

Upregulation of the TLR3 Pathway by Kaposi's Sarcoma-Associated Herpesvirus during Primary Infection[∇]

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Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with several different human malignancies, including Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. KSHV establishes lifelong latency in the host and modulates the host immune response. Innate immunity is critical for controlling de novo viral infection. Toll-like receptors (TLRs) are key components of the innate immune system, and they serve as pathogen recognition receptors that stimulate the host antiviral response. In particular, TLR3 has been implicated in RNA virus recognition. Currently, there is no information regarding how KSHV infection modulates any TLR pathway. We report the first evidence that KSHV upregulates TLR3 expression in human monocytes during primary infection. This is also the first demonstration of a human DNA tumor virus upregulating TLR3, a TLR that thus far has been associated with the recognition of RNA viruses. We found that KSHV upregulates the TLR3 pathway and induces TLR3-specific cytokines and chemokines, including beta 1 interferon (IFN- β 1) and CXCL10 (IP-10). Small interfering RNAs directed against TLR3 greatly reduced the ability of KSHV to upregulate IFN- β 1 and CXCL10 upon infection.

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma, the most common neoplasm of human immunodeficiency virus (HIV)-infected patients (21, 53). KSHV is also the causative agent of two lymphoproliferative diseases, primary effusion lymphoma and multicentric Castleman's disease (1, 21, 39, 53). KSHV, also named human herpesvirus 8, is a gamma-2 herpesvirus that can establish lifelong latency in its host. In order to enter the latent phase following primary infection, the virus must modulate the host innate immune response to allow for the establishment of latency (23, 40, 44).

Toll-like receptors (TLRs) are one of the key components of the innate immune system. TLRs are classified as pattern recognition receptors, as they recognize invading pathogens (2, 3, 55). Currently, there are 10 identified human TLRs. The TLR family of proteins are transmembrane proteins with an immunoglobulinlike extracellular domain and an intracellular domain which contains Toll/interleukin-1 (IL-1) receptor (TIR) domains through which the TLRs initiate their cell signaling cascade (55). All TLRs contain anywhere from 21 to 25 leucine-rich repeats in their extracellular domains which recognize and bind to pathogen-associated molecular patterns on the surface of the incoming pathogen (10). However, not all TLRs are expressed at the cell surface. TLR3, -7, -8, and -9 are all expressed in intracellular compartments (4, 5). Current research has identified TLR2, -3, -4, -7, -8, and -9 as being involved in the recognition of viruses through binding to RNA, DNA, or viral glycoproteins (4, 5, 34). Thus far, TLR3 and TLR7 have been reported to be induced by RNA. TLR3 can

recognize single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), while TLR7 can recognize ssRNA (2, 5, 7, 18, 32, 33, 49, 50, 54). Both of these ligands (ssRNA and dsRNA) are common intermediates in RNA virus replication. TLRs can also recognize DNA viruses and DNA motifs; for example, TLR9 recognizes CpG DNA motifs (8, 30). There is recent evidence implicating a role for TLRs in herpesvirus infection. TLR2 and TLR4 have been shown to recognize several herpesviruses, including herpes simplex virus 1 (HSV-1) and human cytomegalovirus (12, 22, 29, 66). Epstein-Barr virus has recently been shown to upregulate TLR7 (38). Additionally, Zhang et al. have recently reported that TLR3 deficiency in humans leads to the uncontrolled spread of HSV-1 and to HSV-1 encephalitis (68).

There are two basic pathways that TLRs use for signaling, one being dependent on the adaptor protein MyD88 and the other being a MyD88-independent pathway (3, 4). All TLRs, with the exception of TLR3, signal through the MyD88-dependent pathway. TLR3 is unique in that the initial signaling cascade begins with the binding of TRIF (the TIR domain-containing adaptor inducing beta interferon [IFN- β]) to the TIR binding domains within the intracellular domains of TLR3 (31, 67). TRIF, as indicated by its name, has been shown to be responsible for the activation of IFN- β and also for nuclear factor kappa B (NF- κ B), both of which are important immunoregulatory proteins (4, 7). TLR3 can activate cytokines downstream via two separate pathways, one involving interferon regulatory transcription factor 3 (IRF3) and IRF7 and the other involving TRAF6 and the interleukin receptor-associated kinase family of kinases (3, 4, 49). The result of both of these pathways is the upregulation of signature cytokines and chemokines, primarily CXCL10, CCL-2, and IL-6, among others (18, 42). Activation of the interferon response is also a consequence of TLR3 activation, as it is for the activation of

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most TLRs (4). The role of TLRs in response to KSHV infection has not yet been elucidated.

Previous studies have shown that KSHV can successfully infect human monocytes and macrophages both in vitro and in vivo (11, 20). Blasig et al. detected viral replication in KSHV lesions and identified the cells of origin as monocytes within the lesion (11). Caselli et al. showed that coinfection of monocytes with KSHV and HIV greatly increased the replication of HIV in the presence of KSHV (20). Rappocciolo et al. reported that KSHV uses DC-SIGN to enter monocyte-derived macrophages and dendritic cells (43). Because monocytes are infected in vivo by KSHV, we chose to analyze the TLR response to KSHV infection in THP-1 monocytes as well as in primary human monocytes.

In this study, we present evidence that KSHV infection results in the upregulation of TLR3 expression in THP-1 monocytes and primary human monocytes. This is the first evidence that a human DNA tumor virus specifically upregulates TLR3, which until now has only been shown to signal in response to RNA viruses. We also show that as a direct result of this TLR3 activation, there is strong upregulation of signature cytokines and chemokines in KSHV-infected cells, in particular CXCL10, a potent chemoattractant of monocytes, T cells, and neutrophils.

MATERIALS AND METHODS

Cell culture. THP-1 monocytes were maintained in RPMI 1640 medium (Cellgro) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P-S), and 0.05 M 2-mercaptoethanol. Vero cells stably expressing KSHV-green fluorescent protein were the kind gift of Jeff Vieira (63). They were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cellgro) containing 10% FBS, 1% P-S, and 0.005 mg/ml of puromycin (for selection). HEK293 cells and HEK293/TLR3 cells were maintained in DMEM containing 5% FBS and 1% P-S and in DMEM containing 5% FBS, 1% P-S, and 0.005 mg/ml of Blasticidin (InvivoGen), respectively. Sf9 cells were maintained in Sf-900 serum-free medium containing amphotericin B (0.25 µg/ml) and P-S (0.1%). Sf9 cells were grown at 30°C under 5% CO₂ in a monolayer.

Virus production and purification. A baculovirus vector encoding KSHV ORF50/Rta was the kind gift of Jeff Vieira (63). Baculovirus ORF50 was amplified in Sf9 cells for 72 h, after which time the cell debris was pelleted and the supernatant stored at 4°C. Vero cells stably expressing KSHV-green fluorescent protein were then infected with baculovirus ORF50 in DMEM (without phenol red) containing 2% FBS, 1% P-S, and 17 µl of 1 M sodium butyrate to allow reactivation of the latent KSHV. After 72 h, the supernatant was harvested, cell debris pelleted, and the supernatant filtered through a sterile 0.45-µm filter. In order to concentrate the virus, 28 ml of supernatant was layered over an 8-ml cushion of 20% sucrose (optical grade; Sigma), and the virus was pelleted by ultracentrifugation in an SW28 swinging-bucket rotor for 5 h at 4°C in a Beckman ultracentrifuge. The supernatant was decanted, and the virus pellet was resuspended in sterile phosphate-buffered saline in 1% of the original volume. The UV inactivation of KSHV was measured by infecting HEK293 cells with either wild-type (WT) or UV-inactivated recombinant KSHV virus and by determining the green fluorescence for 1 to 5 days postinfection. While the WT KSHV-infected HEK293 cell line showed infected green cells 24 h postinfection, the UV-inactivated virus did not show any infected green cells 5 days postinfection.

THP-1 and primary human monocyte infections. THP-1 cells were infected via a method called "spinoculation." A total of 1×10^6 THP-1 cells were plated in a six-well dish in supplement-free RPMI 1640 medium. Either 2.5 ml (nonconcentrated virus) or 100 µl (concentrated virus) was added to a total volume of 3 ml/well. Polybrene (4 µg/µl, 1,000×) was added to a final concentration of 1×10^6 . The cells were centrifuged in a tabletop centrifuge for 90 min at 2,500 rpm and 30°C. Following centrifugation, the inoculum was removed and replaced with fresh RPMI 1640 medium (no supplement). The infected cells were incubated overnight (16 h) at 37°C. This protocol was also followed for HEK293 and HEK293/TLR3 cell infections with KSHV. In certain instances, virion pellets were treated with 2 µl of RNase A (Sigma) per 100 µl of virus or 2 µl of buffer alone for 1 h at 37°C. For experiments with small interfering RNA (siRNA),

THP-1 cells were nucleofected with a plasmid containing an siRNA specific to TLR3, psiTLR3 (InvivoGen), along with a control plasmid containing an siRNA specific to the luciferase gene, psiLUC (InvivoGen). Twenty-four hours later, these cells were infected with KSHV. This protocol was also used in the analysis of the THP-1 cell response to poly(I:C) treatment following transfection with psiTLR3 and psiLUC. Three micrograms of each siRNA plasmid was transfected into THP-1 cells, followed by treatment with poly(I:C) (75 µg/ml) at 48 h posttransfection. Primary human monocytes were infected in the same manner as described above except that 1×10^5 CD14-positive primary human monocytes (purchased from Lonza, Inc.) were either mock infected or KSHV infected. Following infection, the cells were maintained in 12% FBS plus 10 ng/ml mononuclear phagocyte colony-stimulating factor for 16 h.

First-strand synthesis and quantitative real-time PCR (qPCR). Following infection, RNA from THP-1 cells was isolated using Trizol (Invitrogen). RNA was resuspended in 50 µl of RNase-free water and treated with 1 unit of RQ1 DNase (Promega) for 1 h at 37°C. Following DNase treatment, the RNA samples were phenol-chloroform extracted and precipitated.

RNA was prepared for first-strand synthesis by the resuspension of the RNA pellet in 17 µl of RNase-free water. The first-strand synthesis was carried out using a first-strand synthesis kit (SuperArray catalogue no. C-01) per the manufacturer's instructions. The first-strand reaction mixture was increased to a total volume of 100 µl with distilled water. The SuperArray TLR PCR array (APH-018) was used to analyze the cDNA obtained from infected cells for the activation of the TLRs or any of the downstream genes in each of the pathways. Each 96-well plate contained 84 genes related to the human TLR pathway. Plates were prepared according to the manufacturer's instructions. The plates were run on an ABI 7000 or ABI 7300 machine, using the following protocol: 95°C for 10 min (1 cycle), 95°C for 15 seconds and 60°C for 1 min (data collection point) (40 cycles), followed by a dissociation profile and 95°C for 15 seconds, 60°C for 1 min, and 95°C for 15 seconds (1 cycle). All activation increases were normalized to β-actin expression in the samples. The qPCR profiling of the TLR pathway genes after KSHV infection of THP-1 cells was repeated more than 15 times.

CXCL10 (IP-10) ELISA. An enzyme-linked immunosorbent assay (ELISA) specific for CXCL10 (human IP-10) (BD) was used to analyze the amount of protein secreted from KSHV-infected THP-1 cells. Briefly, plates were coated overnight with capture antibody, washed the following morning, and blocked with phosphate-buffered saline-Tween. Samples were then added and incubated for 2 h at room temperature. After 2 h, the plate was washed and an antibody-enzyme solution was added, per the manufacturer's instructions, for 1 hour. The plate was then washed, and substrate solution (ABTS [2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)]) (Pierce) was added for 30 min, after which time the absorbance at 405 nm was determined. The standard (provided by the manufacturer) was plotted, and the concentration for each sample was determined based on the standard curve.

siRNA inhibition of TLR3. THP-1 cells were nucleofected (amaxa) with either 3 or 8 µg of the psiTLR3 (InvivoGen) or psiLUC (InvivoGen) plasmid. At 48 h posttransfection, cells were harvested for RNA analysis as described above or for protein analysis, in which case cells were harvested using radioimmunoprecipitation buffer. Protein was quantified by the Bradford assay (13a), and equal amounts of protein were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Western blotting was performed with an anti-TLR3 antibody (InvivoGen).

Luciferase assay. Luciferase assays were performed to measure the activation of NF-κB in response to KSHV infection in HEK293 cells stably transfected with TLR3. The plasmid, pGL3-ELAM-luc, was obtained from Addgene (Addgene plasmid 13029). A total of 1×10^5 HEK293 or HEK293/TLR3 cells were subjected to FuGENE 6 transfection with pELAM NF-κB (13). At 24 h posttransfection, the cells were infected via spinoculation as described above. Sixteen hours postinfection, the cells were lysed, and the lysates were analyzed as described previously (13). This assay was also performed for HEK293 and HEK293/TLR3 cells following treatment with poly(I:C) at a concentration of 50 µg/ml. All luciferase values were normalized to the protein amount. The same protocol was used to measure KSHV activation of NF-κB in THP-1 cells. Briefly, THP-1 cells were transfected with the pELAM NF-κB plasmid for 48 h, at which time the cells were infected with KSHV for 24 h. The total protein was isolated from the cells at 24 h postinfection and subjected to a luciferase assay.

Cytokine array analysis. A human cytokine antibody array (RayBiotech) was used to quantify the overall activation pattern following KSHV infection of monocytes. The protocol was performed as described by the manufacturer. Briefly, each array membrane was blocked and then incubated for 2 h at room temperature with undiluted supernatant collected from either KSHV-infected THP-1 cells or mock-infected THP-1 cells. The arrays were then washed and incubated with biotin-conjugated antibody solution, specific to each array, for

TABLE 1. TLR pathway genes activated upon KSHV infection of monocytes

Gene product	Function	Increase (fold) in mRNA level ^a
TLR3	Toll-like receptor	9.71
CXCL10	Chemokine	743.06
IFN- β 1	Interferon	34.3
CCL2	Chemokine	29.18
IRF1	Interferon regulatory factor	12.81
IL-6	Cytokine	6.73
EIF2AK2/PKR	RNA-activated kinase	11.26
MyD88	TLR adaptor molecule	5.92
CD80	B-lymphocyte activation antigen	11.36

^a Over that in mock-infected cells.

2 h. The membranes were washed again and then incubated with a horseradish peroxidase-conjugated streptavidin solution for 2 h, followed by a wash step. Arrays were exposed to detection buffer and then exposed to X-ray film. Quantitation of the individual dots was done using the ImageJ program.

RESULTS

Activation of the TLR3 pathway upon KSHV infection of THP-1 monocytes. To determine the effect of KSHV infection on the TLR pathway in monocytes, we first analyzed the mRNA expression levels of all 10 TLRs in THP-1 cells in response to KSHV infection. A total of 1×10^6 THP-1 monocytes were infected with WT recombinant KSHV virus (rKSHV.219) (63). Infections were carried out via spinoculation, and the RNA was isolated at 16 h postinfection. RNA was reverse-transcribed to cDNA. Subsequent to first-strand synthesis, qPCR was performed using an array containing all 10 human TLRs and 74 additional genes involved in the different TLR signaling pathways. All data were normalized to those for β -actin, used as a control. To ensure that the THP-1 cells were infected with KSHV, qPCR for the latency-associated nuclear antigen (LANA) was performed, and LANA mRNA expression was detected 16 h postinfection only in the KSHV-infected THP-1 cells at a sixfold higher level than that of mock-infected cells (data not shown). LANA mRNA has previously been shown to be expressed very early after primary infection in other cell types (35). Analysis of the TLR qPCR array suggested that TLR3 expression was specifically upregulated in response to KSHV infection of monocytes (Table 1). The upregulation of TLR3 was consistently between 8- and 15-fold greater than that of mock-infected cells. To confirm that our activation of TLR3 was specific to the infectious virus, we UV inactivated KSHV prior to the infection of THP-1 cells and performed the same infection followed by qPCR evaluation. THP-1 cells infected with UV-inactivated KSHV showed no significant upregulation of TLR3 (or any other TLRs), indicating that the activation observed in our experiments is due primarily to infectious virus (Table 1).

In order to confirm the upregulation of TLR3 by KSHV, we also performed conventional reverse transcriptase PCR (RT-PCR) with RNA from WT and UV-inactivated, KSHV-infected THP-1 cells with a different set of TLR3-specific primers. Using this independent assay, we again found that KSHV infection specifically upregulated the expression of TLR3 (Fig. 1A).

Since TLR3 can be activated by RNA molecules, we wanted to test the possibility that our virion preparation and purification

procedures were contaminated with free RNAs. We reasoned that viral RNAs that were present inside the infectious virion (9, 14) would be protected from RNase treatment but that any contaminating, superfluous RNAs that had copurified with our infectious virions would not be protected from RNase treatment. We pelleted KSHV virions through a sucrose cushion and then split the pellet in half. One fraction was treated with RNase and incubated at 37°C for 1 hour while the other fraction was incubated with buffer without RNase. We next infected THP-1 cells with the RNase-treated and untreated virion samples. Cells were harvested at 16 h postinfection, and RNA was isolated and subjected to real-time PCR as described above. Figure 1B shows the relative mRNA expression level of TLR pathway genes in cells infected with RNase-treated virus or untreated virus. As shown in Fig. 1B, the expression levels of all TLR pathway genes, including TLR3, were very similar, and RNase treatment of the virus did not reduce TLR3 or CXCL10 mRNA levels. This suggests that KSHV is truly activating TLR3 expression and that TLR3 upregulation is not due to contaminating RNAs present in the viral preparation. We also detected a very modest (less-than-threelfold) upregulation of mRNAs of other endosomal TLRs, including TLR7, TLR8, and TLR9 (data not shown), but the upregulation was insignificant compared to TLR3 induction levels.

KSHV activates TLR3-responsive CXCL10, IFN- β 1, CCL2 (MCP-1), and IRF-1 genes. Although increased TLR3 gene expression is not a requirement for TLR3 activation, it appears that KSHV specifically upregulates TLR3 at the transcriptional level, leading to the activation of downstream targets of TLR3, such as CXCL10, IFN- β 1, CCL2 (monocyte chemoattractant protein 1 [MCP-1]), and IRF-1.

CXCL10 (or IP-10) is a member of the CXC family of chemokines and is a strong attractant of Th1 lymphocytes and monocytes (57, 58). CXCL10 induction upon the treatment of cells with the dsRNA homolog poly(I:C) is characteristic of TLR3 activation (25, 45, 48). CXCL10 has also been shown to be induced in astrocytes upon treatment with poly(I:C), and CXCL10 secretion upon respiratory syncytial virus infection of MRC-5 cells has been shown to be dependent on TLR3 stimulation (45). Interestingly, Teruya-Feldstein et al. have also found that CXCL10 was associated with Epstein-Barr virus lymphoproliferative diseases, such as lymphomatoid granulomatosis, nasal T-cell or natural killer (NK)-cell lymphomas, and Hodgkin's lymphoma (59, 60).

In this study, we show that CXCL10 is dramatically upregulated in response to KSHV infection of monocytes (Table 1). qPCR analysis showed that the mRNA level of CXCL10 was increased \sim 700-fold more than that of mock-infected THP-1 cells. Furthermore, in cells infected with UV-inactivated KSHV, there was no induction of CXCL10, similar to the outcome for mock-infected cells. To confirm our results, we performed conventional RT-PCR with a different set of CXCL10 primers with RNA isolated from WT and UV-infected THP-1 cells and found that the mRNA levels of CXCL10 were vastly increased in WT KSHV-infected THP-1 cells (Fig. 1A).

To confirm this observation at the protein level, we performed a sandwich ELISA assay for CXCL10. The plates were coated overnight, blocked the following day, and then incubated with supernatant collected from KSHV-infected THP-1 cells. As

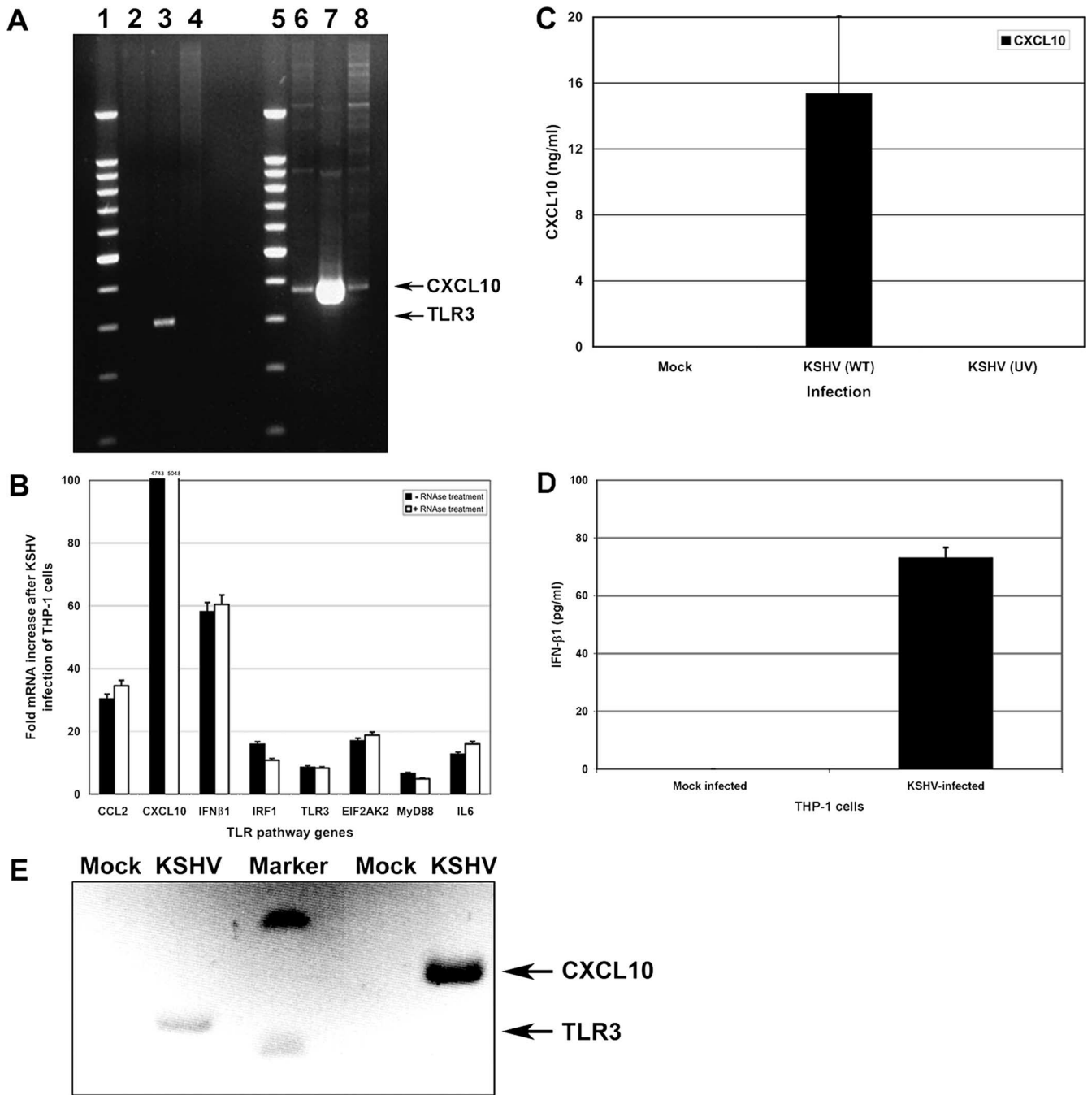


FIG. 1. KSHV activates the gene expression of the TLR3 pathway. (A) Conventional RT-PCR of TLR3 and CXCL10 from mock-infected, KSHV-infected, or UV-irradiated and KSHV-infected THP-1 cells. Lanes: 1, 1-kb ladder; 2, TLR3, mock infected; 3, TLR3, KSHV infected; 4, TLR3, UV irradiated and KSHV infected; 5, 1-kb ladder; 6, CXCL10, mock infected; 7, CXCL10, KSHV infected; 8, CXCL10, UV irradiated and KSHV infected. (B) RNase treatment of KSHV virions does not alter the TLR3 activation profile. Shown are the mRNA increases after the infection of THP-1 cells with WT KSHV and RNase-treated KSHV. There was no significant difference between the mRNA levels of the activated genes in the KSHV-infected cells and those of cells infected with RNase-treated KSHV virus. (C) Comparison of CXCL10 protein secretion levels from mock-infected, KSHV-infected, and UV-irradiated KSHV-infected cells. KSHV-infected cells consistently produced an average of 15 to 20 ng/ml of CXCL10. The mock-infected and the UV-irradiated and KSHV-infected cells did not secrete any CXCL10 protein. (D) IFN- β 1 secretion from mock-infected and KSHV-infected cells. KSHV-infected THP-1 cells produced approximately 65 pg/ml of IFN- β 1. The mock-infected and the UV-irradiated KSHV-infected cells did not secrete IFN- β 1. (E) RT-PCR of TLR3 and CXCL10 from mock- and KSHV-infected CD14-positive primary human monocytes.

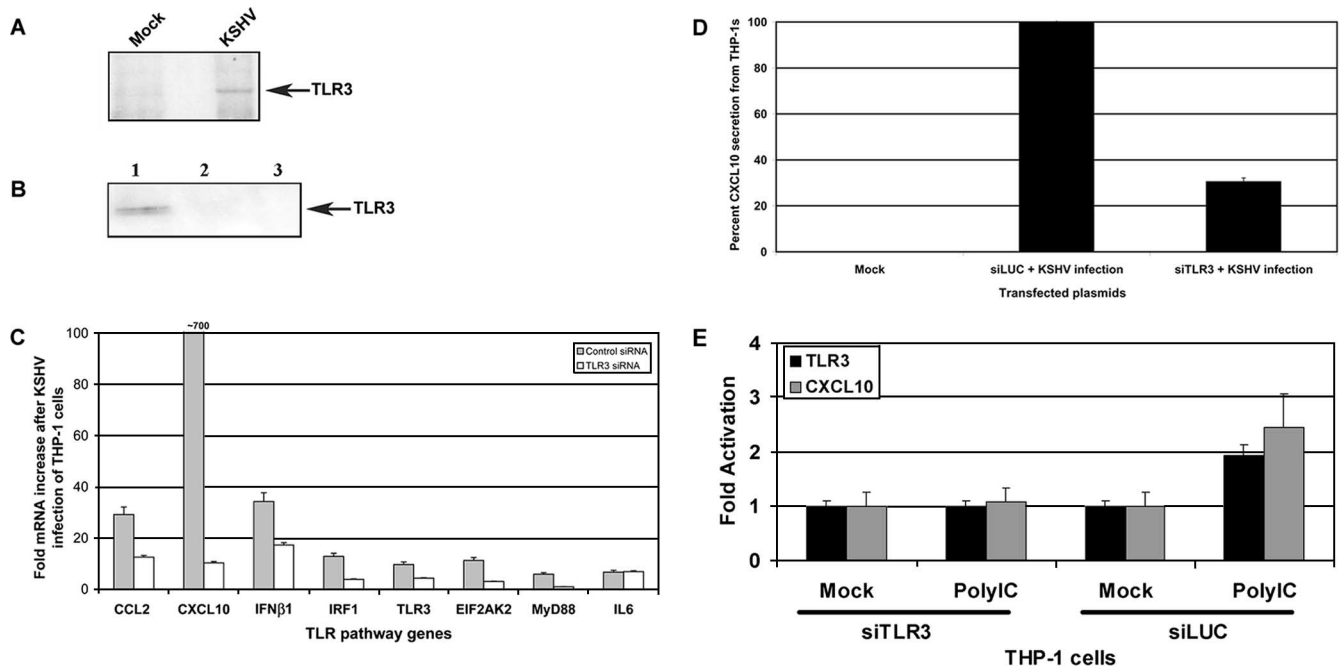


FIG. 2. siRNA against TLR3 diminishes the induction of CXCL10, IFN- β 1, and IRF-1 gene expression from KSHV-infected THP-1 cells. (A) Western blot of TLR3 protein levels in KSHV-infected or mock-infected THP-1 cells. (B) THP-1 cells were nucleofected with a control luciferase siRNA plasmid, psiLUC (lane 1), or a TLR3-specific siRNA plasmid, psiTLR3 (lanes 2 [3 μ g psiTLR3] and 3 [8 μ g psiTLR3]), followed by KSHV infection and Western blotting for TLR3. (C) THP-1 cells were nucleofected with 3 μ g control siRNA or a TLR3-specific siRNA plasmid, psiTLR3. Real-time PCR profiling of TLR pathway genes indicated a significant reduction in the increase of mRNA upon pretreatment of THP-1 cells with the siRNA specific to TLR3, indicating that these downstream genes are specifically activated through TLR3 in response to KSHV infection. (D) THP-1 cells were nucleofected with 3 μ g control siRNA (siLUC) or TLR3-specific siRNA (psiTLR3). Cells were infected with KSHV, and CXCL10 was measured 16 h postinfection. The CXCL10 ELISA shows a 70% decrease in CXCL10 secretion from KSHV-infected THP-1 cells transfected with psiTLR3 compared to cells transfected with psiLUC. (E) THP-1 cells were transfected with 3 μ g of psiTLR3 or psiLUC. At 48 h posttransfection, cells were treated with poly(I:C) for 24 h. RNA was harvested and subjected to RT-PCR using TLR3 and CXCL10 primers.

shown in Fig. 1C, WT KSHV infection induced very high levels of CXCL10 in monocytes, in the range of 10 to 20 ng/ml.

In addition to CXCL10, we found that IFN- β 1, CCL2 (MCP-1), and IRF-1 mRNAs were all upregulated by KSHV infection of THP-1 cells compared to the levels in mock-infected cells (Table 1). IFN- β 1 is a member of the IFN family of proteins and is involved in the innate immune response; specifically, activation of IFN- β 1 is induced upon activation of TLRs. Multiple studies have determined that IFN- β 1 is upregulated in response to TLR3 activation (36, 42, 51, 62). Poly(I:C), a homolog of dsRNA which is known to stimulate TLR3, has been shown to induce IFN- β 1 in multiple cell lines. Astrocytes, human corneal epithelial cells, and human primary uterine epithelial cells all produce elevated levels of IFN- β 1 in response to the TLR3 ligand poly(I:C) (36, 42, 51, 62). We consistently observed an \sim 35-fold increase in the mRNA expression of IFN- β 1 (Table 1) upon infection of THP-1 cells with KSHV. We confirmed this increase of IFN- β 1 mRNA levels in monocytes by performing an IFN- β 1 ELISA (Fig. 1D). In KSHV-infected cells, approximately 70 pg/ml of IFN- β 1 was being secreted into the surrounding media at 16 h postinfection compared to no secretion of IFN- β 1 from mock-infected cells.

CCL2 (MCP-1) is a chemokine that has been implicated in the recruitment of a variety of immunoresponsive cells, including monocytes, T lymphocytes, and NK cells. There are also multiple examples of CCL2 activation in response to the TLR3 ligand

poly(I:C) (25, 51). Thus, TLR3 agonists lead to the upregulation of the chemokine CCL2. We consistently observed a 25- to 30-fold induction in the expression level of CCL2 in response to KSHV infection in THP-1 monocytes (Table 1).

IRF-1 is an interferon regulatory factor that has been shown to control the transcription of CXCL10 through binding to an IFN-stimulated response element (ISRE) on the promoter of CXCL10. IRF-1 has previously been shown to respond to TLR3 stimulation via poly(I:C) treatment in HT29 human intestinal epithelial cells (52). Furthermore, IRF-1 is essential for IFN- β 1 and CXCL10 expression after reovirus infection in human lung fibroblasts (28). This evidence points to a pivotal role for IRF-1 in TLR3 signaling, directly contributing to the upregulation of CXCL10. We consistently detected an \sim 12-fold increase in IRF-1 transcription upon the infection of THP-1 monocytes with KSHV (Table 1).

We have also examined the upregulation of TLR3 and CXCL10 upon KSHV infection of CD14-positive primary human monocytes (Fig. 1E) and found that there was a 15- and 1,200-fold upregulation in the levels of TLR3 and CXCL10, respectively, upon KSHV infection. Altogether, our RNA profiling and protein analyses are consistent with TLR3 upregulation upon KSHV infection of monocytes.

Specificity of TLR3 activation by KSHV. We performed Western blotting for TLR3 expression on KSHV-infected

THP-1 cells. We found that TLR3 protein levels were also increased in KSHV-infected THP-1 cells 16 hours postinfection compared to those in mock-infected cells (Fig. 2A).

We next confirmed that we could suppress the expression of the TLR3 protein via the expression of an siRNA plasmid specific to TLR3. THP-1 cells were nucleofected with different amounts of the TLR3-specific siRNA plasmid, psiTLR3, or a control luciferase-specific siRNA plasmid, psiLUC (Invivo-gen), prior to KSHV infection. After KSHV infection, cells were harvested and the cell lysates were analyzed by immunoblotting with a TLR3-specific antibody. As shown in Fig. 2B, the psiTLR3 plasmid (Fig. 2B, lanes 2 and 3) efficiently reduced the amount of TLR3 protein compared to that in the cells transfected with psiLUC (Fig. 2B, lane 1).

We next tested the specificity of TLR3 activation upon KSHV infection by knocking down the expression of TLR3 with siRNA against TLR3 in THP-1 monocytes prior to KSHV infection. THP-1 cells were nucleofected with 3 μ g of a TLR3-specific siRNA plasmid, psiTLR3 (InvivoGen), or psiLUC. Forty-eight hours posttransfection, THP-1 cells were infected with KSHV as described above. At 16 hours postinfection, the RNA was isolated and analyzed by both qPCR and conventional RT-PCR. Analysis by both methods (qPCR and conventional PCR) showed that in cells transfected with psiTLR3, the level of genes in the TLR3 pathway was significantly reduced compared to that of the cells transfected with psiLUC after KSHV infection. This included TLR3 itself and its downstream targets CXCL10, IFN- β 1, CCL2, and IRF-1 (Fig. 2C). Consistently, the cells transfected with psiTLR3 showed a significant reduction in mRNA expression levels. TLR3 mRNA expression was reduced \sim 3-fold, CXCL10 mRNA expression was reduced \sim 70-fold, IFN- β 1 mRNA expression was reduced \sim 2.5-fold, CCL2 mRNA expression was reduced \sim 3-fold, and IRF-1 mRNA expression was reduced 4-fold (Fig. 2C). Interestingly, we did not find that IL-6 mRNA levels were affected by TLR3-specific siRNA, suggesting that it is not upregulated as a consequence of TLR3 activity.

We also measured CXCL10 in cells that had been transfected with psiTLR3 or the control psiLUC by ELISA. We found that siRNA against TLR3 reduced the amount of CXCL10 protein production by 70% (Fig. 2D). Thus, KSHV specifically upregulates the TLR3 pathway in infected monocytes, and this activation can be inhibited by TLR3-specific siRNA treatment.

For a control, we performed the same analysis using the TLR3 agonist poly(I:C) instead of KSHV infection. THP-1 cells were transfected with 3 μ g of psiTLR3 or psiLUC. Forty-eight hours postinfection, cells were treated with 75 μ g/ml poly(I:C) for 24 h. RNA was harvested and subjected to qPCR using TLR3- and CXCL10-specific primers. We found that the psiLUC-transfected THP-1 cells showed approximately a 2.5-fold increase in CXCL10 and a 2-fold increase in TLR3 mRNA levels after poly(I:C) stimulation (Fig. 2E). This level of activation of TLR3-responsive genes upon poly(I:C) treatment of THP-1 cells corresponds well with what has previously been shown for the poly(I:C) stimulation of THP-1 cells (56). For example, Saitoh et al. have previously shown that the poly(I:C) stimulation of THP-1 cells induces only a twofold activation of an ISRE reporter construct through TLR3 signaling (46). This is in contrast to the poly(I:C) stimulation of HEK293/

TLR3 cells, which induces a $>$ 10-fold induction of the same ISRE reporter construct through poly(I:C)-mediated TLR3 signaling (46). Furthermore, the poly(I:C)-stimulated increase of TLR3 and CXCL10 was ablated in THP-1 cells treated with psiTLR3 (Fig. 2E), and the psiTLR3-transfected THP-1 cells were unable to respond to poly(I:C) stimulation.

KSHV infection induces NF- κ B activation in HEK293 cells expressing TLR3. One endpoint of TLR signaling is the activation of NF- κ B (3, 4). To determine whether KSHV infection of HEK293 cells would induce NF- κ B via the TLR3 signaling pathway, we used HEK293 cells stably transfected with TLR3. Equal amounts of both native HEK293 cells and TLR3-expressing HEK293 cells (HEK293/TLR3) were transfected with an NF- κ B luciferase reporter plasmid. At 24 h posttransfection, both cell lines were infected with KSHV. Cells were harvested at 16 h posttransfection, and luciferase assays were performed. On average, the KSHV-infected HEK293/TLR3 cells showed an approximately fivefold increase in luciferase activity compared to that in mock-infected HEK293/TLR3 cells (Fig. 3A). Additionally, KSHV-infected HEK293/TLR3 cells showed an approximately fivefold increase in NF- κ B compared to that in mock-treated HEK293 cells (Fig. 3B). As a control, we also treated HEK293 and HEK293/TLR3 cells with the TLR3 agonist poly(I:C). The poly(I:C)-treated HEK293/TLR3 cells showed an approximately eightfold increase in luciferase activity compared to that in mock-treated HEK293/TLR3 cells (Fig. 3A), and the same trend occurred in the poly(I:C)-treated HEK293/TLR3 cells compared to the HEK293 cells (Fig. 3B). These data demonstrate that KSHV triggers the TLR3 signaling pathway, leading to the upregulation of the transcription factor NF- κ B. We also infected THP-1 cells transfected with the NF- κ B reporter plasmid as described above. Briefly, THP-1 cells were transfected with 1 μ g of the pELAM-NF- κ B reporter plasmid and incubated for 48 h. The transfected cells were subsequently infected with KSHV and incubated for another 24 h. At 24 h postinfection, the total protein was harvested and subjected to a luciferase assay. We found that KSHV infection induced a 2.5-fold increase in NF- κ B activity in infected THP-1 cells compared to that in mock-infected cells (Fig. 3C).

KSHV infection of THP-1 monocytes upregulates the production of a multitude of cytokines, chemokines, and growth factors. From our TLR profiling, we found that KSHV upregulates the expression of TLR-responsive cytokines/chemokines, and we next queried whether the virus was activating a wider range of cytokines. In order to do this, we analyzed the supernatants of KSHV-infected THP-1 cells for the upregulation of a large number of secreted cytokines, chemokines, and growth factors by using antibody arrays. Infections were carried out as described above, and the supernatants from the KSHV-infected cells were analyzed by a cytokine antibody array. Antibody arrays spotted with 120 cytokine antibodies were blocked, washed, and incubated with the supernatants from the virus-infected THP-1 cells. Following incubation with the supernatants, the blots were washed again and probed with primary antibody, followed by probing with secondary antibody. As shown in Table 2, approximately 12 cytokines, chemokines, and growth factors were upregulated in response to KSHV infection of monocytes. Included among those were CCL2 (MCP-1) and IL-6, both of which were also increased at the

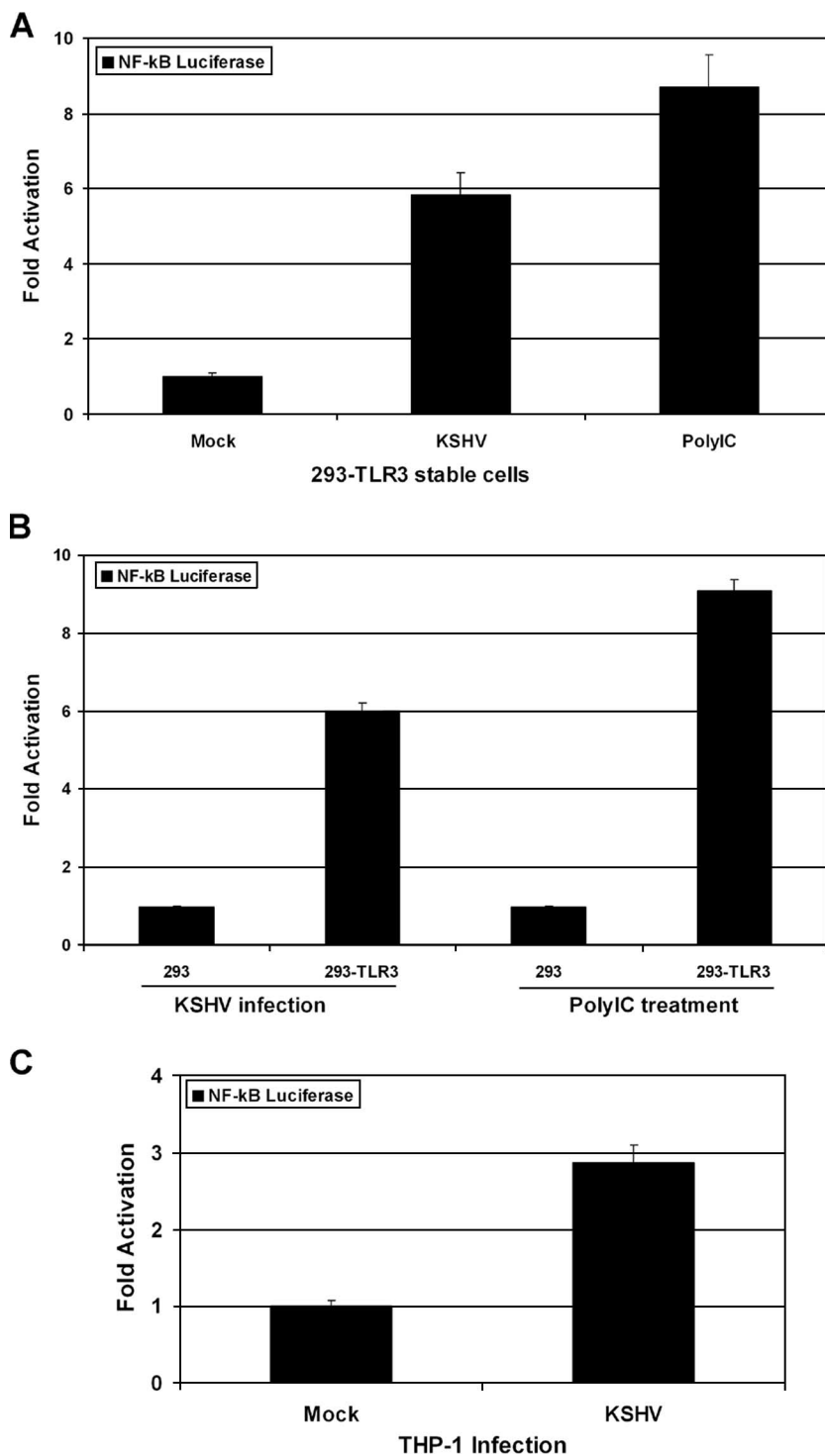


FIG. 3. KSHV infection of HEK293/TLR3 (293-TLR3) cells leads to NF- κ B activation. (A) Activation of an NF- κ B-driven luciferase construct in response to KSHV infection of HEK293/TLR3 stable cells. HEK293/TLR3 stable cells were transfected with an NF- κ B-luciferase reporter plasmid, followed by mock infection or infection with KSHV. KSHV-infected cells showed a fivefold increase in luciferase activity compared to that in the mock-infected cells, indicating the activation of NF- κ B in HEK293/TLR3 cells upon infection with KSHV. Cells treated with the TLR3 agonist poly(I:C) showed an 8.5-fold increase in luciferase activity compared to that in the mock-infected cells, confirming that TLR3 stimulation in HEK293/TLR3 cells leads to NF- κ B activation. (B) Activation of an NF- κ B-driven luciferase construct in response to KSHV infection of HEK293 versus HEK293/TLR3 cells. HEK293 and HEK293-TLR3 cells were transfected with an NF- κ B-luciferase reporter plasmid and infected with KSHV 24 h posttransfection. Cells stably expressing TLR3 showed a fivefold increase in luciferase activity compared to the activity in HEK293 cells after infection with KSHV, indicating a specific role for TLR3 induction of NF- κ B. Similarly, poly(I:C)-treated HEK293/TLR3 cells showed an 8.5-fold increase in NF- κ B activity compared to that in poly(I:C)-treated HEK293 cells. (C) Activation of an NF- κ B-driven luciferase construct in response to KSHV infection of THP-1 cells. KSHV-infected THP-1 cells were transfected with the NF- κ B reporter plasmid for 48 h. The cells were then infected with KSHV for 24 h. Twenty-four hours postinfection, the total protein was harvested and the NF- κ B luciferase activity was measured. The KSHV-infected THP-1 cells showed a 2.5-fold increase in NF- κ B activity compared to that in mock-infected THP-1 cells transfected with the same NF- κ B reporter plasmid.

TABLE 2. Increase in protein levels after KSHV infection of monocytes

Protein ^a	Function	Increase (fold) in protein level
PDGF-BB	Growth factor	2.23
AgRP	Neuropeptide	2.38
GRO- α,β,γ	Chemokines	2.81
GRO- α	Chemokine	2.80
HCC-4	Chemokine	2.85
Oncostatin M	Cytokine	6.00
PIGF	Growth factor	5.50
VEGF	Growth factor	3.00
VEGF-D	Growth factor	8.70
MCP-1	Chemokine	2.70
IL-6	Cytokine	2.40
Leptin	Adipokine	3.50

^a PDGF-BB, platelet-derived growth factor BB; AgRP, agouti-related protein; PIGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGF-D, vascular endothelial growth factor D.

mRNA level in response to KSHV infection of THP-1 monocytes. In addition to CCL2 and IL-6, we found eight other cytokines and growth factors whose expression levels were increased upon KSHV infection. These include platelet-derived growth factor BB, placental growth factor, vascular endothelial growth factor, vascular endothelial growth factor D, and the cytokines/chemokines GRO- α,β,γ , GRO- α , HCC-4, and oncostatin M. Additionally, the neuropeptide agouti-related protein and the adipokine leptin were also activated upon KSHV infection of monocytes.

DISCUSSION

Here we report the first evidence of a role for TLRs in the host response to infection by KSHV. The data presented above demonstrate that KSHV stimulates TLR3 gene expression upon infection of THP-1 and CD14-positive primary human monocytes. This is also the first evidence of a human DNA tumor virus activating the expression of TLR3, a TLR that hitherto has been implicated only as a sensor of RNA viruses. There are several possible explanations for how KSHV is activating TLR3. Most likely is the possibility that a viral gene product may directly activate TLR3 transcription, since the UV inactivation of KSHV did not result in TLR3 upregulation. This is consistent with the need for infectious virus to upregulate TLR3 activation, as has been previously shown for influenza A virus infection of lung epithelial cells. Only viable influenza A virus, not UV-inactivated influenza A virus, could activate TLR3 signaling (27). Another possibility is that since TLR3 resides in the endosomal membrane and the endoplasmic reticulum and since KSHV has previously been shown to enter cells through endocytosis (6), the virus may encounter TLR3 in the endosome. Human cytomegalovirus contains RNAs in the virion (14), and recently, Bechtel et al. have shown that KSHV encapsidates 11 specific viral transcripts in its virion (9). These viral RNAs could activate TLR3 upon viral entry and uncoating in the endosome. It is even plausible that the newly discovered KSHV microRNAs (17, 47) are contained in the KSHV virion and that they activate TLR3. Regardless of the mechanism, recent clinical data prove that TLR3 can control herpesvirus infection, since children with a

TLR3 deficiency were very susceptible to HSV-1-induced encephalitis (68). Hence, the fact that our data demonstrate that KSHV activates the TLR3 pathway may be very relevant with respect to KSHV infection and herpesviral infection in general.

We found that TLR3 upregulation by KSHV in monocytes induces a number of cytokines and chemokines, including IFN- β 1, CCL2, and CXCL10. Previous work has shown that CXCL10 is specifically upregulated through the activation of TLR3 (36, 41, 42, 45). CXCL10 mRNA upregulation was the most significant, with up to a 743-fold increase over that of mock-infected cells. This was much higher than the upregulation of poly(I:C)-treated THP-1 cells. We also observed a concomitant increase in CXCL10 protein levels upon KSHV infection compared to those of mock-infected monocytes. We surmise that CXCL10 may play a role in the recruitment of inflammatory cells to the site of KSHV infection *in vivo*, since CXCL10 is a chemoattractant for monocytes (36, 41, 42, 45). A previous study has established some precedence for a role for CXCL10 in aiding the maintenance of viral latency. Theil et al. showed that CXCL10 (IP-10) mRNA expression levels are strongly induced in HSV-1-infected trigeminal ganglia and that this chronic immune response seems to aid the maintenance of viral latency (61). It is possible that CXCL10 plays a similar role in KSHV infection.

The upregulation of IFN- β 1, a potent inducer of the antiviral response, is a direct result of the activation of the TLR3 pathway by KSHV, as evidenced by the inhibition of IFN- β 1 when cells were transfected with TLR3-specific siRNA prior to KSHV infection. Upregulation of IFN- β 1 can also be directly linked to TLR3 activation (36, 42, 51, 62). CCL2, for which we also observed a strong induction of mRNA levels, is a potent attractor of NK cells and T lymphocytes (19, 26, 37). In the context of KSHV infection, CCL2 (MCP-1) has been shown to play a significant role. Acute KSHV infection of endothelial cells promotes a strong CCL2 induction, leading to increased inflammation and the promotion of angiogenesis (20). All of this evidence taken together suggests that the activation of CCL2 (MCP-1) upon KSHV infection is advantageous to KSHV and may promote angiogenesis and the spread of the virus.

IRF-1 is known to exert transcriptional control over CXCL10, TLR3, and IFN- β 1 (16, 28), all of which were observed to be upregulated in KSHV-infected monocytes. It is possible that TLR3 activation stimulates IRF-1, which in turn activates both CXCL10 and IFN- β 1, leading to the promotion of the inflammatory response and a positive feedback loop.

Why does KSHV induce the TLR3 pathway? One strategy for viruses to establish latency is to stimulate an immune response, which can lead to cytokine release and NF- κ B activation. Indeed, NF- κ B, a downstream target of TLR3 activation, is critical for the establishment of KSHV latency (15). The goal of KSHV is to establish a latent infection that will persist within the host and last for the lifetime of the host. It is also possible that KSHV, via the activation of TLRs and specifically TLR3 in monocytes, initiates the inflammatory response to recruit cells to the site of infection, where it can spread and establish latency. Such a scenario has been reported for West Nile virus (WNV), in which TLR3 appears to enhance the ability of WNV to replicate and spread in the brain (64). Wang et al. suggested that TLR3 mediates an innate immune re-

sponse, which is used by WNV to gain entry into the central nervous system and induce encephalitis (64). We propose that KSHV capitalizes on TLR3's activation of CXCL10 and CCL2 to spread to other cells through CXCL10-induced chemotaxis of immune cells to the site of infection and through CCL2-mediated angiogenesis and vascularization. We find that four downstream TLR3 effectors (CXCL10, IRF-1, CCL2, and IFN- β 1) are specifically upregulated by KSHV infection. These effectors are known to be involved in the initiation or progression of the inflammatory response.

It is important to note that this induction is seen upon primary infection of monocytes. At later times, it is likely that these proinflammatory genes are downregulated, since KSHV encodes a number of viral genes that aid in the suppression of the antiviral response (23, 40, 44). We speculate that activation of an innate immune response by KSHV might actually aid the virus in the establishment of latency. If there were no innate immune response to KSHV infection, the virus would replicate and likely prove fatal for the host, as has been suggested for the infection of human cells by the simian herpes B virus (24, 65). Thus, the activation of the host innate immune response against KSHV not only helps to curb viral replication and viral spread but also induces a selective pressure on the virus to establish a latent state. This allows the virus to evade the subsequent wave of adaptive immune host responses that normally follows an effective innate immune response. Thus, in summary, KSHV can capitalize on the host innate immune response, which is used to the advantage of the virus.

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