of 2 transfection experiments. C. BCBL-1 cells were transfected either with STAT3 siRNA and FITC+ scrambled siRNA [siSTAT3(1)] at 3:1 ratio (the latter, to mark transfected cells), or with FITC- and FITC+ scrambled siRNA at 3:1 ratio (Scrambled). After 36 hours, the FITC+ (i.e. transfected) population was examined by flow cytometry for K8.1+ cells. Numbers indicate the percentage of transfected cells that were spontaneously lytic. A representative of two experiments is shown. D. BCBL-1 cells were treated as in B and cell-free supernatants were used to inoculate primary HUVECs. After 48 hours, HUVECs were fixed, permeabilized, stained with anti-LANA antibody and DAPI, and visualized at 40X magnification. E. BCBL-1 cells were transfected with scrambled siRNA or siRNA to STAT3, harvested at 48 hours post-transfection, and subjected to Western blot analysis using anti-STAT3 and anti-\( \beta \)-actin antibodies.

Figure 4. Overexpression of STAT3 restrains susceptibility to KSHV lytic activation. BCBL-1 cells were transfected with pEGFPN1 plasmid (GFP) or
pEGFPN1-STAT3 plasmid (STAT3-GFP), exposed to VPA after 12 hours, and harvested after another 24 hours for determination of relative amounts of transcripts from KSHV lytic genes ORF50, ORF59, ORF9 and K8.1 by qRT-PCR after normalization to 18S rRNA using the ΔΔCT method (A), for determination of relative amounts of cell-associated KSHV DNA by qPCR (B), for K8.1+ cells by flow cytometry (C), or assayed for infectious virions in cell-free supernatant by inoculation of HUVECs and staining with anti-LANA antibody and DAPI 48 hours later (D). Error bars in A and B: SEM of 3 technical replicates from each of 2 transfection experiments. Numbers in C indicate the percentage of GFP+ (i.e. transfected) cells that were lytic. Lytic (i.e. K8.1+) gates were placed based on a 1% cut-off of similarly treated cells that were stained with isotype control antibody. A representative of two experiments is shown in C. E. BCBL-1 cells were transfected with GFP plasmid or STAT3-GFP plasmid (as in A-D) and harvested at 24 hours post-transfection for Western blot analysis using anti-STAT3 and anti-β-actin antibodies.

Figure 5. Knock-down of STAT3 suppresses KAP1 and suppression of KAP1 causes increase in levels of lytic transcripts. BCBL-1 cells were transfected with scrambled siRNA (Sc) or two different siRNAs to STAT3 [STAT3(1), STAT3(2)] in A and B, scrambled siRNA (Sc) or siRNA to KAP1 in C and D, and harvested 24 hours (C, D) or 48 hours (A, B) later for determination of relative amounts of transcripts from cellular KAP1 (A) and KSHV lytic genes ORF50, ORF59, ORF9 and ORFK8.1 (C) by qRT-PCR after normalization to 18S rRNA using the ΔΔCT method or
subjected to Western blotting using anti-KAP1 and anti-β-actin antibodies (B and D).

Error bars: SEM of 3 technical replicates from each of 2 transfection experiments.

Numbers in B and D indicate relative amounts of KAP1 protein determined by
densitometry, after normalization to β-actin.
Figure 1
Figure 2

A

B

C

Un-treated

LANA

DAPI

VPA

WP1066

VPA+WP1066

WP1066

STAT3

K8.1

0.6

1.5

3.2