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# Constitutive activation of IL-13/STAT6 contributes to KSHV-associated PEL cell proliferation and survival

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ABSTRACT

Activation of the Janus Kinase (JAK) / signal transducer and activator of transcription (STAT) signaling pathway has been associated with numerous human malignancies, including primary effusion lymphomas (PELs). PEL, a cancerous proliferation of B-cells, is caused by the Kaposi's sarcoma-associated herpesvirus (KSHV). Previously we identified constitutive phosphorylation of STAT6 on tyrosine 641 (p-STAT6$^{c}$) in PEL cell lines BC3 and BCBL1, however, the molecular mechanism leading to this activation remains unclear. Here we demonstrate that STAT6 activation tightly correlates with interleukin-13 (IL-13) secretion, JAK1/2 tyrosine phosphorylation, and reduced expression of SHP1 due to KSHV infection. Moreover, p-STAT6$^{c}$ and reduction of SHP1 were also observed in KS patient tissue. Notably, blockade of IL-13 by antibody neutralization dramatically inhibits PEL cell proliferation and survival. Taken together, these results suggest that IL-13/STAT6 signaling is modulated by KSHV to promote host cell proliferation and viral pathogenesis.

**Key Words**: KSHV, IL-13, STAT6 phosphorylation, PEL

31 IMPORTANCE

32 STAT6 is a member of signal transducer and activator of transcription (STAT) family,
33 whose activation is linked to KSHV-associated cancers. The mechanism through
34 which STAT6 is modulated by KSHV remains unclear. In this study, we demonstrate
35 that constitutive activation of STAT6 in KSHV-associated PEL cells, results from
36 interleukin-13 (IL-13) secretion and reduced expression of SHP1. Importantly, we also
37 found that depletion of IL-13 reduces PEL cell growth and survival. This discovery
38 provides a new insight that IL-13/STAT6 plays an essential role in KSHV
39 pathogenesis.

## INTRODUCTION

Cytokines play a critical role in many viral infections. Viruses not only manipulate host cytokine production to favor virus survival, replication and infection, but also help virus-infected cells to modulate host immune response, which potentially results in the development of viral persistent infection, pathogenesis or tumorigenesis (1). Kaposi's sarcoma-associated herpesvirus (KSHV), also named human herpesvirus 8 (HHV-8), is an oncogenic γ-herpes virus that associates with several aggressive malignancies including AIDS-related Kaposi's sarcoma (KS) (2), primary effusion lymphoma (PEL) (3) and multicentric castleman's disease (MCD) (4). Increasing evidences have suggested that KSHV also deregulates an array of host cytokines including: IL-6, IL-8, and IL-1α thereby inducing cell proliferation, malignant transformation (5-8).

Signal transducer and activator of transcription (STAT) proteins are a family of cytoplasmic transcription factors involved in cytokine signal transduction. STAT6, is a key member of STAT family, whose role in the biology of cancer and immune cells has been firmly established (9, 10). STAT6 is activated by cytokine IL-4 or IL-13, via a common receptor chain, namely IL-4Rα. Upon interleukin binding, IL-4Rα dimerizes with IL-4Rγ or IL-13Rα1 to form Type I or Type II IL-4R receptor, respectively. The dimerized receptor recruits and activates phosphorylation of Janus tyrosine kinases (JAK), including JAK1 and JAK2, which in turn phosphorylates tyrosine residues on IL-4R, providing a docking site for the recruitment of STAT6. STAT6 itself becomes phosphorylated at its conserved tyrosine residue Y641 (11), and subsequently translocates into nucleus, where it regulates downstream gene expression through

binding to distinct consensus $TTCN_{3/4}GAA$ regions within the gene promoter (12, 13). To date, at least 35 genes in physiological and pathophysiological processes are activated by STAT6 (12). Regulation of STAT6 signaling is governed by a variety of inhibitory signals including: SOCS-1 (suppressor of cytokine signaling-1), and SHP-1 (SH2-containing phosphatase-1). These proteins suppress IL-4/STAT6 and block STAT6 activation by dephosphorylating activated JAK, respectively (14).

Of significant importance, is the identification of constitutive STAT6 activation in a number of human malignancies (9) including: prostate carcinomas (15), and Hodgkin lymphoma (16). Mechanistically, STAT6 is constitutively activated in primary mediastinal large B-cell lymphomas due to amplification of the JAK2 (13), while in hepatocellular carcinoma, gastric carcinoma, colorectal cancer and hematological malignancies, STAT6 activation results from promoter hypermethylation and silencing of SHP1 or SOCS-1(17-20). Interestingly, in virus-associated diseases, constitutive STAT6 activation occurs through different pathways (21-23). In KSHV-associated cancers, we and other colleagues recently found that IL-4-mediated STAT6 activation is tightly regulated by the virus in order to switch lifecycles from latency to lytic replication (24, 25). These observations strongly suggest that STAT6 may play a role in KSHV-induced oncogenesis.

However, the molecular mechanism leading to constitutive STAT6 activation in PELs remains unclear. In an attempt to better understand the role of constitutively phosphorylated STAT6 in KSHV pathogenesis, we explored the expression pattern of STAT6-related molecules in KSHV-positive and negative B lymphoma cells. In this report, we demonstrate that constitutive activation of STAT6 correlates with IL-13 secretion and JAK1/JAK2 phosphorylation due to down-regulation of SHP1. Furthermore, blockade of IL-13 by antibody neutralization dramatically inhibits PEL



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cell proliferation and survival. These findings provide a new insight into how KSHV usurps STAT6 signaling pathway to promote host cell proliferation and viral pathogenesis.

## MATERIALS AND METHODS

***Cell culture and transfection —*** KSHV/EBV-negative (BJAB and DG75) and KSHV-positive (BC1, BC3, BCP1, BCBL1, and JSC1) B-lymphoma cells, EBV-transformed B cell line LCL1, KSHV-infected BJAB with low passage (K-BJAB$^{Low}$), iSLK and iSLK-Bac16 (K-iSLK, a gift from SJ Gao at University of South California) cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Gibco-BRL). All cell lines were incubated at 37°C in a humidified environmental incubator with 5% $CO_2$. B-cells transfection was performed with Lonza-4D nucleofector$^{TM}$ system in an optimized program CA137.

***Antibodies —*** SOCS1, SHP1, SHP2, and Akt (C-20) antibodies were purchased from Santa Cruz Biotech. Inc.. Rat antibodies to IL-4, IL-13, and κ isotype IgG1 were purchased from BioLegend. Antibodies to β-actin, JAK1 (6G4), JAK2 (D2E12), STAT6, p-STAT6 (Tyr-641) and Phospho-Akt (pAkt, Ser473, 587F11) were from Cell signaling Technology (Beverly, MA). Antibodies to IL-4R (clone 25463, R&D), phosphor-Tyrosine (clone 4G10, Millipore), GAPDH (G8140-01, US Biological) and KAP1 (20C1, Abcam) were used according to the manufacturers specifications.

***Immunoprecipitation and immunoblotting —*** Cells were harvested, washed with ice-cold PBS, and lysed in ice-cold RIPA buffer [10 mM Tris-HCl (pH 7.5). 1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA with protease inhibitors]. Cell lysates were individually subjected to immunoprecipitation (IP) and immunoblotting (IB) or directly

immunoblotting with specific antibodies as described previously (26).

***Virion Purification and Primary infection of KSHV or EBV*** — HEK293-Bac36 (KSHV-green fluorescent protein [GFP]) cells or HEK293-GFP-EBV cells were individually induced with 20 ng/ml of tetradecanoyl phorbol acetate (TPA) and 1.5 mM sodium butyrate (Sigma-Aldrich, St. Louis, MO) for 2 days at 37°C with 5% $CO_2$. After induction, the supernatant of culture medium was collected and filtered through a 0.45μm filter, and viral particles were spun down at 25,000 rpm for 2h at 4°C. The concentrated virus was collected and used for primary infection as described previously (27). The infection was evaluated by interrogating the expression of viral antigen (LANA for KSHV, EBNA1 for EBV) in addition to visualizing GFP expression using fluorescence microscopy.

***Inhibition of Tyrosine Phosphorylation by chemical reagents*** — The kinase inhibitor LY294002, PD98059, and AG490 from Upstate Biotechnology reconstituted in dimethyl sulfoxide (DMSO). Cell lines were seeded at 0.4 to 0.6 $\times 10^5$ cells/mL and treated for 24 hours with different dose of LY294002, PD98059 or AG490. Control cells were treated with equal volumes of DMSO. After treatment, cells were washed in PBS and lysed as described in immunoblotting.

***Flow cytometry of cell cycle*** — Treated cells were harvested, washed in ice-cold PBS, and fixed in cold methanol/acetone, followed by stained with PBS containing propidium iodide (PI) and RNase A as described previously (27). Cell cycle profiles of stained cells were analyzed using FACScan (BD Biosciences, Foster, CA) and FlowJo software.

***Nuclear Acid Extract*** — Genomic DNA from B lymphoma cells was extracted by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. Total



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RNA from cells was extracted by using Trizol, and cDNA was made with a Superscript II reverse transcription kit (Invitrogen, Inc., Carlsbad, CA).

***Quantitative PCR*** — The primers used for PCR are shown in Table 1. The standard real-time PCR protocol was followed as described previously (27). A melting curve analysis was performed to verify the specificities of the amplified products. Some PCR products were subjected to DNA sequencing for sequence analysis.

***Chromatin immunoprecipitation*** — The chromatin immunoprecipitation (ChIP) experiments were done as previously described (27). Briefly, aliquots were incubated with each specific antibody or IgG control overnight at 4°C. Immune complexes were separated into bound and unbound complexes with Protein A-agarose and cross-links were reversed by treatment at 65°C overnight. After treatment with RNase A and proteinase K, samples were extracted once with phenol/chloroform, and the DNA was precipitated and resuspended. The ChIP DNA and 10% Input were amplified by qPCR using specific primers.

***Immunohistochmistry*** — Immunohistochemistry for p-STAT6 and SHP1 were performed on deparaffinized, formalin-fixed tissue sections using an indirect immunoperoxidase method with an automated immunostainer. 3μm paraffin-embedded KS patient tissue was provided from public health clinical center of Fudan University. Usage of redundant cancer sample for research purpose was recognized by the hospital according to Medical Ethics. Briefly, after deparaffination in xylene for 10min three times and rehydration, antigen was retrieved with EDTA ( pH8.0 ) for 10 min at 95°C. Slides were treated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Subsequently, the appropriate primary antibody of LANA, SHP1 or p-STAT6 was added and incubated overnight at 4°C. Next day, after extensively

wash with PBS, the goat anti-mouse/rabbit second antibody IgG conjugated with Horseradish Peroxidase (Kit PV8000, ZSGB-BIO Co., Beijing) was added and incubated for 30min at 25°C. DAB (3,3' diaminobenzidine) was added to stain the slide at room template and counterstained by hematoxylin.

***Statistical analysis*** — Statistical significance of differences between means of at least n=3 experiments was determined using Student's *t*-test.

## RESULTS

### KSHV induces constitutive phosphorylation of STAT6 (p-STAT6$^{C}$)

To answer whether the constitutive activation of STAT6 on Y-641 (p-STAT6$^{c}$) in both BC3 and BCBL1 cell lines, which we observed previously (24), is associated with KSHV infection, we detected levels of p-STAT6$^{C}$ in more KSHV-positive (BC1, BC3, BCP1, BCBL1, JSC1) and negative (DG75, BJAB, iSLK, LCL1) cell lines, including the same genotype cells with and without KSHV infection (K-BJAB$^{Low}$; K-iSLK). Interestingly, the results of immunoblotting against p-STAT6$^{c}$ show that although p-STAT6$^{C}$ is not highly activated in all KSHV-positive cell lines, the presence of KSHV in both BJAB and iSLK cell lines does induce p-STAT6$^{C}$ (1.8-fold and 2.4-fold increase, respectively) to some extent (Figure 1A). In contrast, LCL1 cells with EBV infection alone did not present p-STAT6$^{C}$.

To address whether activation of JAK kinases or other kinases are important for the constitutive activation of p-STAT6$^{c}$, we individually treated BC3 and BCBL1 cells with the JAK inhibitor AG490, PI3K inhibitor LY294002 or MEK inhibitor PD98059, followed by immunoblotting assays against p-STAT6$^{c}$ and phosphorylated Akt. As shown in Figure 1B, the results indicate that JAK but not PI3K or MEK kinases

contribute to the constitutive activation of STAT6. Consistent with previous reports that JAK is able to phosphorylate PI3K, which in turn phosphorylates Akt, we observed reduction of Akt phosphorylation at serine 473 following AG490-induced JAK inhibition. Conversely, PI3K inhibitor only blocks phosphorylation of Akt instead of STAT6 (Figure 1B). These findings indicate that constitutive activation of STAT6 is due to the cascade phosphorylation of JAK.

To further validate our findings, we looked levels of JAK1/2 and the phosphorylated JAK1/2 in the KSHV positive and negative cell lines by immune-precipitation and immunoblotting assays. While there was no significant difference of native JAK1 or JAK2 expression in KSHV positive and negative cells, a higher level of phosphorylated JAK1 or JAK2 was consistently observed in all the KSHV-positive cell lines (except for BC1, the absence of p-JAK could be due to lower expression of IL-4Rα as observed below) including EBV-negative BJAB cells with KSHV infection than that in the KSHV-negative cell lines (Figure 1C). Intriguingly, we also observed that an activated form of the IL-4Rα receptor (a key upstream regulator of JAK) was consistently elevated in all KSHV positive cell lines (Figure 1C, low panel), which further supports our hypothesis that constitutive p-STAT6 in PEL cells stems from activation of JAK and is due to KSHV infection.

**Low expression of SHP1 in PEL cells associates with p-STAT6[C]**

To further elucidate pathways changes contributing to constitutive phosphory-lation of STAT6 in PEL cells, we performed immunoblotting assay to detect the protein levels of three key negative regulators — SOCS1, SHP1 and SHP2. As shown in Figure 2A, similar to the pattern of phosphorylated JAK1 and JAK2 were observed in the KSHV-positive and negative cell lines, lower expression of SHP1 but

not SHP2 or SOCS1 was also consistently observed in KSHV-positive cells, particularly about 2.6-fold and 2.1-fold decrease in BJAB and iSLK cells after KSHV infection, respectively (Figure 2A, right panel). This leads to our speculation that the absence of p-STAT6$^{c}$ (Figure 1A) in the KSHV and EBV-positive cell lines including BC1 and JSC1 could be specimen-specific, resulting from, co-infection of EBV may directly block the constitutive phosphorylation of STAT6 induced by KSHV.

To exclude the possibility that the reduced expression of SHP1 was due to mutation or a deletion within its promoter instead of KSHV infection, we analyzed the transcriptional level and promoter sequences of SHP1 along with SHP2, SOCS1 and SOCS3 as control in the KSHV-positive and negative cells. Surprisingly, the sequence analysis revealed that no recurrent mutations or deletions up to 1kb upstream region of SHP1 promoter occurred in the KSHV-positive cells, although a site mutation at -307 position of SHP1 promoter from BC3 and JSC1 cells was observed (Figure 2B). In contrast, no mutation within SHP2, SOCS1 or SOCS3 promoter was observed (data not shown). Unexpectedly, the results of analysis of transcriptional levels of SHP1 and SHP2 showed that there was no significant difference in SHP1 or SHP2 expression in KSHV positive versus negative cells (Figure 2C). This finding supports the notion that constitutively activated STAT6 in PEL cell lines is associated with KSHV-mediated reduced SHP1 expression at the protein, rather than transcriptional, level.

**Activation of p-STAT6$^{C}$ is induced by KSHV in PEL cells**

Our previous study showed that inhibition of KAP1 results in reduction of KSHV episome copy number and viral protein expression in PEL cells (27). To prove that constitutively activated STAT6 is due to KSHV in PEL cells, we compared the p-

232 STAT6$^{C}$ levels in the BC3 cells with or without loss of KSHV induced by KAP1
233 knockdown. In agreement with our hypothesis, our results demonstrate that KAP1
234 knockdown mediates loss of KSHV associates with subsequent reduction of p-
235 STAT6$^{C}$ (Figure 3A, compare lane 1, 4 with lane 2, 5, respectively). Moreover, p-
236 STAT6$^{C}$ was only partially rescued when endogenous level of knocked-down KAP1
237 supplemented by transiently expression of exogenous KAP1 (Figure 3A, compare
238 lane 2, 5 with lane 3, 6, respectively). The reduced expression of SHP1 was only
239 reversed by loss of KSHV but not introduction of exogenous KAP1 (Figure 3A,
240 compare lane 2, 5 with lane 3, 6, respectively), further supporting the direct link
241 between KSHV and p-STAT6$^{C}$ activation in PEL cells.

242 Previously studies showed that KSHV primary infection usually contains mixture
243 of latent and lytic replication status within 7 days, and quickly shuts off lytic replication
244 at about 3 days post-infection and establishes dominantly latency at about 5 days
245 post-infection (26, 28-30). To further confirm that phosphorylated STAT6 is regulated
246 by KSHV infection, we performed primary infection of human peripheral blood
247 mononuclear cells, followed by immunoblotting analysis against STAT6 and
248 phosphorylated STAT6. KSHV dramatically induced phosphorylation of STAT6 within
249 5 days following infection, however the phosphorylated STAT6 was relatively lower on
250 day 5 (Figure 3B). In KS patient tissues, we also observed moderately higher levels
251 of phosphorylated STAT6 and lower expression of SHP1 when compared that in the
252 normal tissue (Figure 4 A and B).

253 Given p-STAT6$^{C}$ in both BC3 and BCBL1 cells – infected with KSHV alone - but
254 not in JSC1 or BC1 cells infected with both KSHV and EBV, we speculate that the
255 discrepancy in p-STAT6$^{C}$ stems from EBV infection. To prove our hypothesis, BC3
256 and BCBL1 cells were individually infected with EBV virus carrying a GFP-

fluorescence *in vitro* followed by immunoblotting against p-STAT6$^{C}$, STAT6 and SHP1. We observed a reduction in levels of p-STAT6$^{C}$ in BC3 cells after EBV infection, although SHP1 expression was not significantly affected (Figure 5A and B, similar data from BCBL1 cells not shown). Consistent with the previous studies (31), we also could observe that EBV rapidly induce both latent and lytic genes expression during early infection, and maintain relatively higher expression of latent genes to establish dominantly latency which similar to LCL at 21 days post-infection (Figure 5C). Therefore, our results suggest that co-infection of EBV reduces KSHV-induced p-STAT6$^{C}$ activation.

**IL-13 is essential for p-STAT6$^{C}$ activation and proliferation of PEL cells**

Since phosphorylation of STAT6 signaling is primarily activated through IL-4 or IL-13 cytokine, to determine if these two cytokines contribute to p-STAT6$^{c}$, we performed the immunoblotting analysis for these interleukins in KSHV-positive and negative cells. Strikingly, our results demonstrate that IL-13 not IL-4 was highly expressed and correlates with p-STAT6$^{c}$ in the BC3 and BCBL1 cells, as well as BJAB and iSLK with KSHV infection (Figure 6A and B). To further confirm these findings, we performed *in vitro* antibody neutralization against human IL-13 or IL-4 in culture media and monitored phosphorylation level of STAT6. Neutralization of IL-13 in BC3 and BCBL1 strongly blocked p-STAT6$^{c}$ activation, whereas no significant changes were following IL-4 neutralization (Figure 6C). Altogether, these findings imply that constitutive activation of STAT6 by KSHV in PEL cells is driven by IL-13 expression and secretion, along with low expression of SHP1 and phosphorylated JAK status.

To explore whether mutations of IL-13 promoter leads to high expression of IL-13

in both BC3 and BCBL1 cells, we amplified and sequenced DNA isolated from KSHV-positive (BC3, BCBL1, and JSC1) and negative (BJAB) B lymphoma cells. While several mutations within IL-13 promoter were detected, there was no correlation between the mutation observed and IL-13 expression (Figure 6D). To elucidate why expression of IL-13 and not IL-4 was associated with p-STAT6 activation, we hypothesized that p-STAT6$^{c}$ may selectively bind to IL-13 promoter through specific binding sites. To evaluate our hypothesis, we analyzed the promoter sequences of both IL-13 and IL-4, and identified four potential STAT6-binding sites located at the IL-13 promoter but only one site at the IL-4 promoter (Figure 6D). We validated this finding using chromatin-immunoprecipitation (ChIP) assays, which demonstrated increased affinity of p-STAT6 antibody towards the IL-13 promoter (Figure 6E).

To determine the biological consequences of IL-13-mediated STAT6 activation, we evaluated the effect of IL-13 or IL-4 neutralization along with non-specific antibody control on the proliferation of KSHV positive PEL cells (BC3 and BCBL1) as well as BJAB and BJAB with KSHV infection (K-BJAB$^{Low}$). The growth rate of PEL cells and K-BJAB$^{Low}$ (but not BJAB) cells following IL-13 but not IL-4 neutralization was significantly less ($p$<0.05) than that in response to non-specific IgG isotype or untreated control, as shown in Figure 7A. The inhibitory effect of IL-13 neutralization on PEL cell proliferation causes a moderate increase in cell apoptosis (30.2% vs 38.8%), although this effect was further enhanced (38.8% vs 61.5%) following cell starvation (Figure 7B). Taken together, these results indicate that IL-13 is essential for activation of STAT6 and PEL cell proliferation.

## DISCUSSION

JAK-STAT is one of the most important pathways induced by cytokines. Upon

ligand binding to its cognate receptor, receptor-associated JAKs are phosphorylated and in turn successively activates STAT (32). It has been reported that constitutive activation of STAT6 is widely associated with tumor development, including virus-associated cancers (13, 15, 16, 33, 34). KSHV, a recently discovered oncogenic herpesvirus, has been shown to escape host immune surveillance through modulating the JAK-STAT pathway (35, 36). However, the specific molecular mechanism through which signal pathway modulation remains largely uncharacterized. Our previous studies showed that KSHV blocks IL-4-mediated STAT6 activation (24). In the present investigation, we demonstrated that STAT6 was constitutively activated (p-STAT6$^{c}$) in KSHV-associated PEL cells to some extent. Moreover, the moderately activation of p-STAT6$^{c}$ was tightly associated with IL-13 autocrine secretion and reduced expression of SHP1 caused by KSHV infection. Together, these pathway changes lead to PEL cell proliferation and survival (Figure 8). Consistent with previous reports that STAT6-associated IL-4 receptor is highly expressed in KS tissues (37, 38). We also found that IL-4 receptor was highly expressed in all PEL cells. These findings indicate that constitutive activation of STAT6 is a common feature in KSHV-associated cancers, although the level of p-STAT6$^{c}$ is relatively lower than that stimulated by IL-4 cytokine.

Recent publications have shown that cytokine induction of a Th2-cell immune response is one of the mechanisms that inhibiting an anti-tumor immune response. Among Th2 cytokines, IL-4 has been documented as the most critical cytokine to for Th2-induction. However, another related cytokine, IL-13, has also been identified as a critical mediator of anti-tumor immunity (39). The phosphorylation of STAT6 is primary activated by cytokine IL-4 and IL-13. To decipher how STAT6 is constitutively activated in different tumors, several groups have shown that IL-13 is frequently

expressed and antibody neutralization of IL-13 result in a dramatic decrease of phosphorylated STAT6 (15, 40). In this study, we also demonstrate that constitutively activation of STAT6 is induced by highly expressed IL-13 in PEL cells independent of IL-4, moreover, treatment of PEL cells with antibodies against IL-13 diminished phosphorylated STAT6. In addition, these results suggest that neutralization with IL-13 antibodies results in reduction of phosphorylated STAT6 and blocks the cell proliferation and survival of PEL cells. This work therefore supports the hypothesis that activated STAT6 could accelerate cell proliferation by down-regulating cyclin-dependent kinase inhibitor p27 (9).

Despite IL-13's role in the regulation of STAT6 activation, functional dysregulation of its upstream activator or negative regulator may also contribute to constitutively active STAT6. In MedB-1 cells derived from primary mediastinal large B-cell lymphoma, phosphorylated STAT6 was a result of a mutation of negative regulator SOCS1, an upstream activator of JAK2 (34, 41). In KSHV-associated PEL cells, we found that the negative regulator SHP1 is significantly down-regulated in PEL cell lines when compared to controls. In contrast, no difference of SOCS1 and SHP2 protein expression was observed in these cells. This implies that inhibition of SHP1 may contribute to the activation of STAT6 in PEL cells. Alternatively, increased STAT6 phosphorylation has been associated with promoter hypermethylation of SHP1 or SOCS-1, as reported in many cancers (42). To further determine hyper-methylation or promoter mutation of negative regulator resulted in the activated STAT6 in PEL cells, we amplified and sequenced SOCS1, SOCS3, SHP1 or SHP2 promoters, as well as treated PEL cells with de-methylation reagent in different concentration and analyzed the transcriptional profile of SOCS1, SOCS3, SHP1 or SHP2. Our results demonstrate that de-methylation did not affect gene expression,

and that no promoter mutation with consistent impact on gene expression was identified (data no shown).

It has been reported that virus could directly or indirectly activate STAT6 upon infection. For instances, Herpesvirus saimiri (HVS)-encoded Tip (Tyrosine kinase-interacting protein) could interact with STAT6 and induce phosphorylation in T cell (22); SHP1 expression was selectively down-regulated in HTLV-1 transformed T-cell lines and Tax transactivates IL-13 overexpression (43, 44); and EBV Zta protein is able to induce the expression of IL-13 and promote the proliferation of EBV-infected B cells (45). To address whether KSHV infection triggered constitutive activation of STAT6 in PEL cells, we down-regulated KSHV episome by knocking down KAP1 followed by determined the level of phosphorylated STAT6. The results confirmed our hypothesis and showed that loss of KSHV due to KAP1 knockdown reduced STAT6 phosphorylation while rescuing KAP1 expression via transfection only partially recovered the levels of phosphorylated STAT6. We verified these findings by infecting PBMCs with KSHV. Interestingly, due to the facts that dynamic status of latency and lytic replication at different stages during primary infection (26, 28-30), STAT6 phosphorylation was up-regulated at the early stages following infection and subsequently down-regulated, suggesting that higher level phosphorylated STAT6 could be required for establishment but not maintenance of KSHV latency. Consistent with our and other colleague's previous works (24, 25), the robust activation of STAT6 at early stage during KSHV primary infection could contribute to viral lytic replication, and gradually reduced to a lower extent (probably due to shut off potent IL-4 induction and remain mild IL-13 induction as we observed so far) along latent establishment. Altogether, these results highlight a critical role for constitutive activation of STAT6 in PEL cells following KSHV infection.

Recent studies have shown that co-infection of two pathogens could compete for cytokine-mediated regulation of STAT6 (25). In this study, although we did not see any phosphorylated STAT6 in JSC1 cells with both KSHV and EBV infection, immunoblotting results showed a decrease in SHP1 in JSC1 which is consistent with KSHV-positive PEL cell lines BC3 and BCBL1. Moreover, infection of BC3 or BCBL1 with EBV *in vitro* reduced phosphorylated STAT6. These results imply that EBV co-infection could alter KSHV-induced STAT6 activation, and the EBV/KSHV dual positive PEL cells may have different regulatory pathway to bypass the effect of IL-13/STAT6. However, details of this mechanism require a more thorough investigation.

In summary, we demonstrated that STAT6 is constitutively activated in PEL cells due to secretion of IL-13, and KSHV-mediated down-regulation of SHP1. This indicates that constitutively activation of IL-13/STAT6 signaling is another mechanism utilized by KSHV to promote pathogenesis and tumorigenesis during latency infection, and STAT6 could be a candidate target for tumor therapy.

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538 TABLE

539 **Table 1**. Primers used for PCR

| Target | Primer sequence | Product (bp) |
|---|---|---|
| SHP1 | Sense: 5'- GTCGGAGTACGGGAACATCACC -3'<br>Antisense : 5'-CCCAGGGATTTATTTACAAGAGGAG -3' | 387 |
| SHP2 | Sense : 5'- ATGAGGAGACACGGGTAGGACT -3'<br>Antisense : 5'- GCTATGTGTGAAAGTTGATCCC -3' | 303 |
| SOCS1 | Sense : 5'-GACTGCTAGCCATGGTAGCACACAACCAGGTGGCAG -3'<br>Antisense : 5'- AGTCCTCGAGTCAAATCTGGAAGGGGAAGGAGCTC -3' | 640 |
| SOCS3 | Sense : 5'- GACTGCTAGCCATGGTCACCCACAGCAAGTTTCCC -3'<br>Antisense : 5'- AGTCCTCGAGTTAAAGCGGGGCATCGTACTGGTC -3' | 687 |
| GAPDH | Sense : 5'- TGCACCACCAACTGCTTAG -3'<br>Antisense : 5'- GATGCAGGGATGATGTTC -3' | 190 |
| IL-4 | Sense : 5'-ACCTCCCAACTGCTTCCC -3'<br>Antisense : 5'- GCTGCTTGTGCCTGTGGA -3' | 298 |
| IL-13 | Sense : 5'- GTGGACCCAGGGATGACA- 3'<br>Antisense: 5'- CTCCTGGTGTCCACTGCT- 3' | 297 |
| SHP1p | Sense : 5'- ATA*GGTACC*TTGGTTTGGCGGTGTTGATGTTT -3'<br>Antisense : 5'- AT*AAGCTT*GGGAATGAGGAGGTGCAGCTAGTCT -3' | 1132 |
| IL-13p | Sense : 5'- CCGTTACATAAGGCCACCCCC -3'<br>Antisense : 5'- TCCAGTGTCGCATAAAGGAAAGAGTT -3' | 1596 |
| IL-4-ChIP | Sense : 5'- GGCCTCTCCCTTCTATGCAAA -3'<br>Antisense : 5'- GGGCCAATCAGCACCTCTCT -3' | 211 |
| IL-13-ChIP | Sense : 5'- GCCCTCCACAGCACTCATTC -3'<br>Antisense : 5'- GTGGCTGGAAGTAGTGTGCAC -3' | 237 |

540 Note: primers for EBV latent and lytic genes see reference (31).
541

542 FIGURE LEGENDS

543 **Figure 1**. KSHV induces constitutively phosphorylation of STAT6 (p-STAT6$^{c}$). (**A**) The
544 relative level of p-STAT6$^{c}$/STAT6 in the KSHV-positive (BC1, BC3, BCP1, BCBL1,
545 JSC1, K-BJAB$^{Low}$, K-iSLK) and KSHV/EBV-negative (DG75, BJAB, LCL1, iSLK) cell
546 lines. (**B**) p-STAT6$^{c}$ is blocked by JAK inhibitor in PEL cells. The representative of
547 immunoblotting data of treated BC3 cells is shown in lower panel. (**C**) The levels of
548 IL-4R$\alpha$ expression and phosphorylated JAK (p-JAK1 and p-JAK2) are elevated in the
549 KSHV-infected cells. The relative protein level is quantitated in the histogram (low
550 panel). RD, relative density.

551 **Figure 2**. KSHV down-regulates SHP1 expression. (**A**) Low expression of SHP1 in
552 PEL cells. Cell lysates from equal amount of KSHV-positive and negative cells were
553 subjected to immunoblotting analysis with antibodies as indicated in the figure.
554 GAPDH was used as internal control. (**B**) Analysis of SHP1 promoter sequence from
555 B lymphoma and PEL cells. (**C**) Transcriptional level of SHP1 and SHP2 in the KSHV-
556 positive and negative cells. Total RNA was extracted from cultured cells for
557 quantitative PCR analysis for SHP1 and SHP2. GAPDH was used as internal control.
558 RD, relative density.

559 **Figure 3**. p-STAT6$^{c}$ is associated with SHP1 down-regulation by KSHV infection. (**A**)
560 Loss of KSHV episome due to inhibition of KAP1 reduced the level of p-STAT6$^{c}$. Cell
561 lysates from BC3 cells (clone 1 and 2) with constitutively knockdown of KAP1
562 (shKAP1), luciferase control (shKAP1) or supplement with exogenous KAP1 with
563 FLAG tag were subjected to immunoblotting as indicated. The relative density (RD) of
564 p-STAT6$^{c}$ was quantitated and shown in the middle panel. (**B**) Immunoblotting
565 analysis. Whole cell lysate of human PBMC cells with GFP-KSHV infection for 3 or 5

days or mock (uninfected), were subjected to immunoblotting with antibodies against p-STAT6$^{c}$, STAT6, and GAPDH. The relative density of p-STAT6$^{c}$/STAT6 and LANA is individually quantitated, and relative trend line is predicted and shown at the bottom panel.

**Figure 4**. Expression levels of p-STAT6$^{c}$ and SHP1 in KS patient tissues. (**A**) Representative images of Immunohistochemistry assays of KS patient tissue and normal skin tissue against p-STAT6$^{c}$, SHP1 and LANA. The larger magnification (x400) of image is shown at the lower panels. (**B**) The relative intensity of p-STAT6$^{c}$ and SHP1 in KS (n=6) and normal skin tissue (n=3) was individually quantitated by nuclear and cytoplasmic staining (double positive percentage: +,10-20%; +/-, 1-10%; -, <1%) of 100 cells.

**Figure 5**. EBV infection reduces p-STAT6$^{C}$ expression in PEL cells. (**A**) Cell lysate from BC3 cells infected with GFP-EBV for 2, 7 or 21 days or mock were subjected to immunoblotting as indicated in figure. (**B**) The relative ratio of p-STAT6$^{C}$/STAT6 along EBV infection. The results were presented by the average relative fold compared with mock from 3 independent experiments. A representative image of BC3 cells with GFP-EBV infection at each time point was shown on the top panel. (**C**) Latent (EBNA1, EBNA2 and LMP1) and lytic (BZLF1, BALF5 and BcLF1) gene expression during GFP-EBV infection. The relative mRNA levels of each genes including GAPDH as control were examined by quantitative PCR. The fold change was calculated by the $\Delta\Delta$Ct method.

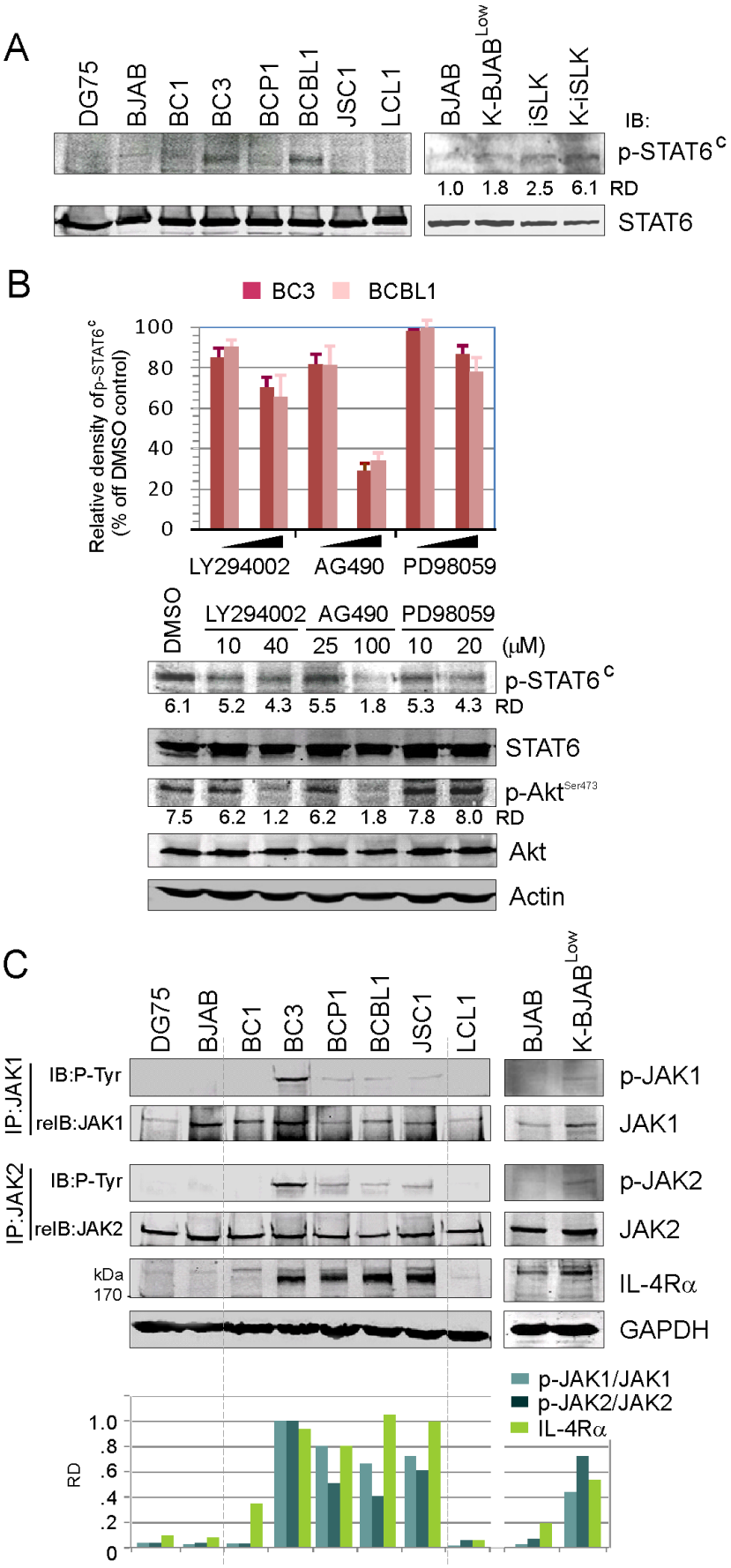
**Figure 6**. IL-13 expression is correlated with p-STAT6$^{c}$ in PEL cells with KSHV infection alone. (**A**) Equal amount of KSHV-positive and negative B lymphoma cells were subjected to immunoblotting analysis with antibodies as indicated in the figure.

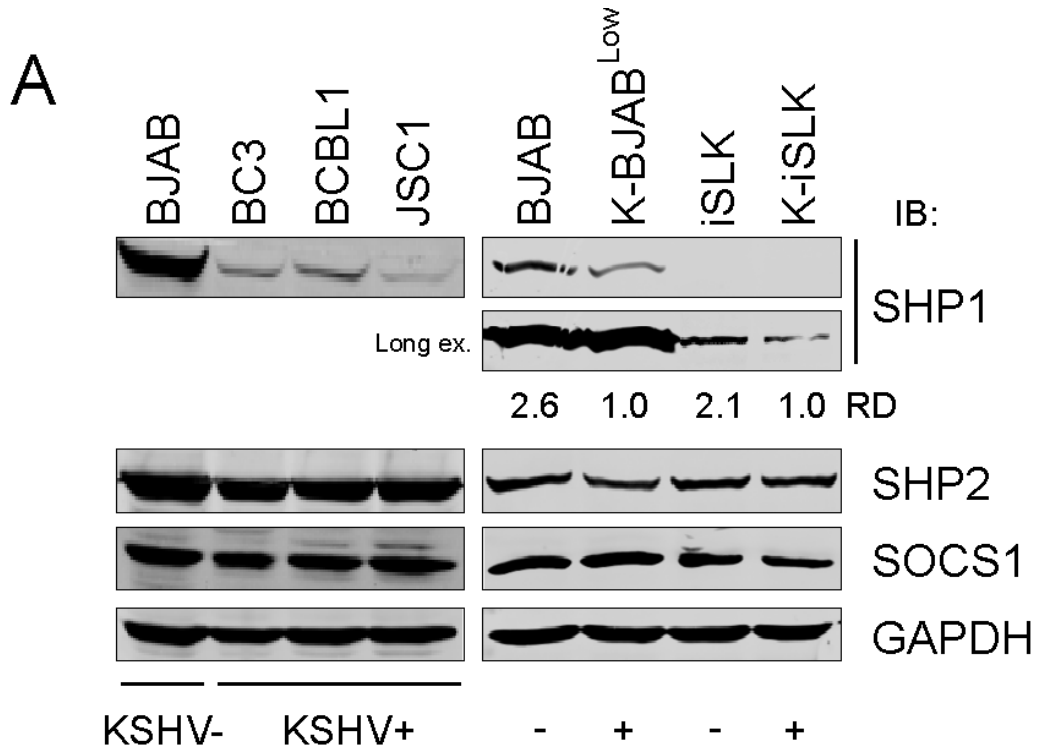
(**B**) The transcription level of IL-13 not IL-4 is upregulated by KSHV. (**C**) Cytokine IL-13 not IL-4 depletion reduces the level of p-STAT6$^{c}$. PEL cells were individually incubated with or without antibodies against IL-4, IL-13 or same isotype IgG control for 12 hrs, followed by immunoblotting analysis as indicated in the figure. (**D**) The putative STAT6-binding sites within IL-13 and IL-4 promoters were indicated on the top panel. DNA sequencing reveals four hot mutation spots within IL-13 promoter from PEL cells. nd, not determined. (**E**) p-STAT6$^{c}$ has higher affinity with IL-13 promoter than IL-4 promoter. Chromatin immunoprecipitation (ChIP) with p-STAT6$^{c}$ from BC3 cells was performed, and the relative density (RD) of p-STAT6$^{c}$ bound to IL-13 or IL-4 promoter was detected by quantitative PCR. The specific amplicon was verified by agrose electrophoresis and shown on the top panel.

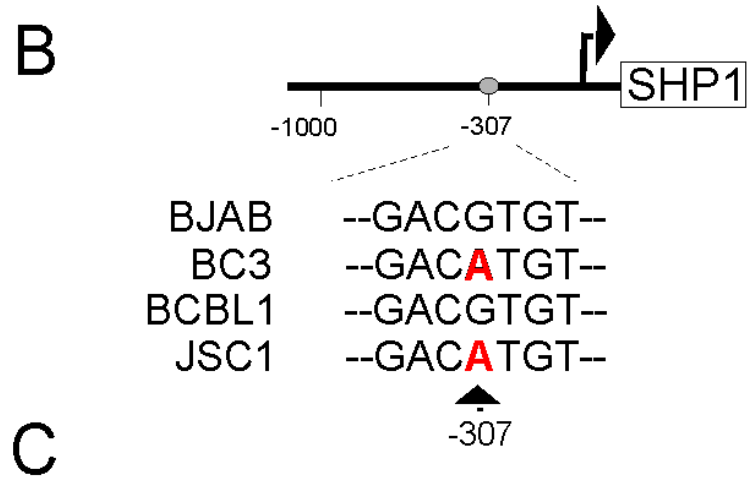
**Figure 7.** IL-13 is crucial for triggering PEL cell proliferation and survival. (**A**) Equal amount (2 million) of BC3, BCBL1, BJAB, and K-BJAB$^{Low}$ cells were individually treated with either 20 μg/ml anti-IL-4, anti-IL-13 antibody or an IgG isotype control. Proliferation was measured at 24, 48, and 72 hours by cell vitality counter. The proliferation rate of the treated cells was presented by percentage of the corresponding untreated control, and calculated as the mean of triplicate samples. The statistical significance was evaluated and $p<0.05$ indicated as double asterisks. (**B**) IL-13 enhances PEL cell survival. BC3 cells were individually subjected to treatment with anti-IL-13 or control antibody (as shown in panel A) in combination with or without sera starve (0.1%) overnight, followed by analysis of cell cycle profile. The average percentage of different phases (sub-G1, G1, S, G2/M) from three repeats in presented in a histogram.

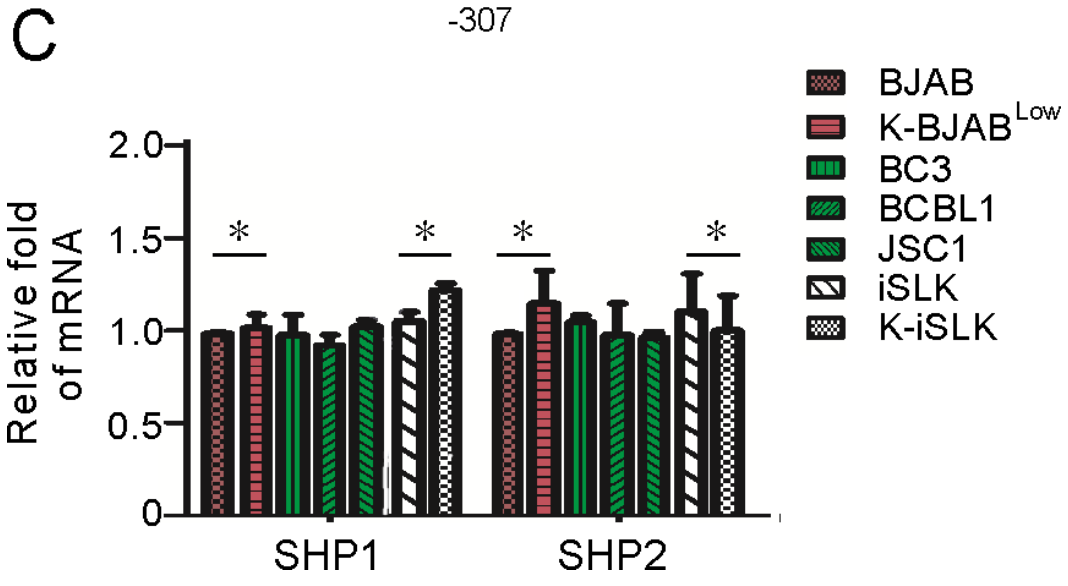
**Figure 8.** A schematic representation of constitutive activation of IL-13/STAT6

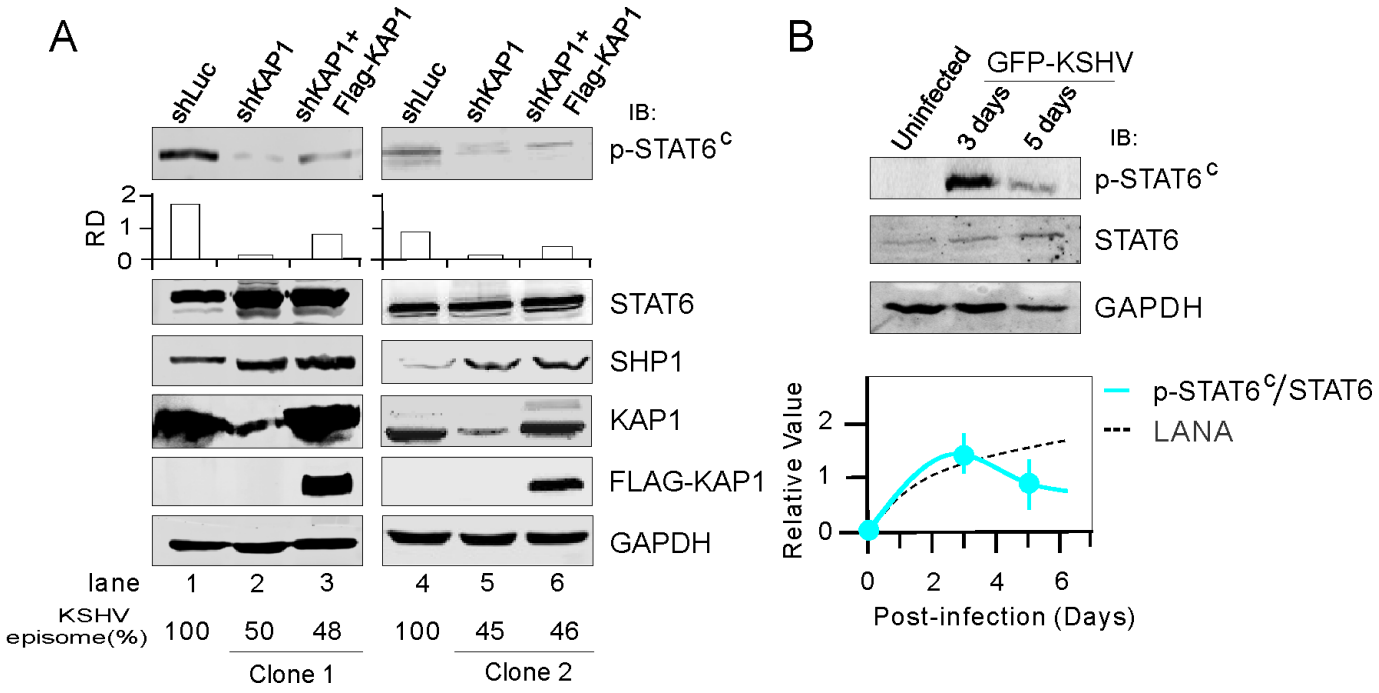
signaling in PEL cells. In KSHV-associated B lymphoma cells, KSHV not only significantly blocks IL-4-induced activation of STAT6 (high) for suppressing immune cell growth and activation (Cai et al, JVI 2010), it also down-regulates SHP1 and constitutively activates IL-13-mediated phosphorylation of STAT6 (low) to a certain extent via selectively induction of IL-13 not IL-4 expression, for enhancing host cell proliferation and survival. However, EBV co-infection dramatically blocks KSHV-induced activation of IL-13/STAT6 signaling.



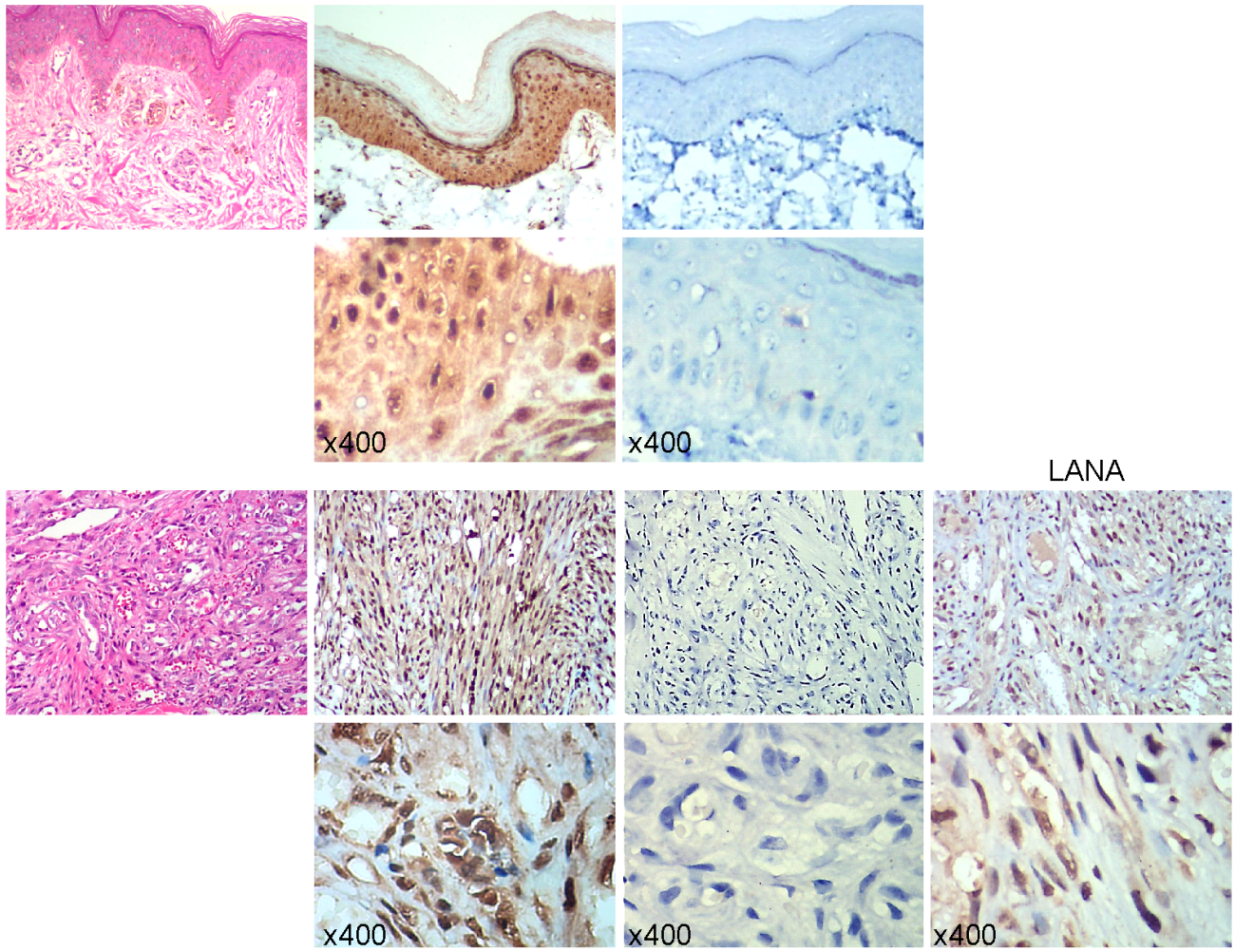




A




B

| | SHP1 | p-STAT6 c |
|---|---|---|
| Skin Tissue | ++++ | − |
| KS | ++ | +/− |