TLR4-mediated inflammation promotes KSHV-induced cellular transformation and tumorigenesis by activating the STAT3 pathway

**Running title:** TLR4-mediated inflammation promotes cellular transformation

Marion Gruffaz¹, Karthik Vasan¹, Brandon Tan¹, Suzane Ramos da Silva¹, and Shou-Jiang Gao¹

¹Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA

**Note:** Supplementary data for this article are available at Cancer Research Online.

**Corresponding Author:** Shou-Jiang Gao, Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA90033, USA. Phone: 323-332-8028; Fax: 323-442-1710. shoujiag@usc.edu (S.-J.G.)

**Grant Support:** Shou-Jiang Gao, National Cancer Institute, CA096512; Shou-Jiang Gao, National Cancer Institute, CA124332; Shou-Jiang Gao, National Cancer Institute, CA132637; Shou-Jiang Gao, National Cancer Institute, CA213275; Shou-Jiang Gao, National Cancer Institute, CA177377; Shou-Jiang Gao, National Cancer Institute, CA197153; Shou-Jiang Gao, National Institute of Dental & Craniofacial Research, DE025465.

**Key words:** KSHV; Kaposi's sarcoma; Cellular transformation; Tumorigenesis; Toll-like receptor 4, TLR4; Opportunistic bacterial infections; Inflammation; Inflammatory cytokines; STAT3.
Abstract

Toll-like receptors (TLR) are conserved immune sensors mediating antimicrobial and antitumoral responses, but recent evidence implicates them in promoting carcinogenesis in certain cancers. Kaposi's sarcoma (KS) is caused by infection of Kaposi's sarcoma-associated herpesvirus (KSHV) and is characterized by uncontrolled neoangiogenesis and inflammation. Here we show that TLR4 is upregulated in KSHV-infected spindle tumor cells in human KS lesions. In a model of KSHV-induced cellular transformation, KSHV upregulated expression of TLR4, its adaptor MyD88, and coreceptors CD14 and MD2. KSHV induction of TLR4 was mediated by multiple viral microRNAs. Importantly, the TLR4 pathway was activated constitutively in KSHV-transformed cells resulting in chronic induction of IL-6, IL-1β and IL-18. Accordingly, IL-6 mediated constitutive activation of the STAT3 pathway, an essential event for uncontrolled cellular proliferation and transformation. TLR4 stimulation with lipopolysaccharides or live bacteria enhanced tumorigenesis while TLR4 antagonist CLI095 inhibited it. These results highlight an essential role of the TLR4 pathway and chronic inflammation in KSHV-induced tumorigenesis, which helps explain why HIV-infected patients, who frequently suffer from opportunistic bacterial infections and metabolic complications, frequently develop KS.
Introduction

Kaposi’s sarcoma-associated herpesvirus (KSHV) is an oncogenic virus etiologically linked to Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL), and a subset of multicentric Castleman’s disease (MCD), malignancies commonly found in immunocompromised patients (1). More recently, KSHV has been associated with an inflammatory cytokine syndrome (KICS) (2). Because of the lack of relevant models mimicking the complex spectrum of KSHV-associated malignancies, the mechanisms involved in these pathologies remain unclear.

KSHV has a biphasic lifecycle consisting of a lifelong latent infection punctuated by viral lytic reactivation (3). While a small subset of cells undergo spontaneous lytic replication in KSHV-associated malignancies, most tumor cells are latently infected by KSHV, suggesting an essential role of latent infection in these malignancies (1). During latent infection, only a restricted number of genes are expressed including LANA (ORF73), vFLIP (ORF71), vCyclin (ORF72), and 12 precursor microRNAs (miRNAs), which effectively evade the host immune controls (3).

KS, the most common malignancy in AIDS patients, is characterized by proliferating vascular spindle tumor cells expressing mixed cellular markers of vascular and lymphatic endothelial cells as well as mesenchymal precursor cells, reflecting the promiscuous origins of these cells (1). While KS tumors contain vast angiogenesis and abundant inflammatory cytokines, which are essential for the development of KS tumors, the mechanism underlying these pathological processes remain unclear.

The Toll-like receptors (TLRs) family consists of 10 human (TLR1 to TLR10) and 12 rodents (TLR1 to TLR9, and TLR11 to TLR13) transmembrane members (4).
TLRs detect nucleic acids, glycosylated proteins, lipoproteins and lipids from a wide range of microbes including viruses, bacteria and fungi, leading to an antiviral response, activation of NF-κB, MAPK, PI3K-AKT and IRF3 pathways, and release of cytokines, chemokines and growth factors (4).

TLR4 recognizes bacterial motifs from Gram-negative bacteria such as lipopolysaccharides (LPS), and cellular components such as saturated fatty acids, HMGB1, fetuin A, heat shock proteins, angiotensin II, fibrinogen, heparan sulfate, ceramide and ethanol (5). TLR4 signaling requires MD2 and CD14 coreceptors for LPS binding. Following association with soluble lipid-binding protein (LPB), CD14 interacts with LPS, and facilitates its transfer to the complex TLR4/MD2 to initiate the recruitment of intracellular adaptors (6). TLR4 is highly conserved in mammals, and is the only TLR that can trigger a signal through both MyD88 and TRIF molecules (4, 5).

Besides their protective effect, new evidences have now shown that activation of TLRs could promote certain diseases (7). Upregulation of TLR4 in tumors and infiltrating immune cells could activate NF-κB, PI3K-AKT and STAT3 pathways, and induce proinflammatory cytokines such as IL-6, IL-1β, IL-18 or TNF-α (5, 7). In colorectal cancer, activation of the TLR4-MyD88 pathway is correlated with a high level of IL-6, which is associated with poor prognosis (8). TLR4 activates NF-κB and PI3K-AKT pathways to facilitate cell adhesion, and enhance the metastatic potential of colon tumor cells (9). By activating the STAT3 pathway through proinflammatory cytokines such as IL-6, TLR4 signaling is associated with liver injury and hepatocarcinogenesis (10). TLR4 deficient mice are strongly protected against colon cancer, suggesting a role of TLR4 in carcinogenesis in vivo (11).

KSHV counters host innate immunity to facilitate lytic replication (12). In contrast, KSHV hijacks host innate immune pathways to promote latent infection. For
example, KSHV activates the complement pathway to activate the STAT3 pathway and promote cell survival (13). Furthermore, KSHV induces a proinflammatory immune response to promote KS development during latency. In fact, the KS tumor microenvironment is dysregulated and associated with abundant proangiogenic and proinflammatory cytokines (14). High levels of IL-6, TNF-α and IL-10 are present in sera of KS patients whereas IL-1β transcripts are upregulated in KS biopsies (15).

HIV-infected patients are subjected to opportunistic bacterial infections due to immunosuppression, which could persistently activate TLR4 (16). Moreover, metabolic syndrome is commonly observed in AIDS patients and associated with high levels of fatty acids in the sera, which could activate the TLR4 pathway (17). In this study, we aim to determine whether persistent TLR4 activation contributes to KSHV-induced inflammation and cellular transformation. In KS lesions, we have observed TLR4 upregulation in the spindle tumor cells expressing KSHV latent protein LANA. In culture, we have confirmed TLR4 upregulation in KSHV-transformed cells. Significantly, TLR4 activation results in chronic inflammation associated with upregulation of proinflammatory cytokines including IL-6, which promotes cellular transformation by activating the STAT3 pathway. Finally, TLR4 activation by exogenous LPS stimulation or live bacterial infection enhances KSHV-induced tumorigenesis and tumor dissemination in mice, and this effect is counteracted by a TLR4 antagonist CLI095.

Materials and Methods

Cell culture

Early passages (<10) of primary rat embryonic metanephric mesenchymal stem cells (MM or Mock), and MM cells infected by KSHV (KMM or WT), and different
mutant viruses with deletion of vFLIP (ΔFLIP), vCyclin (ΔCyclin) or a cluster of 10 pre-miRNAs (ΔmiR) are grown previously described (18-21). ΔmiR cells stably expressing 12 individual pre-miRs (-K1 to -K12) were also grown under the same condition (20, 21). Telomerase-immortalized human microvascular endothelial cells (TIME) and KSHV-infected TIME cells (TIME-KSHV) were maintained as previously described (13). All cell lines were routinely tested for mycoplasma contamination using LookOut® Mycoplasma qPCR Detection Kit (Sigma MP0035-1KT).

MM and KMM cells generated in 2012, and MM cells infected by different recombinant viruses and ΔmiR cells expressing different KSHV pre-miRs generated in 2014, all in our laboratory. TIME cells were obtained from Don Ganem in 2007. TIME-KSHV cells were generated in our laboratory in 2014. No further authentication has been performed with these cells. All the cells were used in less than 10 passages from the time they were generated or obtained.

Reagents

Ultrapure LPS from E. coli K12 (tlrl-peklps) and CLI095 (tlrl-cli95) (Thermo Fisher Scientific) were resuspended in PBS and DMSO, respectively. Stattic (Sigma s7947) and SC144 (Sigma sml0763) were resuspended in DMSO. Neutralizing gp80 antibody was from Biolegend (115807).

Cell proliferation assay

Cells were plated at a density of 50,000 cells/well for 16 h, treated with the indicated reagents were counted using a Malassez chamber.

Softagar assay
Softagar assay was carried out as previously described (19).

RNA extraction and RT-qPCR

Total RNAs were extracted with TRI Reagent (Sigma T9424). Reverse transcription (RT) was performed with 500 ng of total RNA using Maxima H Minus First Strand cDNA synthesis kit (Thermo Fisher Scientific K1652). cDNAs diluted 10 times was used for qPCR using KAPA SYBR Fast qPCR kit (Kapa Biosystems K4602) with specific primers for β-actin, TLR4, CD14, MD2, MyD88, IL-6, IL-1β and IL-18 (Supplementary Table S1). β-actin gene was used for calibration. All the sequences of primers used for RT-qPCR are listed in Supplementary Table S1).

Western blot analysis

Western blot analysis was performed as previously described (21). Primary antibodies included mouse monoclonal antibodies to TLR4 (Santa Cruz 293072), STAT3 (CST 9139) and phospho-p65 (CST 3036), and rabbit polyclonal antibodies to CD14 (Abcam 106285), phospho-STAT3 (CST 9145), p65 (CST 8242), IκBα (Sigma I0505), MyD88 (Abcam 2064), MD2 (Santa Cruz 20668), AKT (CST 4691), phospho-AKT (CST 2965) and β-tubulin (CST 2148).

Luminex assay

Supernatants of culture cells were harvested and concentrated 10 times using Amicon Ultra column 10kDa (EMD Millipore UFC501008). Rat IL-6, IL1-β and IL-18 cytokines were detected by Luminex assay (Bio-Rad Bio-plex Pro Cytokine).

siRNA transfection
Cells seeded at a density of $10^5$ cells/well overnight were transfected with different siRNAs at 5 µM (Ambion Silencer Pre-designed siRNA) using Dharmafect II Reagent (Dharmacon 2002). The sequence of each siRNA is listed in Supplementary Table S2. Silencer Negative Control #1 siRNA (Ambion AM4636) was used as a negative control.

**shRNA lentivirus production and lentiviral transduction**

Lentivirus production was carried out as previously described (22). Different shRNAs targeting TLR4 gene or a scrambled shRNA were inserted into pLKO.1 lentiviral vector containing the Blasticidin selection marker. The sequence of each shRNA is listed in Supplementary Table S3. Transduced cells were selected with Blasticidin at 5 µg/ml for 2 days, reseeded and used for the experiments.

**Immunofluorescence staining of tissue sections**

Formalin-fixed, paraffin-embedded tissue array consisting of 14 KS specimens and 1 normal tissue were obtained from the AIDS and Cancer Specimen Resource (ACSR). Sections were de-paraffinized with xylene and rehydrated by successive incubation in 95% and 75% ethanol solutions. Samples were incubated in 3% hydrogen peroxide in methanol for 10 min and processed for antigen retrieval at 95°C for 30 min in 1 mM EDTA at pH 8.0. Dual immunostaining was performed using a rat anti-LANA antibody (Abcam LN35) and a mouse anti-TLR4 antibody (Santa Cruz 293072). Alexa488- and Alexa568-conjugated secondary antibodies (Thermo Fisher Scientific) were used to reveal the signals. Nuclei were counterstained with DAPI. Tissue sections without incubation with primary antibodies were used as negative controls. For each specimen, three images of representative areas were acquired.
using a confocal fluorescence microscopy with a 60x objective (Nikon Eclipse C1). A total of 166 to 842 cells were manually counted per specimen. The scoring of the expression of TLR4 was performed using a modified Histo-score (H-score), which included a semi-quantitative assessment of both fraction of positive cells and intensity of staining (21). The intensity score was defined as no staining (0), weak (1), moderate (2), or strong (3) staining. The fraction score was based on the proportion of positively stained cells (0-100%). The intensity and fraction scores were then multiplied to obtain H-scores, which represented the overall levels of TLR4 expression.

**Animal experiments**

To test the effect of LPS and CLI095 on tumor progression, athymic female nude mice (5 weeks old) purchased from Envigo were subcutaneously engrafted with KMM cells into both flanks of each animal at $10^7$ cells per site. At day 3 post-engraftment, mice were randomly divided into 4 groups, each treated with PBS, LPS (5 µg), CLI095 (50 µg), or both LPS and CLI095 in 100 µl for 3 times a week by intraperitoneal injection. Tumor volumes were monitored once a week and calculated based on the formula (length×width×height). The mice were terminated by CO$_2$ inhalation following by cervical dislocation when the tumor volume reached 1 cm$^3$.

For live bacterial infection with *E. coli* strain C25 (23), athymic female nude mice (5 weeks old) were intraperitoneally engrafted with KMM cells at 3x10$^6$ cells per mouse. At day 3 post-engraftment, mice were randomly divided into 3 groups: *E. coli*, *E. coli* + CLI095, and PBS alone. For the *E. coli* and *E. coli* + CLI095 groups, mice were intraperitoneally infected 3 times a week, each at $10^7$ bacteria per mouse in 100 µl while for the PBS group, mice were injected with PBS. For the *E. coli* + CLI095
group, mice were injected 3 times a week, each with 50 µg of CLI095 in 100 µl while for the PBS and E. coli groups, mice were injected with PBS. At week 12 post-engraftment, in vivo bioimaging of the GFP-expressing tumor cells were performed using IVIS Spectrum In Vivo Imaging System (Perkin Elmer). Analysis of was done with In Vivo Image Software (Perkin Elmer) and the GFP signals were quantified and expressed in ROI based on the \([p/s]/[\mu W/cm^2]\) formula. All animal experiments were approved by the University of Southern California Institutional Animal Care and Use Committee (IACUC) under the protocol number #20312.

**Statistical analysis**

Data were expressed as mean ± standard error from at least three independent experiments. Statistical analysis was performed using two-tailed t-test and p-value ≤0.05 was considered significant. Statistical symbols “*”, “**” and “***” represent p-values ≤0.05, 0.01 and 0.001, respectively while “NS” indicates “not significant”. For animal survival study, Kaplan-Meier survival analysis was performed and statistical significance was calculated using the log-rank test.

**Results**

**TLR4, MyD88, CD14 and MD2 are upregulated in KSHV-infected and -transformed cells, and TLR4 is upregulated in human KS tumors**

Primary rat embryonic metanephric mesenchymal stem cells (MM or Mock), and MM cells infected by KSHV (KMM or WT) are the only available model for efficient KSHV-induced cellular transformation of primary cells (18). To investigate the roles of TLRs in KSHV-induced cellular transformation, we examined the expression levels of TLRs (TLR1 to TLR10) in this model. RT-qPCR showed that,
compared to MM cells, mRNA levels of most TLRs (TLR1-3 and TLR5-10) were slightly upregulated in KMM cells, ranging from 2- to 8-fold (Fig. 1A). In contrast, TLR4 mRNA level was 45-fold higher in KMM than MM cells. Furthermore, mRNA levels of TLR4 coreceptors CD14 and MD2, and adaptor MyD88 were 4-, 18- and 2-fold higher in KMM than MM cells, respectively (Fig. 1B). Consistent with the mRNA results, the protein levels of all components of the TLR4 signaling complex were higher in KMM than MM cells (Fig. 1C). We further examined telomerase-immortalized human microvascular endothelial cells (TIME) and KSHV-infected TIME cells (TIME-KSHV). TLR4, MyD88, CD14 and MD2 had higher mRNA and protein expression levels in TIME-KSHV than TIME cells though the extent of upregulation varied according to individual mRNAs and proteins (Fig. 1D and 1E).

To determine whether TLR4 upregulation was present in human tumors, we examined TLR4 protein in KS lesions. As KS tumors are highly heterogeneic consisting of LANA-positive spindle tumor cells and LANA-negative non-tumor cells, we dually stained for TLR4 and LANA proteins (Fig. 1F). We observed a significant TLR4 upregulation in LANA-positive but not LANA-negative cells (Fig. 1G). Among 14 specimens, 13 were positive for LANA (Supplementary Fig. S1). All LANA-positive specimens had TLR4 upregulation in LANA-positive cells, and of those, 9 were statistically significant. Among LANA-positive specimens, the average TLR4 signal was positively correlated with the percentage of LANA-positive cells (r=0.5807 and p=0.003) (Fig. 1H).

Taken together, we showed that TLR4 was upregulated in KSHV-infected and -transformed cells in culture as well as in KS tumor cells.
The TLR4 pathway is functional and hyper-responsive in KSHV-transformed cells

We stimulated KSHV-transformed cells with LPS and examined the activation of NF-κB and AKT pathways (Fig. 2A). We detected phosphorylation of NF-κB p65 subunit and degradation of IκBα at 15 min after LPS stimulation in KMM cells. At 5 min after LPS stimulation, we observed strong AKT phosphorylation in KMM cells. In contrast, there was no NF-κB activation, and only weak AKT phosphorylation in MM cells following LPS stimulation (Fig. 2A).

TLR4 activation often induces proinflammatory cytokines (4). The mRNA levels of proinflammatory cytokines IL-6, IL-1β and IL-18 were upregulated at the basal level in KMM cells compared to MM cells (Fig. 2B). LPS stimulation further induced IL-6, IL1-β and IL-18 mRNAs in KMM cells but only had weak effect on MM cells. Furthermore, IL-6 cytokine was constitutively secreted in unstimulated KMM cells, and induced to a higher level following LPS stimulation (Fig. 2C). We did not detect secretions of IL-1β and IL-18 cytokines in unstimulated KMM cells, which could be due to the absence of activation of inflammasome or limited sensitivity of the assays. However, after LPS stimulation, both IL-1β and IL-18 cytokines were detected in the supernatants of KMM cells (Fig. 2C). In contrast, IL-6, IL-1β and IL-18 cytokines were not secreted by MM cells with and without LPS stimulation. These results indicated that KMM cells were in a chronic inflammatory status due to constitutive TLR4 activation, which could be further amplified following stimulation with a TLR4 ligand.

TLR4- and CD14-mediated inflammation is essential for KSHV-induced cell proliferation and cellular transformation
We performed knockdown of TLR4 and CD14 with specific siRNAs and observed their efficient knockdown at both mRNA and protein levels in KMM cells while those of MM cells remained at low or undetectable levels (Fig. 3A-3D). Knockdown of TLR4 or CD14 significantly reduced the proliferation of KMM cells but had a negligible effect on MM cells (Fig. 3E-3F). In parallel, we observed reduced basal mRNA levels of IL-6, IL-1β and IL-18 in KMM while those of MM cells remained at low levels (Fig. 3G). We confirmed reduced secretion of IL-6 cytokine by KMM cells following TLR4 or CD14 knockdown (Fig. 3H). To examine the role of TLR4 in cellular transformation, we performed stable knockdown with specific shRNAs. The three shRNAs efficiently downregulated TLR4 at both mRNA and protein levels (Fig. 3I). Significantly, stable knockdown of TLR4 efficiently inhibited the formation of colonies in softagar (Fig. 3J) and reduced IL-6 secretion by KMM cells (Fig. 3K). These results indicated that TLR4 and its downstream pathways mediated KSHV-induced inflammation as well as cell proliferation and cellular transformation.

**TLR4 antagonist CLI095 inhibits the proliferation and cellular transformation of KSHV-transformed cells**

We used TLR4 antagonist CLI095 to block the TLR4 pathway (24). At the basal level, CLI095 inhibited the proliferation of KMM cells but had no effect on MM cells (Fig. 4A). CLI095 also inhibited the formation of colonies in softagar (Fig. 4B). On the other hand, stimulation with LPS increased the proliferation of KMM cells but had no effect on MM cells (Fig. 4C). LPS also enhanced the formation of colonies of KMM cells in softagar (Fig. 4D). Consistent with these results, CLI095 reduced the mRNA levels of IL-6, IL1-β and IL-18 (Fig. 4E), and secretion of IL-6 cytokine by KMM cells (Fig. 4F). These results confirmed that the TLR4 pathway mediated the
basal chronic inflammation state of KSHV-transformed cells, which was essential for KSHV-induced cell proliferation and cellular transformation.

**KSHV miRNAs mediates TLR4 upregulation and chronic inflammation in KSHV-transformed cells**

Similar to KS tumor cells, KSHV is predominantly latent following cellular transformation, expressing latent genes LANA, vFLIP and vCyclin, and 12 precursor miRNAs (Jones et al., 2014). To identify the viral factor(s) mediating TLR4 upregulation, we examined MM cells infected by ΔFLIP, ΔCyclin or ΔmiR mutant viruses. Compared to KMM cells (WT), deletion of vFLIP or vCyclin reduced TLR4 mRNA level by 50%, hence TLR4 levels in ΔFLIP and ΔCyclin cells remained 16-fold higher than that of MM cells (Mock) (Fig. 5A). Deletion of the miRNA cluster completely abolished TLR4 upregulation, reducing TLR4 level to that of Mock cells. Consequently, TLR4 protein level in ΔmiR cells was reduced to that of MM cells (Fig. 5A). Consistently, deletion of the miRNA cluster completely abolished KSHV-induced upregulation of IL-6, IL-1β and IL-18, reducing their levels to those of Mock cells (Fig. 5B). Deletion of vFLIP or vCyclin also reduced IL-6, IL-1β and IL-18 levels by 30-70% with the exception of ΔFLIP mutant cells, which had a 2.5-fold increase of IL-6 compared to WT cells (Fig. 5B). These results indicated that the miRNA cluster was the primary viral factor mediating KSHV induction of TLR4 and proinflammatory cytokines.

To determine if the TLR4 pathway were functional in cells infected by different mutant viruses, we examined TLR4-mediated NF-κB activation. Deletion of the miRNA cluster rendered the cells no longer responsive to LPS stimulation while deletion of vFLIP or vCyclin had no effect (Fig. 5C). LPS could no longer stimulate
the mRNA expression of IL-6, IL-1β and IL-18 in ΔmiR cells (Fig. 5D). Similar to the Mock cells, ΔmiR cells no longer produced secreted IL-6 cytokine, and LPS failed to stimulate the secretion of IL-6, IL-1β and IL-18 cytokines in these cells (Fig. 5E). In parallel, we found that TLR4 stimulation with LPS was no longer able to increase the proliferation rate of ΔmiR cells (Fig. 5F).

To identify the specific viral miRNA(s) that mediated TLR4 upregulation, we complemented the ΔmiR cells with individual pre-miRNAs (pre-miR-K1 to -K12) (20, 21). Complementation with pre-miR-K1, -K3 and -K11 partially restored the mRNA expression of TLR4, IL-6, IL-1β and IL-18 (Fig. 5G). Hence, pre-miR-K1, -K3 and -K11 contributed to KSHV-induced TLR4 upregulation and chronic inflammation in KSHV-transformed cells.

All together, these results highlighted an important role of viral miRNA cluster in KSHV-induced TLR4 pathway and inflammation-mediated cellular transformation.

The essential role of TLR4 in KSHV-induced cellular transformation is mediated by IL-6R/STAT3 axis

The transcription factor STAT3, activated by IL-6 superfamily cytokines, is associated with numerous types of cancer (25). Since we detected constitutive IL-6 secretion in KMM cells at the basal level and after LPS stimulation (Fig. 2C), we examined STAT3 activation. The level of STAT3 activation measured by the phosphorylation status was higher in KMM than MM cells (Fig. 6A). Addition of Stattic, an inhibitor of STAT3, blocked STAT3 phosphorylation (Fig. 6A). Significantly, Stattic inhibited cell proliferation and formation of colonies of KMM cells in soft agar but had no effect on MM cells (Fig. 6B and 6C), indicating that KSHV-transformed cells were addicted to the STAT3 pathway.
We investigated the dependence of STAT3 activation on the TLR4 pathway. Treatment with LPS induced STAT3 activation while addition of CLI095 inhibited both the basal and LPS-induced STAT3 activation in KMM cells (Fig. 6D). Of interest, we observed dual peaks of STAT3 activation following LPS treatment with the first detected at 15 min and the second detected at 2 h post-treatment in both MM and KMM cells albeit the extent of STAT3 activation by LPS was more robust in KMM than MM cells (Fig. 6E). These results indicated that the TLR4 pathway mediated STAT3 activation in KMM cells.

KSHV encodes an IL-6 homolog vIL-6 (ORF-K2), which also activates the STAT3 pathway. To exclude the involvement of vIL-6 in LPS-induced STAT3 activation, we first examined vIL-6 expression following LPS stimulation. While IL-6 expression was responsive to LPS stimulation, vIL-6 expression was not (Fig. 6F), hence was unlikely involved in STAT3 activation. Next, we inhibited both subunits of IL-6 receptor (IL-6R) gp80 and gp130, and examined the effect on STAT3 activation. IL-6 binding to IL-6R and STAT3 activation requires both subunits gp80 and gp130 while vIL-6 binding to its receptor and STAT3 activation only requires gp130 (26). Treatment with a gp80 neutralizing antibody was sufficient to block LPS activation of STAT3 in KMM cells (Fig. 6G). The neutralizing antibody also inhibited the proliferation of KMM cells but not that of MM cells (Fig. 6H). These results confirmed that IL-6 but not vIL-6 was involved in STAT3 activation. In parallel, we treated the cells with a gp130 subunit inhibitor SC144 (27). SC144 blocked LPS activation of STAT3 in KMM cells (Fig. 6I), and inhibited the proliferation of KMM cells but only had marginal effect on MM cells (Fig. 6J). Finally, SC144 also inhibited the formation of colonies of KMM cells in softagar (Fig. 6K).
Together, these results indicated that KSHV-induced cellular transformation depended on the IL-6R/STAT3 pathway, which was activated by the TLR4 pathway through induction of proinflammatory cytokine IL-6.

**Exogenous stimulation of TLR4 enhances tumorigenesis**

Our results showed that exogenous stimulation of TLR4 with LPS activated NF-κB, AKT and STAT3 pathways, increased the expression and secretion of proinflammatory cytokines, and enhanced cell proliferation and efficiency of colony formation in softagar of KSHV-transformed cells. We further used a mouse Kaposi’s sarcoma model to test the role of TLR4 activation in tumorigenesis (18). KMM cells were subcutaneously engrafted into both flanks of nude mice. The mice were randomized into 4 groups 24 h later, and then treated every other day with PBS, LPS, CLI095, or both LPS and CLI095 for 16 weeks. We monitored the growth of tumors and the survival of mice (Fig. 7A and 7B). Mice treated with LPS alone had a significant faster tumor growth rate and a lower survival rate than the control group treated with PBS had. Mice treated with both CLI095 and LPS displayed a tumor growth rate and a survival rate similar to those of PBS control group, hence confirming the specificity of TLR4 engagement in LPS-mediated tumorigenesis. Although we showed that CLI095 alone efficiently inhibited cell proliferation and cellular transformation in cell culture (Fig. 4A and 4B), we did not observe any significant difference in tumor initiation, tumor growth and animal survival rate between mice treated with PBS and CLI095 (Fig. 7A and 7B), which could be due to the low bacteria load in and surrounding the animals.

We examined the expression of IL-6, IL-1β and IL-18 in the KS-like tumors (Fig. 7C) and livers (Fig. 7D). Mice treated with LPS alone had significant higher
levels of IL-6, IL-1β and IL-18 in both tumors and livers compared to the control group treated with PBS. This LPS-induced inflammation was abolished in mice treated with both CLI095 and LPS, attesting the specificity of TLR4 activation and the efficiency of anti-inflammatory effect of CLI095 \textit{in vivo}. Again, no significant difference in level of IL-6, IL1-β or IL-18 was observed between mice treated with PBS and CLI095.

To further confirm the role of the TLR4 pathway in KSHV-induced tumorigenesis in a more physiological context, we induced a chronic infection by challenging the tumor-carrying mice with live bacteria. Mice were first engrafted with KMM cells for 3 days, then inoculated with live bacteria \textit{E. coli}, the combination \textit{E. coli} and CLI095, or PBS alone as a control for 3 times a week for 12 weeks. We performed live-imaging to monitor the progression of the tumors based on the GFP signal (Fig. 7E), and quantified the tumor burden (Fig. 7F). Mice infected with live \textit{E. coli} had a stronger GFP signal than those treated with PBS, and injection with CLI095 reduced the GFP signal to the level similar to those treated with PBS, hence confirming the specific role of TLR4 activation in the enhancing effect of bacteria on tumor growth. Moreover, mice infected with live \textit{E. coli} had more disseminated tumors than those treated with PBS had, and CLI095 prevented this effect (Fig. 7G). Together, these results highlighted the essential role of TLR4 in the growth and spread of KSHV-induced tumors, which could be counteracted by TLR4 antagonist CLI095.

\section*{Discussion}

We have investigated the close relationship between chronic inflammation and KSHV-induced tumorigenesis using a model of KSHV-induced cellular transformation (Fig. 7H). KSHV upregulates TLR4, its adaptor MyD88, and coreceptors CD14 and...
MD2. The TLR4 pathway promotes KSHV-induced cellular transformation and KS-like tumor through the STAT3 pathway as a result of chronic inflammation in response to endogenous ligand stimulation and hyper-responsive to exogenous ligand stimulation. Importantly, we have detected TLR4 upregulation in tumor cells in KS lesions, confirming the clinical relevance of our findings.

Chronic inflammation is a common feature of KSHV-associated malignancies (14). KS is an angiohyperplastic-inflammatory lesion mediated by proinflammatory cytokines including IL-6, IL-1β, IL-18 and TNF-α (28). PEL defined as a lymphomatous effusion in the body cavity produces a large amount of proinflammatory cytokines such as IL-6, IL-10 and oncostatin (29). MCD, a B cell lymphoproliferative disorder, is characterized by inflammatory symptoms attributed to the overproduction of proinflammatory cytokines, particularly IL-6 (30). Finally, patients with KICS display highly inflammatory MCD-like symptoms associated with the secretion of IL-6 without any pathological features of MCD (2). Our results indicate that by upregulating TLR4, KSHV could induce inflammation in these malignancies.

Through downstream signaling, TLRs mediate the induction of innate and adaptive immune responses, which are critical for host antimicrobial and antitumor immunities (4, 7). Besides normal tissues, TLRs are often expressed in tumor cells, and control hyper-proliferation by inducing apoptosis and/or interferon response. However, inflammation mediated by TLRs could also display a complex dual role during tumorigenesis (5, 7). TLRs could enhance immunosuppression and tumorigenesis through chronic inflammation by regulating proinflammatory cytokines such as IL-6, IL1-β and TNF-α, suppressive cytokines such as IL-10 and TGF-β, and regulatory T-cells. Many studies have linked TLR4 activation with tumorigenesis (5, 9,
Furthermore, upregulated levels of TLRs are efficient biomarkers of cancer progression. In particular, TLR4 expression is highly correlated with poor prognosis for patients with colorectal and colon cancers (8), and with tumor aggressiveness in patients with laryngeal and breast cancers (31, 32). In our study, we have demonstrated that TLR4 is upregulated in LANA-positive tumor cells in KS lesions (Fig. 1).

The dual role of TLRs in KSHV-associated malignancies could be illustrated by the complex host-virus interactions during KSHV lifecycle. During early stage of KS, while most tumor cells are latently infected by KSHV, spontaneous lytic cells are readily detectable in the tumors (33). In fact, KSHV load is correlated with the progression of early KS in HIV-infected patients (34, 35). These lytically-infected cells are essential for the tumor spread and sustaining a proinflammatory microenvironment by directly producing KSHV-encoded cytokines or indirectly inducing cellular cytokines in the replicating or newly infected cells (3). As a result of lytic replication, it is expected that KSHV lytic proteins and virions could trigger host innate immune responses such as the complement and interferon pathways. However, KSHV encodes multiple proteins to subvert the host innate immune responses (12). It is expected that effective inhibition of multiple innate immune response pathways could maximize viral productive replication during acute infection or lytic reactivation, hence contributing to the spread and progression of KS tumors.

In late stage of KS, almost all KS tumor cells are latently infected by KSHV with minimal lytic activity expressing a few latent genes including LANA, vCyclin, vFLIP and a cluster of 12 pre-miRNAs, which are essential for maintaining KSHV latency, and providing oncogenic and survival signals for tumor cells (3). In current study, we have used a tumor model with tight viral latency, which recapitulates the
pathological and virological features of late KS tumors (18). We have previously shown that all viral latent products are essential for the development of KS-like tumors in this model. LANA and vCyclin promote cellular transformation and tumorigenesis by hijacking the BMP-smad1-Id1 pathway and inhibiting p27Kip1-mediated contact inhibition, respectively (19, 36) while vFLIP and miRNAs promote cell survival and homeostasis by activating the NF-κB pathway (20, 21). Of interest, instead of inhibiting the innate immune pathways, KSHV appears to hijack the pathways to promote viral latency and cell survival. We have shown that KSHV hijacks the complement pathway to promote cell survival by downregulating cell surface complement regulatory proteins CD55 and CD59 (13). Here, we have demonstrated that KSHV miRNA cluster, particularly miR-K1, -K3 and -K11, mediates the upregulation of TLR4 to induce chronic inflammation, and promote cellular transformation and tumorigenesis (Fig. 5). Since KSHV miRNA cluster mediates activation of the NF-κB and PI3K/AKT pathways (20, 37, 38), it is possible that TLR4 upregulation could be due to the constitutive activation of these pathways. Indeed, we have shown that miR-K1 could target IκBα protein, a master negative regulator of the NF-κB pathway (37) while miR-K3 targets GRK2 to induce CXCR2 and release AKT from GRK2 inhibition leading to maximum AKT activation (38). Moreover, miR-K11 is an ortholog of cellular oncogenic miR-155 (39). miR-155 could promote the expansion of B cells in vivo (40), and play a critical role in immunity and inflammation in macrophages (41). Interestingly, PTEN, a negative regulator of PI3K-AKT pathway, is a target of miR-155 and BHRF1 miR-cluster of EBV, another malignant herpesvirus (42).

It was reported that overexpression of vFLIP could induce the secretion of cytokines including IL-10, IL-6, IL-2, IL-13, interferon-γ, TNF-α, MCP1 and RANTES.
in endothelial cells (43). However, we have shown that deletion of vFLIP from the KSHV genome doesn't affect the basal inflammation and induction of proinflammatory cytokines (Fig. 5A-5C).

We have shown that TLR4 promotion of cellular transformation is mediated by the STAT3 pathway (Fig. 6). Numerous KSHV genes can activate the STAT3 pathway. LANA physically interacts with STAT3 and enhances STAT3 transcriptional activity (44) while vGPCR constitutively activates STAT3 independent of IL-6R (45). Kaposin B and miR-K6-3p activates STAT3 by inducing its phosphorylation and targeting negative regulator SH3BGR, respectively (46, 47). vIL-6 also activates the STAT3 pathway through the gp130 subunit (48). In contrast to vIL-6, activation of the STAT3 pathway by cellular IL-6 requires both gp80 and gp130 of IL-6R. We have shown that activation of the STAT3 pathway depends on both gp80 and gp130 subunits in KSHV-transformed cells. LPS stimulation of TLR4 enhances STAT3 activation, and this depends on the induction of cellular IL-6 but not vIL-6. Interestingly, IL-6 is present in KS tumors (28), and abundant in PEL, MDC and KICS (2, 29, 30). Therefore, without excluding the role of vIL-6, we hypothesized that as a result of TLR4 activation, cellular IL-6 plays a major role in KSHV-induced inflammation and cellular transformation.

TLR4 can be activated by cellular and microbial ligands (5, 7). It is unclear what endogenous and/or exogenous ligand(s) might be involved in the activation of TLR4 pathway at the basal level in KSHV-transformed cells. Firstly, KSHV infection might modify the secretome of the cells leading to secretion of endogenous TLR4 ligands. Secondly, among potential exogenous microbial ligands, bacterial endotoxins are powerful stimulators of TLR4. Because endotoxins are ubiquitous in the environment including air, skin, gut and oral cavity etc, the use of FBS with a low
level of endotoxins would be biologically relevant. We have chosen to use certified heat-inactivated FBS with <1 EU/ml of endotoxins to supplement the culture media instead of endotoxin-free serum. Therefore, we can’t exclude a role of endotoxin in the activation of TLR4. Lastly, different classes of lipids are present in FBS. Saturated free fatty acids (FFA) can induce TLR4 activation, and promote inflammation and angiogenesis in cancer cells (49). Regardless the origins of TLR4 ligands, we have carefully examined the role of TLR4 pathway in KSHV-transformed cells in comparison with the uninfected cells. Hence, the observed essential role of the activated TLR4 pathway in KSHV-induced cellular transformation is likely specific.

We have demonstrated that TLR4 stimulation with an exogenous ligand LPS and a live bacterial infection enhances the tumorigenesis of KSHV-transformed cells (Fig. 7). Most of the untreated AIDS-KS patients suffer from severe immunodeficiency, and therefore are susceptible to opportunistic infections. Among them, LPS-containing bacteria could play an important role in the progression of KSHV-associated malignancies by promoting inflammation and tumorigenesis through TLR4 stimulation. Furthermore, HIV-infected patients often develop metabolic syndromes producing high levels of triglycerides in blood (50). Among them, unsaturated fatty acids could act as ligands for TLR4 (5), and promote KSHV-induced malignancies. Therefore, our results highlight the importance of co-infections such as bacteria and metabolic syndromes in KSHV-associated malignancies in HIV-infected patients. Further studies are required to delineate the complex interactions of HIV, KSHV, opportunistic infections, metabolic syndromes and innate immune responses in AIDS-KS patients, which should reveal their roles in the initiation and progression of AIDS-associated malignancies.
Acknowledgements

We thank the AIDS and Cancer Specimen Resource (ACSR) for providing the KS tissue array, Dr. Philip Sherman for providing the E. coli C25 strain, Dr. Keigo Machida for his advice on the animal experiments, and members of Dr. Gao’s laboratory for technical assistance and helpful discussions.

References


FIGURES

Figure 1. Upregulation of TLR4, MyD88, CD14 and MD2 in KSHV-infected and -transformed cells, and in human KS tumors. A, Expression of individual TLRs (TLR1-10) analyzed by RT-qPCR in MM (Mock) and KMM (KSHV) cells. B-C, Expression of TLR4, CD14, MyD88 and MD2 analyzed by RT-qPCR (B) and Western blot (C) in MM and KMM cells. D-E, Expression of TLR4, CD14, MyD88 and MD2 analyzed by RT-qPCR (D) and Western blot (E) in TIME (Mock) and TIME-KSHV (KSHV) cells. F, Expression of TLR4 and LANA proteins in KS tumors analyzed by dual immunofluorescence staining on a tissue array containing 14 KS and 1 normal tissue specimens. The sections were counterstained with DAPI, and pictures were taken with a confocal microscopy (Magnification 600x). Boxed area is enlarged. G, Relative expression of TLR4 in LANA-positive and -negative cells in KS tumors in the tissue array described in (F) (n=14). (H) Positive correlation of average percentage of LANA-positive cells with average TLR4 level in KS tumors based on analysis described in (G).

Figure 2. Hyper-responsiveness of the TLR4 pathway in KSHV-transformed cells. A, LPS-induced phosphorylation of NF-κB p65 subunit and AKT, and IκBα degradation in KMM but not MM cells. Cells were treated with 1 µg/ml LPS for the specified times, and analyzed by Western blot. B, Expression of IL-6, IL-1β and IL-18 mRNAs analyzed by RT-qPCR in MM and KMM cells with or without stimulation with 1 µg/ml LPS for 4 h. C, IL-6, IL-1β and IL-18 cytokines in supernatants of MM and KMM cells with or without stimulation with 1 µg/ml LPS for 24 h analyzed by Luminex assays.
Figure 3. TLR4 and CD14 are essential for the proliferation, formation of colonies in softagar, and induction of proinflammatory cytokines of KSHV-transformed cells. A-D, TLR4 (A, C) and CD14 (B, D) expression following siRNA knockdown analyzed by RT-qPCR (A, B) and Western Blot (C, D) in MM and KMM cells. E-F, Cell proliferation following siRNA knockdown of TLR4 (E) or CD14 (F) in MM and KMM cells. G, IL-6, IL-1β and IL-18 expression following siRNA knockdown of TLR4 or CD14 analyzed by RT-qPCR in MM and KMM cells. H, IL-6 cytokine in the supernatants of MM and KMM cells following siRNA knockdown of TLR4 or CD14 analyzed by Luminex assay. I, TLR4 expression following shRNA knockdown analyzed by RT-qPCR and Western blot in MM and KMM cells. J, Formation of colonies in softagar following shRNA knockdown of TLR4. Efficiencies of colony formation are quantified. Representative fields are shown. K, IL-6 in the supernatants of MM and KMM cells following shRNA knockdown of TLR4 analyzed by Luminex assay.

Figure 4. CLI095 inhibits cell proliferation, formation of colonies in softagar, and induction of inflammation cytokines of KSHV-transformed cells. A, Cell proliferation of MM (Mock) and KMM (KSHV) cells treated with DMSO or 10 µg/ml of CLI095 analyzed by cell counting. B, Formation of colonies of KMM cells treated with DMSO or 10 µg/ml of CLI095 analyzed by softagar assay. Efficiencies of colony formation are quantified. Representative fields are shown. C, Cell proliferation of Mock and KSHV cells treated with PBS or 500 ng/ml LPS analyzed by cell counting. D, Formation of colonies of KMM cells treated with PBS or 500 ng/ml LPS analyzed by softagar assay. Efficiencies of colony formation are quantified. Representative fields are shown. E, IL-6, IL-1β and IL-18 expression analyzed by RT-qPCR in Mock and
KSHV cells treated with DMSO or 10 µg/ml CLI095 for 24 h. F, IL-6 in the supernatants of Mock and KSHV cells treated with DMSO or 10 µg/ml CLI095 for 24 h analyzed by Luminex assay.

**Figure 5.** KSHV-induced upregulation of TLR4 and inflammation are mediated by KSHV miRNAs. A, TLR4 expression in MM (Mock), and MM cells infected by KSHV (KMM, WT), and mutant viruses with deletion of vFLIP (ΔFLIP), vCyclin (ΔCyclin) or a cluster of 10 pre-miRNAs (ΔmiR) analyzed by RT-qPCR and Western Blot. B, IL-6, IL-1β and IL-18 expression in different cells described in (A) analyzed by RT-qPCR. C, Activation of the NF-κB pathway in different cells described in (A) following treatment with PBS or 1 µg/ml LPS for 15 min analyzed by Western blot using anti-phospho-p65 and -p65 antibodies. D, IL-6, IL-1β and IL-18 expression in Mock, WT or ΔmiR cells following treatment with PBS or 1 µg/ml LPS for 4 h analyzed by RT-qPCR. E, IL-6, IL1-β and IL-18 cytokines in the supernatants of Mock, WT or ΔmiR cells following treatment with PBS or 1 µg/ml LPS for 24 h analyzed by Luminex assays. F, Cell proliferation of WT and ΔmiR cells treated with PBS or 500 ng/ml LPS analyzed by cell counting. G, Expression of TLR4, IL-6, IL-1β and IL-18 analyzed by RT-qPCR in Mock, WT, ΔmiR cells or ΔmiR cells complemented with individual KSHV pre-miRNAs (K1 to K12). Statistical analyses were performed by comparing ΔmiR cells expressing the individual miRNAs with ΔmiR cells.

**Figure 6.** IL-6R/STAT3 axis mediates TLR4 promotion of cell proliferation and cellular transformation of KSHV-transformed cells. A, STAT3 phosphorylation level in MM (Mock) and KMM (KSHV) cells treated with DMSO or 2 µM and 5 µM Stattic for 4 h analyzed by Western blot using anti-phospho-STAT3 and -STAT3 antibodies.
Cell proliferation of Mock and KSHV cells treated with DMSO or 2 µM Stattic analyzed by cell counting. C, Formation of colonies of KMM cells treated with DMSO or 5 µM Stattic analyzed by softagar assay. Efficiencies of colony formation are quantified. Representative fields are shown. D, STAT3 phosphorylation level in KMM cells treated with DMSO or 10 µg/ml CLI095 for 24 h, and then stimulated with PBS or 1 µg/ml LPS for 2 h analyzed by Western blot using anti-phospho-STAT3 and -STAT3 antibodies. E, STAT3 phosphorylation level in Mock or KSHV cells treated with 1 µg/ml LPS for the specified times, and analyzed by Western blot using anti-phospho-STAT3 and -STAT3 antibodies. F, IL-6 and vIL-6 expression in KMM cells treated with PBS or 1 µg/ml LPS for 4 h or 24 h analyzed by RT-qPCR. G, Activation of the STAT3 pathway in KMM cells treated with indicated concentrations of gp80 neutralizing antibody for 24 h and then stimulated with PBS or 1 µg/ml LPS for 2 h analyzed by Western blot using anti-phospho-STAT3 and -STAT3 antibodies. H, Cell proliferation of Mock or KSHV cells treated with indicated concentrations of gp80 neutralizing antibody analyzed by cell counting. I, STAT3 phosphorylation level in KMM cells treated with indicated concentrations of SC144 for 24 h, and then stimulated with PBS or 1 µg/ml LPS for 2 h analyzed by Western blot using anti-phospho-STAT3 and -STAT3 antibodies. J, Cell proliferation of Mock or KSHV cells treated with indicated concentrations of SC144 analyzed by cell counting. K, Formation of colonies of KMM cells treated with DMSO or 2 µg/ml SC144 analyzed by softagar assay. Efficiencies of colony formation are quantified. Representative fields are shown.

Figure 7. Exogenous TLR4 stimulation promotes tumorigenesis, which is inhibited by TLR4 antagonist CLI095 in a KS-like mouse model. A, Tumor growth in nude mice.
treated with PBS, LPS, CLI095, or both CLI095 and LPS. The number “#” indicates
the number of mice terminated in LPS group at each specific time point. B, Animal
survival curves of tumor-bearing nude mice treated with PBS, LPS, CLI095, or both
CLI095 and LPS. Mice were terminated when tumors reached 1 cm³. C-D,
Expression of proinflammatory cytokines (IL-6, IL-1β and IL-18) in tumors (C) or livers
(D) from nude mice treated with PBS, LPS, CLI095, or both CLI095 and LPS for 16
weeks analyzed by RT-qPCR. Statistical analyses were performed by comparing
different groups of mice with the PBS group. E, Live bioimaging of tumor-bearing
nude mice either treated with PBS alone (PBS) or infected with E. coli, then treated
with either PBS (E. coli) or CLI095 (E. coli + CLI095) 3 times a week for 12 weeks. F,
Quantification of signals based on images acquired in (E) using Living Image
Software. The relative intensities are expressed in ROI based on the [p/s]/[µW/cm²]
formula. “n” represents the number of mice in each group. G, Tumor spreading in
different groups of mice described in (E) quantified based on the number of individual
foci detected for each mouse. Results are expressed in percentage and detection of
≥2 foci are considered for tumor spreading. “n” represents the number of mice in
each group. H, Schematic illustration of the role of TLR4 signaling in KSHV-mediated
transformation through the activation of the STAT3 pathway. KSHV miRNAs induce
TLR4 upregulation in KSHV transformed cells. Activation of TLR4 pathway by various
ligands such as LPS or E.coli induces the activation of NF-κB and Akt pathways, and
subsequently the expression and secretion of pro-inflammatory cytokines (IL-6, IL-1β
and IL-18). In particular, IL-6 stimulates STAT3 pathway through gp130/gp80
receptors to promote a pro-oncogenic program in the latently infected cells.
FIGURE 1

A

Relative expression level

***

Mock KSHV

TLR1 TLR2 TLR3 TLR4 TLR5 TLR6 TLR7 TLR8 TLR9 TLR10

B

Relative expression level

Mock KSHV

***

TLR4 CD14 MD2 MyD88

C

Mock KSHV

TLR4 CD14 MyD88 β-tubulin MD2

D

Relative expression level

***

Mock KSHV

TLR4 CD14 MD2 MyD88

E

Mock KSHV

TLR4 CD14 MyD88 β-tubulin

F

TLR4 LANA

DAPI Merge: LANA-positive cells Merge: LANA-negative cells

G

Relative TLR4 expression level

***

LANA - +

H

Relative TLR4 expression level

r = 0.5807 P = 0.003

LANA-positive cells (%)

Downloaded from cancerres.aacrjournals.org on January 2, 2021. © 2017 American Association for Cancer Research.
FIGURE 2

A

<table>
<thead>
<tr>
<th>LPS (Min)</th>
<th>Mock</th>
<th>KSHV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- p-p65
- p65
- IκBα
- p-Akt
- Akt
- β-tubulin

B

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-1β</th>
<th>IL-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT LPS</td>
<td>**</td>
<td>**</td>
<td>NS</td>
</tr>
<tr>
<td>NT LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSHV</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-1β</th>
<th>IL-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT LPS</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>NT LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KSHV</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TLR4-mediated inflammation promotes KSHV-induced cellular transformation and tumorigenesis by activating the STAT3 pathway

Marion Gruffaz, Karthik Vasan, Brandon Tan, et al.

Cancer Res Published OnlineFirst October 19, 2017.