Latent Kaposi’s Sarcoma-Associated Herpesvirus Infection of Monocytes Downregulates Expression of Adaptive Immune Response Costimulatory Receptors and Proinflammatory Cytokines

Sean M. Gregory,*b Ling Wang,* John A. West,* Dirk P. Dittmer,*b and Blossom Damania*ab
Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA,* and Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USAab

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), is a member of the gamma-herpesvirus subfamily. KSHV is the etiological agent of Kaposi’s sarcoma (KS) (8), primary effusion lymphoma (PEL), and multicentric Castleman’s disease (MCD) (1, 8, 36). KS is a highly inflammatory and angiogenic vascular tumor defined by characteristic spindle cells, which are believed to originate from endothelial cells. PEL and MCD are both B cell lymphoproliferative diseases.

Like other herpesviruses, KSHV establishes latent infection in its host. A number of PEL cell lines have been established where most of the cells are latently infected, with only a small population of cells undergoing spontaneous lytic reactivation (28, 38). During latency, a limited number of viral proteins are expressed, including the latency-associated nuclear antigen (LANA), vFLIP, vCyclin, kaposin, vIRF3, K1, and vIL-6 (7, 12, 35, 37). Maintenance of the viral genome is absolutely dependent on the LANA protein, which tethers the latent viral episome to the host cell chromosome, ensuring that the viral genome is replicated with the host genome and is not diluted out of the expanding population of latently infected cells (10, 13). The LANA protein has been shown to be expressed in latently infected B cells and endothelial cells, as well as in the KSHV-positive tumors associated with these cell types (3, 10, 13).

KSHV can successfully infect human monocytes and macrophages in vitro and in vivo (4–6, 24). Rappoccio et al. demonstrated that KSHV uses the receptor DC-SIGN to enter macrophages and dendritic cells (DCs) (31, 32). Kerur et al. showed that in the THP-1 acute monocytic leukemia cell line, KSHV primary infection was dependent on αβ1 integrins and that it was preceded by endosomal entry, which activated FAK, Src, PI3K, NF-κB, and ERK1/2 signaling (20). Coinfection of monocytes with KSHV and HIV increased the replication of HIV in the presence of KSHV (6). Monocytes present in KS lesions have been shown to support viral replication (5). Additionally, KSHV has been found to infect CD34+ stem cell precursors in vitro, suggesting that stem cells or later-stage committed progenitor cells may be infected and subsequently differentiate into B cells, monocytes, and other lineages to replenish depleted pools of infected, differentiated cells (45).

Immunological detection of KSHV infection by T cells requires T cell receptor (TCR) cognate interactions with the antigen-presenting cell (APC) major histocompatibility complex (MHC) surface molecules displaying KSHV peptide. On inactivated T cells, CD28 is expressed at low levels. Upon TCR-MHC contact and APC surface receptor CD40 ligation by CD40 ligand (CD40L), CD28 expression is upregulated. Concomitantly, the costimulatory molecules CD80 and CD86 on the APCs, which interact with CD28 to increase the immune response, are upregulated. Despite the existence of KSHV-specific T cells and immune control in healthy individuals, latent virus is unable to be eliminated from the host (41, 42). It is known that KSHV lytic proteins K3 and K5 actively downregulate CD80 and MHC class I (MHC I) surface expression; however, the suppression of adaptive immune molecules during latency also contributes to evasion of the host response (22). Cells involved in immunity that are tropic for KSHV may facilitate suppression of host immune responses.

Given that KSHV infects monocytes in vivo, we established a latently infected monocytic cell line by using the monocytic leukemia cell line THP-1 to characterize viral gene expression in latently infected monocytes (2, 39). THP-1 cells are susceptible to human cytomegalovirus (HCMV) infection and support viral latency (43). Importantly, although we have previously shown that KSHV can infect primary human monocytes (44), we could not establish a long-term latent culture in these cells because of the primary nature of the monocytes. In contrast, THP-1 cells enable...
the establishment of a monocytic latent cell line that harbors KSHV and which can be passaged over a long period.

**MATERIALS AND METHODS**

Production of recombinant rKSHV.219 virus. Vero cells containing latent rKSHV.219 (KSHV-Vero) and a recombinant baculovirus KSHV Orf50 (Bac50) were kindly provided by Jeffrey Vieira (40). KSHV.219 expresses green fluorescent protein (GFP) and also contains a puromycin resistance gene as a selectable marker. Insect SF9 cells were grown in SF900-II serum-free medium at 28°C. SF9 cells were infected with baculovirus expressing KSHV Orf50 (Bac50) for 3 days, after which time the baculovirus-containing supernatant was clarified by centrifugation (1,500 rpm for 10 min). KSHV-Vero cells were then infected with Bac50 and treated with 2 mM sodium butyrate for 3 days. Supernatant was harvested, and cells were removed by centrifugation (1,500 rpm for 10 min). Supernatants were subsequently passed through a 0.45-μm filter.

Establishment of the KSHV-THP-1 cell line. THP-1 cells were cultured in RPMI with 10% fetal bovine serum (FBS) and were maintained at 37°C in a 5% CO₂ environment. The rKSHV.219 produced from KSHV-Vero cells was used to infect THP-1 cells. We first made an infection cocktail containing complete RPMI medium and rKSHV.219 supernatants (volume ratio of 1:2) with 4 μg/ml Polybrene. THP-1 cells were centrifuged at 1,500 rpm for 5 min, and then 2 × 10⁵ THP-1 cells were resuspended in 3 ml infection cocktail and added to one well of a 6-well plate. The cells in the 6-well plate were spun for 90 min at 30°C for 2,500 rpm; then, the supernatants were removed and the cells were resuspended in 3 ml complete RPMI medium. The cells were incubated at 37°C for 72 h and then selected in complete RPMI medium containing 1 μg/ml puromycin for 3 to 5 weeks to establish stable KSHV-THP-1 cells. Once THP-1 cells were 100% KSHV positive, KSHV-THP-1 cells were main- tained in 0.1 μg/ml puromycin, which is necessary for O2-tetracanoylphorbol-13-acetate (TPA)-induced reactivation. THP-1 control cells and KSHV-THP-1 cells were subsequently passaged.

Immunofluorescence assays. THP-1 or KSHV-THP-1 cells (1 × 10⁶) were air dried on microscope slides and then fixed with 0.4% formaldehyde for 30 min. Cells were washed twice with phosphate-buffered saline (PBS) for 5 min each, permeabilized with PBS, 0.1% Triton X-100 for 20 min, and washed twice with PBS, 1% bovine serum albumin (BSA) for 5 min each. Incubation with primary antibody anti-HHV8 open reading frame 73 (ORF73) (Advanced Biotechnologies) (1:500) was performed for 16 h at 4°C. Cells were washed twice with PBS, 1% BSA for 5 min each and incubated with anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (Sigma), 1:500, for 1 h at room temperature. Cells were washed twice with PBS, 1% BSA for 5 min each and then stained with 4',6-diamidino-2-phenylindole (DAPI) for 2 min. Next, cells were washed with PBS, 1% BSA for 5 min, followed by double-distilled water (ddH₂O) for 5 min. Microscope slides were mounted using Vectashield (Vector Laboratories) mounting medium and visualized using a Nikon Microphot FXA upright fluorescence microscope.

Quantitative real-time PCR (qPCR). DNase-treated total cellular RNAs (1 μg) from THP-1 or KSHV-THP-1 cells were reverse transcribed into cDNA using a reverse transcription system from Promega. The real-time PCR was done with an ABI 7300 real-time PCR machine using SYBR green PCR master mix (Applied Biosystems). PCR was carried out with 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All fold activations were normalized by GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression in the samples, and all of the PCR products were resolved using 1% agarose gels.

Viral gene profiling. THP-1 or KSHV-THP-1 cells (1 × 10⁶) were harvested, and total RNA was isolated with an RNeasy kit (Qiagen) according to the manufacturer’s instructions and then DNase treated using a DNA-free RNA kit (Zymo Research). The cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems) as directed by the manufacturer. Detailed methods for real-time qPCR arrays and primer names were described previously (11, 12). The primer names is as published before and indicates the ORF name in KSHV followed by position of the forward primer. In addition, the original primer set was replaced with improved primers for orf11 (KS10008-1), orf20 (KS100121-1), orf33 (KS10034-1), orf50 (KS10052-1), orf53 (KS10055-1), orf63 (KS10069-1), orf65 (KS10070-1), orf71 (KS10076-1), orf K5 (KS10014-1), and orf K15 (KS100821-1). Primer sequences are available from the UNC ViroNomics core. Cycle threshold (C_T) values were determined by automated analysis. The threshold was set to five times the standard deviation (SD) of the nontemplate control (NTC). For each qPCR run, dissociation curves were analyzed to verify that identical primer-specific, single reaction products were generated in each run. Genes with multiple primer pairs indicate two independent primer sets. Samples were normalized to GAPDH levels, centered by median of gene, and ordered by hierarchical clustering using ArrayMiner (Optimal Design, Inc., Brussels, Belgium) software with standard correlation metrics (11, 12). Further statistical analysis was conducted using the R programming environment (v 2.5.1).

Flow cytometry. To assess the expression of cell surface receptors, THP-1 and KSHV-THP-1 monocytes were suspended at 1 × 10⁶ cells/ml in staining buffer (1 × PBS, 2% BSA) and labeled with 20 μl of mouse allophycocyanin-conjugated anti-human CD86 (BD Pharmingen), mouse allophycocyanin-conjugated anti-human CD83 (BD Pharmingen), or isotype control allophycocyanin-conjugated IgG1 kappa (BD Pharmingen) antibody for 30 min at 4°C, protected from light. Cells were then thoroughly washed twice with staining buffer by centrifugation at 1,500 rpm at 4°C. Next, labeled cells were fixed using 1% formaldehyde for 30 min at 4°C, followed by washing with staining buffer. Samples were resuspended in 250 μl of staining buffer and analyzed using a BD FACs Calibur (BD Biosciences) flow cytometer with data analysis using Summit version 4.3 (Dako). Data were acquired for a minimum of 25,000 total events. Cell sorting was performed by staining cells as described above for CD86, and results were analyzed by the UNC Flow Cytometry Core facility using iCyt/Sony Reflection under sterile conditions.

Western blotting. THP-1 or KSHV-THP-1 cells were washed in ice-cold 1× PBS and lysed by 3× dry ice-ethanol fractionations in radioimmounoprecipitation assay (RIPA) lysis buffer. Cell lysates were centrifuged at 13,000 rpm, 4°C for 10 min. Protein quantifications were performed using the Bradford protein quantification assay. Whole-cell lysate (25 μg) was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by wet transfer at 30 V overnight at 4°C. Transfers were verified by Ponceau S staining, followed by blocking in 5% nonfat dry milk (NFDM), 1× PBS, 0.2% Tween 20. Following three washes in 1× PBS, 0.2% Tween 20 for 10 min each at room temperature, membranes were incubated with primary antibody overnight at 4°C. Following 4°C Rabbit monoclonal anti-human CD86 (Novus Biologicals) or rabbit monoclonal anti-human CD80 (Novus Biologicals) was diluted in 5% NFDM at 1:500 or 1:10,000, respectively. Following incubations, membranes were washed three times and incubated with peroxidase-conjugated, anti-rabbit IgG (Cell Signaling) in 5% NFDM at 1:2,000 for 1 h at room temperature. Following the three washes, membranes were developed using Amersham ECL Plus Western blotting detection reagents (GE Healthcare). Viral protein K8.1 was detected using mouse K8.1 monoclonal antibody (ABI Biologicals) diluted 1:1,000 in 5% NFDM overnight at 4°C, followed by peroxidase-conjugated, anti-mouse IgG as described above.

Cytokine expression analysis. THP-1 or KSHV-THP-1 cells (1 × 10⁶) were plated in 12-well plates containing complete medium. After incubation at 37°C for 24 h, supernatants were harvested and cleared of cell debris by centrifugation at 1,250 rpm for 10 min. Supernatants were analyzed for cytokine expression using Millipore Lumix multianalyte technology according to the manufacturer’s instructions.

Effect of Latent KSHV Infection in Monocytes
viral interleukin-6 (IL-6) primers and GAPDH endogenous controls. PCR products were analyzed by 1.2% agarose gel electrophoresis. Viral K8.1 protein expression was analyzed 120 h after TPA treatment as described above.

RESULTS
KSHV can successfully infect human monocytes and macrophages in vitro and in vivo (4–6, 25). We infected THP-1 cells with rKSHV.219, expressing green fluorescent protein (GFP) driven by the CMV promoter, red fluorescent protein (RFP) under a lytic viral promoter, and the puromycin resistance gene (40). Seventy-two hours postinfection, cells were added to puromycin-containing selection medium to maintain KSHV infection and to achieve a 100% KSHV-infected monocytic cell line as monitored by GFP expression (Fig. 1A).

To confirm KSHV infection of THP-1 cells, we performed immunofluorescence assays on KSHV-infected and uninfected THP-1 cells for LANA protein expression (Fig. 1B). Briefly, KSHV-THP-1 or THP-1 cells suspended in PBS were spotted on slides, air dried, and fixed with paraformaldehyde. Cells were stained with an antibody directed against LANA followed by a TRITC-conjugated secondary antibody. Cells were also stained with DAPI to demarcate the nucleus. As can be seen in Fig. 1B, uninfected THP-1 cells did not show any LANA staining, while the KSHV-THP-1 cells showed characteristic nuclear speckled staining for LANA protein (19).

In order to determine the profile of KSHV viral genes expressed in the THP-1 monocytic cell line, qPCR was performed on KSHV-THP-1 cells and uninfected THP-1 cells. qPCR primer pairs were designed for each KSHV ORF as previously described (11). Equal amounts of total poly(A) mRNA from THP-1 and KSHV-THP-1
cells were used as starting material. Figure 2A shows cycle threshold (C_T) values for each KSHV gene versus qPCR cycle threshold (C_T) value. Dotted line represents limit of detection for viral genes. (B) Density distribution (on the vertical axis) of relative log fold change in gene expression (on the horizontal axis) in KSHV-THP-1 cells compared to that in uninfected THP-1 cells for each KSHV-specific primer pair. Also shown is the 95% CI (10^{4.9} \rightarrow 10^{5.6}) between the genes that are highly expressed (≥100-fold above uninfected control) and those that are moderately expressed (<100-fold) or that were undetectable under both conditions and the difference between these two groups based on nonparametric test. (C) Heat map depicting KSHV transcription in KSHV-THP-1 cells compared to that in uninfected control for each open reading frame. Gene expression is represented by the following colors: red, high; white, intermediate; blue, undetectable. Each condition depicts two biological replicates. Rows indicate the primers, which were labeled using the name of the KSHV orf followed by a position of the forward primer.

FIG 2 KSHV gene expression profile of KSHV-THP-1 cells. (A) Analysis of log KSHV gene expression versus qPCR cycle threshold (C_T) value. Dotted line represents limit of detection for viral genes. (B) Density distribution (on the vertical axis) of relative log fold change in gene expression (on the horizontal axis) in KSHV-THP-1 cells compared to that in uninfected THP-1 cells for each KSHV-specific primer pair. Also shown is the 95% CI (10^{4.9} \rightarrow 10^{5.6}) between the genes that are highly expressed (≥100-fold above uninfected control) and those that are moderately expressed (<100-fold) or that were undetectable under both conditions and the difference between these two groups based on nonparametric test. (C) Heat map depicting KSHV transcription in KSHV-THP-1 cells compared to that in uninfected control for each open reading frame. Gene expression is represented by the following colors: red, high; white, intermediate; blue, undetectable. Each condition depicts two biological replicates. Rows indicate the primers, which were labeled using the name of the KSHV orf followed by a position of the forward primer.

The positive controls, were present at a ratio of 1, i.e., at equal expression levels in infected and uninfected cells. Figure 2B shows the density distributions, i.e., the number of genes with a given expression level above that for uninfected control cells. Fewer than 10 transcripts were expressed at levels significantly greater than 100 times that of the uninfected control cells (Fig. 2B, dotted line); the
remainder of genes were undetectable or present at low levels (<100-fold of level for mock-infected cells). The difference between these two groups of genes was significant to a P value of $2.4 \times 10^{-9}$, with a 95% confidence interval (95% CI) between the means of expression of $10^{4.99}$ and $10^{3.64}$. Relative expression levels are depicted by heat maps comparing KSHV-THP-1 and THP-1 cells (Fig. 2C). Lytic transcripts detected at high to moderate expression levels likely reflect the low number of cells spontaneously reactivating, similar to results for other KSHV-infected cell lines in culture (33). Importantly, KSHV-THP-1 cells were capable of reactivation after treatment with 20 ng/ml TPA (Fig. 3A), as evidenced by measuring viral IL-6 mRNA levels after 48 h and 72 h compared to GAPDH mRNA levels as the endogenous control. No signal was obtained in the absence of reverse transcription (−RT) or absence of template (NTC). Lytic viral protein expression of KSHV K8.1 could also be detected in the TPA-reactivated cells (Fig. 3B).

Primary infection of dendritic cells and macrophages with KSHV has been shown to downregulate DC-SIGN, and in B cells KSHV infection decreased MHC I (31, 32). Hence, we investigated whether latently infected monocytes show downregulation of monocyte activation markers. We found that surface expression of CD86 was reduced from approximately 31% in THP-1 cells to 4% for KSHV-THP-1 cells (average fold change, 5.02 ± 2.4) (Fig. 4). Additionally, we observed that surface expression of CD83 was downregulated from 12% to 1.20% in KSHV-THP-1 cells compared with that in uninfected control cells (average fold change, 5.1 ± 4.0). Since KSHV-infected monocytes are prevalent in KS lesions, reduced expression of costimulatory molecules on the surface of antigen-presenting cells, such as monocytes, during latency may dampen host immune responses against KSHV-infected cells.

In order to confirm coreceptor downregulation, we performed reverse transcription-qPCR (RT-qPCR) for these markers as well as additional costimulatory markers CD80 and CD1a (Fig. 5). We
found that several genes associated with macrophage/dendritic cell activation were downregulated compared to levels in uninfected THP-1 control cells (Fig. 5A). By densitometry, we found that genes for costimulatory molecules CD80, CD86, CD1a, and CD83 were downregulated 2.9-, 8.1-, 2.7-, and 4.1-fold, respectively. The downregulation of CD86 and CD80 basal protein expression levels in KSHV-THP-1 cells was confirmed by Western blot analysis (Fig. 5B).

There is a heterogeneous level of CD86 coreceptor expression on THP-1 cells. Our findings may allow for the alternative possibility that KSHV selectively enters cells with low CD86 expression and that KSHV-infected latent THP-1 cells display a low CD86 expression due to this fact. To rule out this possibility, THP-1 cells were stained for CD86, and the cells that expressed the highest levels of CD86 coreceptor were isolated using fluorescence-activated cell sorting (FACS). This population is denoted as CD86hi THP-1 (Fig. 6A). Sorted CD86hi THP-1 cells were then infected with KSHV and analyzed by flow cytometry for GFP expression (Fig. 6B). CD86hi THP-1 cells were 68.6% GFP positive 24 h postinfection, suggesting that KSHV can enter the fraction of THP-1 cells with the highest level of CD86 surface expression prior to establishing latency. Hence, it is more likely that CD86 is downregulated by KSHV and that this effect is not an artifact of KSHV preferentially entering THP-1 cells that express the smallest amount of CD86 on their surface.

We also investigated cytokine expression in KSHV-THP-1 cells compared to that in THP-1 controls by Luminex multianalyte analysis. THP-1 or KSHV-THP-1 cells were incubated for 24 h and cell-free supernatants collected for analysis. Overall, we analyzed the expression of 15 proinflammatory cytokines, and for the most part, expression was unchanged (data not shown). However, a significant difference in tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) was observed (Fig. 7A). TNF-α and IL-1β expression levels were 7.73 ± 0.56 pg/ml and 4.90 ± 0.59 pg/ml, respectively, in THP-1 cells, and these cytokines were undetectable in KSHV-THP-1 cells. In order to confirm that these cytokines were downregulated at the transcription level, RT-PCR analysis was performed. Figure 7B shows that transcription of both genes is suppressed in KSHV-infected THP-1 cells. TNF-α and IL-1β are involved in upregulating the transcription of genes involved in inflammation, hematopoiesis, and immune responses, including costimulatory molecules, and therefore their downregulation may contribute to inhibition of adaptive immunity.

**DISCUSSION**

KSHV establishes a lifelong persistent infection in the human host. The virus can establish latency in a number of different cell types, including B cells, monocytes, and epithelial and endothelial cells. Previous studies have analyzed cellular and viral gene expression in lytically and latently infected B cells and endothelial cells (7, 12, 27, 29). Here we report viral gene profiling of a KSHV latently infected monocytic cell line. We observed that KSHV-THP-1 cells predominantly expressed a latent viral gene profile with low levels of lytic replication, which we attribute to spontaneous reactivation. We found that KSHV-THP-1 cells display reduced expression of several cellular proteins involved in inflammation and immunity, compared to expression in uninfected cells. This suggests that viral proteins may downregulate expression of genes that could lead to detection of the virus by the host immune system.
KSHV-specific cytotoxic T lymphocyte (CTL) responses are reduced in KS patients compared to responses in KSHV-positive asymptomatic individuals, suggesting that failure to mount a CTL-mediated immune response contributes to development of disease (17). After recognition of a T cell’s cognate antigen in the context of major histocompatibility complex (MHC) on the surface of APCs, engagement of CD80 or CD86 provides a necessary costimulatory signal for CTL activation (16, 34). Monocytes help dictate CTL action, since they contribute to their stimulation by upregulating costimulatory molecules, such as CD80 and CD86, upon pathogen detection. Furthermore, monocytes are recruited to sites of T cell activation and are capable of differentiating into macrophages and dendritic cells, which are potent antigen-presenting cells necessary for an effective cell-mediated immune response (14).

Previous studies have shown that viral infection reduces expression of several cell surface immune receptors. Varicella-zoster virus (VZV) infection of mature DCs results in selective downregulation of expression of functional immune molecules, including MHC I, CD80, CD83, and CD86 (25). Herpes simplex virus 2 (HSV-2) infection causes the downregulation of MHC I and II, CD40, CD80, and CD86 on mature DCs (25), although HSV-1 infection of mature DCs results in downregulation of only CD83 (21). Human cytomegalovirus (HCMV) has been shown to downregulate MHC I and II, CD40, CD80, and CD83, and CD86 on monocyte-derived mature DCs generated by treatment with lipopolysaccharide (LPS) and TNF-α (26, 30); at the same time, productive infection in THP-1 cells induces IL-1β and TNF-α (15, 18). In addition, during acute HIV-1 infection, lymphoid tissue has reduced CD80 and CD86 expression (23). KSHV infection of myeloid-derived macrophages and dendritic cells results in a reduction in differentiation and antigen presentation to CTLs (9, 32). Similarly, our study shows that KSHV latent infection of monocytic THP-1 cells leads to significant downregulation of CD1A, CD80, CD83, and CD86. Thus, it appears that human herpesviruses have multiple strategies to interfere with detection by the host immune system and that KSHV is no exception. Infection of monocytes and suppression of cellular mechanisms of immune recognition during latency suggest that KSHV specifically targets APCs to prevent activation of antiviral responses in order to support long-term dormant infection.

ACKNOWLEDGMENTS

We thank Veera Vieira for providing rKSHV.219 and Stuart Krall for tissue culture assistance. This work was supported by NIH grants DE018328 to B.D. and DE018304 to D.P.D. S.M.G. was supported in part by NIH training grants T32 AI007419 and T32 AI007001. B.D. is a Leukemia & Lymphoma Society Scholar and Burroughs Wellcome Fund Investigator in Infectious Disease. Viral expression profiling was conducted by the UNC Virology core and supported by grant PO1 CA019014. Luminex cytokine analysis was performed by the UNC CGBID Immune-technologies core, supported by grant P30 DK34987.

REFERENCES

infected endothelial, fibroblast, and B cells: insights into modulation events early during infection. Cancer Res. 64:72–84.


