TLR4-Mediated Inflammation Promotes KSHV-Induced Cellular Transformation and Tumorigenesis by Activating the STAT3 Pathway

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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 sarcoma is caused by infection of Kaposi 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 Kaposi sarcoma 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 miRNAs. Importantly, the TLR4 pathway was activated constitutively in KSHV-transformed cells, resulting in chronic induction of IL6, IL1β, and IL18. Accordingly, IL6 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 Kaposi sarcoma. Cancer Res; 77(24); 7094–108. ©2017 AACR.

Introduction

Kaposi sarcoma–associated herpesvirus (KSHV) is an oncogenic virus etiologically linked to Kaposi sarcoma, primary effusion lymphoma (PEL), and a subset of multicentric Castleman disease (MCD), malignancies commonly found in immunocompromised patients (1). More recently, KSHV has been associated with an inflammatory cytokine syndrome (KICS; ref. 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 miRNAs, which effectively evade the host immune controls (3).

Kaposi sarcoma, 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 Kaposi sarcoma tumors contain vast angiogenesis and abundant inflammatory cytokines, which are essential for the development of Kaposi sarcoma tumors, the mechanisms underlying these pathologic processes remain unclear.

The Toll-like receptor (TLR) family consists of 10 human (TLR1 to TLR10) and 12 rodent (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 NFκB, 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, HSPs, 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 NFκB, PI3K-AKT, and STAT3 pathways, and

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Materials and Methods
Cell culture
Early passages (<10) of primary rat embryonic mesenchymal stem cells (MM or Mock), and MM cells infected by KSHV (KMM or WT), and different mutant viruses with deletion of vFLIP (AFLIP), vCyclin (ΔCyclin), or a cluster of 10 pre-miRNAs (ΔmiR) are grown as described previously (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 described previously (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 (lrl-plied) and CLI095 (lrl-cl95) (Thermo Fisher Scientific) were resuspended in PBS and DMSO, respectively. Static (Sigma, s7947) and SCI44 (Sigma, smi0763) were resuspended in DMSO. Neutralizing gp80 antibody was from Biologend (115807).

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

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

RNA extraction and qRT-PCR
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, IL6, IL1β, and IL18 (Supplementary Table S1). β-Actin gene was used for calibration. All the sequences of primers used for qRT-PCR are listed in Supplementary Table S1.

Western blot analysis
Western blot analysis was performed as described previously (21). Primary antibodies included mouse mAbs to TLR4 (Santa Cruz Biotechnology, 293072), STAT3 (Cell Signaling Technology, 9139), and phospho-p65 (Cell Signaling Technology, 3036), and rabbit polyclonal antibodies to CD14 (Abcam, 106285), phospho-STAT3 (CST 9145), p65 (Cell Signaling Technology, 8242), IκBα (Sigma, 10505), MyD88 (Abcam 2064), MD2 (Santa Cruz Biotechnology, 20668), AKT (Cell Signaling Technology, 4691), phospho-AKT (Cell Signaling Technology, 2965), and β-tubulin (Cell Signaling Technology, 2148).

Luminex assay
Supernatants of culture cells were harvested and concentrated 10 times using Amicon Ultra column 10 kDa (EMD Millipore UFC501008). Rat IL6, IL1β, and IL18 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 μmol/L (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 described previously (22). Different shRNAs targeting TLR4 gene or a scrambled shRNA were inserted into pLKO.1 lentiviral vector containing
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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 Kaposi sarcoma specimens and 1 normal tissue were obtained from the AIDS and Cancer Specimen Resource (ACSR). Sections were deparaffinized with xylene and rehydrated by successive incubation in 95% and 75% ethanol solutions. Samples were incubated in 3% hydrogen peroxide in methanol for 10 minutes and processed for antigen retrieval at 95°C for 30 minutes in 1 mmol/L 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 Biotechnology 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 60× 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 semiquantitative 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 10^6 cells per site. At day 3 postengraftment, 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. At day 3 postengraftment, mice were randomly divided into three groups: E. coli, E. coli + CLI095, and PBS alone. For the E. coli and E. coli + CLI095 groups, mice were intraperitoneally injected 3 times a week by intraperitoneal injection. Tumor volumes were monitored once a week and calculated on the basis of the formula (length × width × height). The mice were terminated by CO2 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) purchased from Envigo were subcutaneously engrafted with KMM cells at 3 × 10^6 cells per mouse. At day 3 postengraftment, mice were randomly divided into three 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 three 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 postengraftment, in vivo bioimaging of the GFP-expressing tumor cells were performed using IVIS Spectrum In Vivo Imaging System (Perkin Elmer). Analysis of war was done with In Vivo Image Software (Perkin Elmer) and the GFP signals were quantified and expressed in ROI based on the [p/s]/[μL/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 ± SE from at least three independent experiments. Statistical analysis was performed using two-tailed t test and P < 0.05 was considered significant. Statistical symbols *, **, *** and **** represent P values ≤ 0.05, 0.01, and 0.001, respectively, whereas "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 Kaposi sarcoma 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. qRT-PCR showed that, compared with 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 E).

To determine whether TLR4 upregulation was present in human tumors, we examined TLR4 protein in Kaposi sarcoma lesions. As Kaposi sarcoma tumors are highly heterogeneous consisting of LANA-positive spindle tumor cells and LANA-negative nonmalignant 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; 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 Kaposi sarcoma 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 NFκB and AKT pathways (Fig. 2A). We detected phosphorylation of NFκB p65 subunit and degradation of IkBα at
15 minutes after LPS stimulation in KMM cells. At 5 minutes after LPS stimulation, we observed strong AKT phosphorylation in KMM cells. In contrast, there was no NF\textsubscript{\textkappa}B 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 IL6, IL1\textbeta, and IL18 were upregulated at the basal level in KMM cells compared with MM cells (Fig. 2B). LPS stimulation further induced IL6, IL1\textbeta, and IL18 mRNAs in KMM cells but only had weak effect on MM cells. Furthermore, IL6 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 IL1\textbeta and IL18 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 IL1β and IL18 cytokines were detected in the supernatants of KMM cells (Fig. 2C). In contrast, IL6, IL1β, and IL18 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–D). Knockdown of TLR4 or CD14 significantly reduced the proliferation of KMM cells but had a negligible effect on MM cells (Fig. 3E and F). In parallel, we observed reduced basal mRNA levels of
Figure 3.

TLR4 and CD14 are essential for the proliferation, formation of colonies in soft agar, and induction of proinflammatory cytokines of KSHV-transformed cells. A–D, TLR4 (A and C) and CD14 (B and D) expression following siRNA knockdown analyzed by qRT-PCR (A and B) and Western blot analysis (C and D) in MM and KMM cells. E and F, Cell proliferation following siRNA knockdown of TLR4 (E) or CD14 (F) in MM and KMM cells. G, IL6, IL1β, and IL18 expression following siRNA knockdown of TLR4 or CD14 analyzed by qRT-PCR in MM and KMM cells. H, IL6 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 qRT-PCR and Western blot in MM and KMM cells. J, Formation of colonies in soft agar following shRNA knockdown of TLR4. Efficiencies of colony formation are quantified. Representative fields are shown. K, IL6 in the supernatants of MM and KMM cells following shRNA knockdown of TLR4 analyzed by Luminex assay.
IL6, IL1β, and IL18 in KMM, while those of MM cells remained at low levels (Fig. 3G). We confirmed reduced secretion of IL6 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 soft agar (Fig. 3J) and reduced IL6 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 soft agar (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 soft agar (Fig. 4D). Consistent with these results, CLI095 reduced the mRNA levels of IL6, IL1β, and IL18 (Fig. 4E), and secretion of IL6 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 Kaposi sarcoma tumor cells, KSHV is predominantly latent following cellular transformation, expressing Figure 4. CLI095 inhibits cell proliferation, formation of colonies in soft agar, 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 soft agar assay. Efficiencies of colony formation were 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 soft agar assay. Efficiencies of colony formation were quantified. Representative fields are shown. E, IL6, IL1β, and IL18 expression analyzed by qRT-PCR in Mock and KSHV cells treated with DMSO or 10 μg/mL CLI095 for 24 hours. F, IL6 in the supernatants of Mock and KSHV cells treated with DMSO or 10 μg/mL CLI095 for 24 hours 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 qRT-PCR and Western blot analysis. B, IL6, IL1β, and IL18 expression in different cells described in A analyzed by qRT-PCR. C, Activation of the NFκB pathway in different cells described in A following treatment with PBS or 1 μg/mL LPS for 15 minutes analyzed by Western blot using anti-phospho-p65 and -p65 antibodies. D, IL6, IL1β, and IL18 expression in Mock, WT, or ΔmiR cells following treatment with PBS or 1 μg/mL LPS for 4 hours analyzed by qRT-PCR. E, IL6, IL1β, and IL18 cytokines in the supernatants of Mock, WT, or ΔmiR cells following treatment with PBS or 1 μg/mL LPS for 24 hours 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, IL6, IL1β, and IL18 analyzed by qRT-PCR 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.
latent genes LANA, vFLIP, and vCyclin, and 12 precursor miRNAs (Jones and colleagues, 2014). To identify the viral factor(s) mediating TLR4 upregulation, we examined MM cells infected by ΔFLIP, ΔCyclin, or ΔmiR-mutant viruses. Compared with 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 IL6, IL1β, and IL18, reducing their levels to those of Mock cells (Fig. 5B). Deletion of vFLIP or vCyclin also reduced IL6, IL1β, and IL18 levels by 30%–70% with the exception of ΔFLIP-mutant cells, which had a 2.5-fold increase of IL6 compared with 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 whether the TLR4 pathway was functional in cells infected by different mutant viruses, we examined TLR4-mediated NFκB 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 IL6, IL1β, and IL18 in ΔmiR cells (Fig. 5D). Similar to the Mock cells, ΔmiR cells no longer produced secreted IL6 cytokine, and LPS failed to stimulate the secretion of IL6, IL1β, and IL18 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; refs. 20, 21). Complementation with pre-miR-K1, -K3, and -K11 partially restored the mRNA expression of TLR4, IL6, IL1β, and IL18 (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 IL6R/STAT3 axis

The transcription factor STAT3, activated by IL6 superfamily cytokines, is associated with numerous types of cancer (25). As we detected constitutive IL6 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 Static, an inhibitor of STAT3, blocked STAT3 phosphorylation (Fig. 6A). Significantly, Static inhibited cell proliferation and the formation of colonies of KMM cells in softagar, but had no effect on MM cells (Fig. 6B and C), 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 minutes and the second detected at 2 hours posttreatment 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 IL6 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 vIL6 expression following LPS stimulation. While IL6 expression was responsive to LPS stimulation, vIL6 expression was not (Fig. 6F), hence was unlikely involved in STAT3 activation. Next, we inhibited both subunits of IL6 receptor (IL6R) gp80 and gp130, and examined the effect on STAT3 activation. IL6 binding to IL6R and STAT3 activation requires both subunits gp80 and gp130 while vIL6 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 IL6 but not vIL6 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 IL6R/STAT3 pathway, which was activated by the TLR4 pathway through induction of proinflammatory cytokine IL6.
Exogenous stimulation of TLR4 enhances tumorigenesis

Our results showed that exogenous stimulation of TLR4 with LPS activated NFkB, 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 sarcoma model to test the role of TLR4 activation in tumororigenesis (18). KMM cells were subcutaneously engrafted into both flanks of nude mice. The mice were randomized into 4 groups 24 hours 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 B). 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 B), 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 B), which could be due to the low bacteria load in and surrounding the animals.

We examined the expression of IL6, IL1B, and IL18 in the Kaposi sarcoma-like tumors (Fig. 7C) and livers (Fig. 7D). Mice treated with LPS alone had significant higher levels of IL6, IL1B, and IL18 in both tumors and livers compared with 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 in vivo. Again, no significant difference in level of IL6, IL1B, or IL18 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 physiologic 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 E. coli, the combination 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 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 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.

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 adaptors MyD88, and coreceptors CD14 and MD2. The TLR4 pathway promotes KSHV-induced cellular transformation and Kaposi sarcoma–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 Kaposi sarcoma lesions, confirming the clinical relevance of our findings.

Chronic inflammation is a common feature of KSHV-associated malignancies (14). Kaposi sarcoma is an angiohyperplastic-inflammatory lesion mediated by proinflammatory cytokines including IL6, IL1B, IL18, and TNFα (28). PEL defined as a lymphomatous effusion in the body cavity produces a large amount of proinflammatory cytokines such as IL6, IL10, and oncostatin (29). MCD, a B-cell lymphoproliferative disorder, is characterized by inflammatory symptoms attributed to the overproduction of proinflammatory cytokines, particularly IL6 (30). Finally, patients with KICS display highly inflammatory MCD-like symptoms associated with the secretion of IL6 without any pathologic 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 hyperproliferation by inducing apoptosis and/or IFN 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 IL6, IL1B, and TNFα.
suppressive cytokines such as IL10 and TGFβ, and regulatory T cells. Many studies have linked TLR4 activation with tumorigenesis (5, 9, 10). 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 Kaposi sarcoma 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 Kaposi sarcoma, 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 Kaposi sarcoma 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 IFN 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 Kaposi sarcoma tumors.

In late stage of Kaposi sarcoma, almost all Kaposi sarcoma 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 this study, we have used a tumor model with tight viral latency, which recapitulates the pathologic and virologic features of late Kaposi sarcoma tumors (18). We have previously shown that all viral latent products are essential for the development of Kaposi sarcoma–like tumors in this model. LANA and vCyclin promote cellular transformation and tumorigenesis by hijacking the BMP-Smad1–5/8 pathway and inhibiting p27KiP1-mediated contact inhibition, respectively (19, 36) while vFLIP and miRNs promote cell survival and homeostasis by activating the NFκB 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). As KSHV miRNA cluster mediates activation of the NFκB 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 14-3-3 protein, a master negative regulator of the NF-κB pathway (37), while miR-K3 targets GRK2 to induce CXCR2 and releases 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 IL10, IL6, IL2, IL13, IFNγ, TNFα, MCP1, and RANTES in endothelial cells (43). However, we have shown that deletion of vFLIP from the KSHV genome does not affect the basal inflammation and induction of proinflammatory cytokines (Fig. 5A–C).

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 IL6R (45). Kaposin B and miR-K6-3p activates STAT3 by inducing its phosphorylation and targeting negative regulator SH3BGR, respectively (46, 47). vIL6 also activates the STAT3 pathway through the gp130 subunit (48). In contrast to vIL6, activation of the STAT3 pathway by cellular IL6 requires both gp80 and gp130 of IL6R. 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 IL6 but not vIL6. Interestingly, IL6 is present in Kaposi sarcoma tumors (28), and abundant in PEL, MDC, and AIDS-Kaposi sarcoma (29, 30). Therefore, within a latently infected cell, we hypothesized that as a result of TLR4 activation, cellular IL6 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. First, 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 cannot exclude a role of endotoxin in the activation of TLR4. Finally, 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-Kaposi sarcoma 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 coinfections 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-Kaposi sarcoma patients, which should reveal their roles in the initiation and progression of AIDS-associated malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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