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Activation of Plasmacytoid Dendritic Cells by Kaposi's Sarcoma-Associated Herpesvirus[▽]

John A. West, Sean M. Gregory, Vijay Sivaraman, Lishan Su, and Blossom Damania*

Lineberger Comprehensive Cancer Center and Department of Microbiology & Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with multiple human malignancies, including Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Following primary infection, KSHV typically goes through a brief period of lytic replication prior to the establishment of latency. Plasmacytoid dendritic cells (pDCs) are the major producers of type 1 interferon (IFN), primarily in response to virus 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. pDCs express exclusively TLR7 and TLR9, and it is through these TLRs that the type 1 interferon response is activated in pDCs. Currently, it is not known whether KSHV is recognized by pDCs and whether activation of pDCs occurs in response to KSHV infection. We now report evidence that KSHV can infect human pDCs and that pDCs are activated upon KSHV infection, as measured by upregulation of CD83 and CD86 and by IFN-α secretion. We further show that induction of IFN-α occurs through activation of TLR9 signaling and that a TLR9 inhibitor diminishes the production and secretion of IFN-α by KSHV-infected pDCs.

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (8, 9). KSHV, also known as human herpesvirus 8 (HHV-8), is a gammaherpesvirus belonging to the *Rhadinovirus* genus. Like other herpesviruses, KSHV can establish a lifelong infection in the human host. KSHV exhibits two different phases in its life cycle, a latent phase and a lytic phase. It persists in the host cell as a viral nuclear episome during the latent phase. During the lytic phase of its life cycle, it replicates its viral genome to produce viral progeny. In most cell types, primary infection is usually followed by lytic replication, but within 3 to 4 days following primary infection, KSHV typically enters a latent state (20). KSHV is tropic for many different cell types, including endothelial cells, monocytes, B cells, dendritic cells (DCs), and hematopoietic progenitor cells (5, 6, 27, 36). Several recent studies have shed light on the requirements for KSHV infection of macrophages and dendritic cells. DC-SIGN was identified as the receptor for KSHV present on dendritic cells and macrophages (30). Pretreating cells with an antibody against DC-SIGN blocked KSHV infection of these cell types (30). DC-SIGN was also identified as being critical for KSHV infection of activated B cells isolated from blood and tonsils (30). Infection of dendritic cells was subsequently shown to lead to increases in several cytokines and chemokines, including the following: interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), macrophage inflammatory protein 1α (MIP-1α), and IL-12 p40, among others (30).

Toll-like receptors (TLRs) play a vital role in the innate immune response to viral infection, recognizing specific patterns on invading pathogens (3). Currently, 10 human TLRs have been identified, and for 9 of these a well-defined function has been established. A subset of the TLR family, including TLRs 3, 7, 8, and 9, is expressed primarily in the endosomal compartment of cells that express these proteins (1, 2). The TLR expression profile is different depending on the cell type. Specifically, human plasmacytoid DCs (pDCs) express only 2 of the 10 human TLRs, TLR7 and TLR9 (18). TLR7 has been shown to recognize single-stranded RNA, while TLR9 recognizes CpG DNA sequences (1, 4, 14). Both types of nucleic acid are common by-products of viral infection. TLR7 and TLR9 have both been shown to play key roles in activating the innate immune response against invading viruses.

pDCs are a rare cell type in the blood, comprising approximately 0.4% of the total peripheral blood mononuclear cell (PBMC) population (24). pDCs are a subset of the professional antigen-presenting dendritic cell population; however, the primary role of pDCs is to produce type 1 interferon (IFN) in response to virus infection (21, 24). Both RNA and DNA viruses have been shown to activate or stimulate pDCs, resulting in type 1 IFN production. These viruses include herpes simplex viruses 1 (HSV-1) and 2 (HSV-2), Sendai virus, influenza virus, human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV) (10, 12, 15, 17, 25, 29, 32). Each of these viruses stimulate IFN production through activation of the TLR pathway in pDCs. There is also evidence that pDCs can play a helper role in herpesvirus infection (34) and can be among the primary responders to herpesvirus infection. Intranasal inoculation of mice with murine herpesvirus 68 (MHV-68) led to the recruitment of pDCs to the lung and subsequently led to the activation of DCs, even in the absence of a type 1 IFN response, suggesting that pDCs can activate additional immune effector cells following herpesvirus infection. pDCs also produce IFN

* Corresponding author. Mailing address: Lineberger Comprehensive Cancer Center, CB#7295, University of North Carolina, Chapel Hill, NC 27599. Phone: (919) 843-6011. Fax: (919) 966-9673. E-mail: damania@med.unc.edu.

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in response to synthetic oligonucleotide ligands, such as A-type CpG oligonucleotides [those which contain a poly(G) tail] (12). As mentioned above, pDCs express 2 of the 10 known human TLRs, TLR7 and TLR9. Both TLR7 and TLR9 are expressed in the endosome, a compartment in which virus entry commonly takes place. Therefore, exposure of viral nucleic acids within the endosome during primary infection would likely lead to an immediate and strong induction of type 1 IFN. TLR7 and TLR9 have both been shown to play important roles in the elicitation of the type 1 IFN response seen after infection of pDCs. TLR9 recognizes HSV-2 (25), TLR7 recognizes HIV (26), and both TLR7 and -9 are involved in recognition of EBV (29).

We previously reported that KSHV modulates the TLR signaling pathway (13, 35). We found that KSHV activates TLR3 upon primary infection of monocytes and that TLR7/8 stimulation of latent KSHV-infected B lymphocytes results in viral reactivation (13, 35). Here we investigated whether KSHV could activate and stimulate an IFN response from human pDCs and whether TLR recognition/signaling was required for this response. Although we reported that TLR3 was activated during primary infection of human monocytes, TLR3 is not thought to be expressed in pDCs (37). Hence, we queried whether TLR7 and TLR9, the only two TLRs known to be expressed in human pDCs, could recognize KSHV during primary infection. In addition, we wanted to determine if KSHV could infect and replicate in pDCs, as shown for some viruses. We report here that KSHV infection of pDCs leads to a strong IFN-α response and that this response requires infectious virus particles and is due partly to activation of TLR9. We also report that the cell surface markers CD83 and CD86 are upregulated in pDCs following KSHV infection. Finally, we confirmed the presence of both lytic and latent KSHV gene transcripts in pDCs.

MATERIALS AND METHODS

Cell culture. Purified pDCs were maintained in RPMI 1640 medium (Cellgro) containing 10% human AB serum (Invitrogen), 1% penicillin-streptomycin (P-S), and 10 ng/ml of IL-3 (PeproTech). Vero cells stably expressing KSHV-green fluorescent protein (KSHV-GFP) were the kind gift of Jeff Vieira (33). They were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cellgro) containing 10% fetal bovine serum (FBS), 1% P-S, and 0.005 mg/ml of puromycin (for selection). Both Vero cells and pDCs were grown at 37°C under 5% CO_2. 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 in a monolayer.

Virus production and purification. Virus was produced as described previously (35). In brief, baculovirus ORF50, a kind gift of Jeff Vieira (33), was amplified in Sf9 cells for 72 h, after which 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 2 mM 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, 30 ml of supernatant was layered over an 5-ml cushion of 20% sucrose (optical grade; Sigma), and the virus was pelleted by ultracentrifugation in an SW28 swinging-bucket rotor for 3 h at 4°C in a Beckman ultracentrifuge. The supernatant was decanted, and the virus pellet was resuspended in sterile phosphate-buffered saline (PBS) in 1% of the original volume. The UV inactivation of KSHV was measured as previously described (35).

Human plasmacytoid dendritic cell isolation and infection. Human pDCs were isolated from buffy coats (Gulf Coast Labs) by use of a Diamond plasmacytoid dendritic cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Following isolation, cells were stained with CD303 (BCDA-2)–fluorescein isothiocyanate (FITC) and/or CD123-phycoerythrin (CD123-PE) (both from Miltenyi Biotec) antibody according to the manufacturer's protocol. Flow cytometry was carried out on a MACSQuant analyzer (Miltenyi Biotec). On average, we were able to obtain 95% to 98% purity throughout the course of the experiments. pDC infections were carried out as follows. Freshly isolated pDCs were counted and plated into either 96-well plates or 8-well coverslip-bottom chamber slides (Lab-Tek) (for microscopy). On average, between 1×10^5 and 2×10^5 cells were incubated in RPMI plus 1% P-S and IL-3 (10 ng/ml), with either 100 μl of Dulbecco's PBS (D-PBS) (mock) or 100 μl of concentrated KSHV (approximately 2×10^7 genomes/ml), for 1 h at 37°C. For most assays, except the immunofluorescence assay, pDCs were infected with KSHV and centrifuged for 30 min at 1,500 rpm at 30°C. After infection, human AB serum was added to all wells (mock and infected) to the correct final concentration. When investigating CD83 and CD86 activation by KSHV infection, IL-3 was not added to the medium, as it has previously been shown to be necessary to drive pDCs toward a dendritic cell morphology characterized by CD86 and CD83 upregulation (reviewed in reference 24). The medium was refreshed at either 16 h postinfection or 24 h postinfection. For all microscopy and flow cytometry analyses, cells were harvested and used fresh. For RNA, DNA, or protein analysis, cells were harvested and flash frozen prior to use. All supernatants were flash frozen prior to analysis.

IFN-α ELISA. An enzyme-linked immunosorbent assay (ELISA) specific for IFN-α (human origin; PBL Interferon Source) was used per the manufacturer's instructions to analyze the amount of IFN-α secreted from KSHV-infected pDCs. Briefly, standards and samples were added to precoated microtiter wells, covered, and incubated for 1 h at room temperature. The plate was washed with a diluted wash buffer concentrate, 100 μl of diluted antibody concentrate was added, and the plate was incubated for 1 h at room temperature. The plate was then washed three times, followed by incubation with 100 μl of diluted horseradish peroxidase (HRP) for 1 h at room temperature. Following HRP incubation, the plate was washed four times and then incubated with 100 μl of tetramethylbenzidine (TMB) substrate for 15 min, uncovered and in the dark. After 15 min, 100 μl of stop solution was added and the plate was analyzed on a microplate reader to determine the absorbance at 450 nm. The standard (provided by the manufacturer) was plotted, and the concentration for each sample was determined based on the standard curve.

Cytokine array analysis. A human cytokine antibody array (RayBiotech) was used as described previously (35) to quantify the overall cytokine activation pattern following KSHV infection of pDCs. The protocol was performed as described by the manufacturer. Briefly, each array membrane was blocked and then incubated overnight at 4°C with supernatant collected from either KSHV-infected pDCs or mock-infected pDCs (diluted to the minimum volume specified by the manufacturer). The arrays were then washed and incubated with biotin-conjugated antibody solution, specific to each array, for 2 h at room temperature. 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 then exposed to detection buffer for 2 min and exposed to X-ray film. Quantitation of the individual dots was done using the ImageJ program.

TLR9 inhibition. The TLR9 inhibitor G-ODN was purchased from Invivogen. G-ODN was resuspended in water per the manufacturer's instructions, at a stock concentration of 500 μM. G-ODN was added to pDCs at a final concentration of 25 μM or 40 μM in serum-free medium. G-ODN was added simultaneously with either KSHV or CpG DNA (ODN 2395) (Invivogen) per the manufacturer's instructions. CpG was used at 10 μM (from a stock of 500 μM) and served as a positive control for IFN-α induction.

Fluorescence microscopy. Confocal fluorescence microscopy was performed on a Zeiss LSM5 Pa laser scanning microscope. pDCs were infected in 8-well chamber slides as described above, and live cells were imaged at 24 and 48 h postinfection in differential interference contrast (DIC) and FITC channels.

Flow cytometry. Flow cytometry was performed on a MACSQuant analyzer (Miltenyi Biotec). To analyze the purity of isolated pDCs, freshly isolated cells were stained with CD303 (BCDA-2–FITC) and/or CD123-PE for 10 to 20 min on ice, washed with PBS, and fixed prior to analysis on the MACSQuant instrument. For analysis of CD83 and CD86 upregulation, mock-infected and infected pDCs were harvested, washed with D-PBS, and then stained with CD83-allophycocyanin (CD83-APC) and CD86-APC (BD Pharmingen) on ice for 20 min. The antibody was then washed out (three times) with PBS, and cells were fixed in fluorescence-activated cell sorter (FACS)/fixing buffer (0.02% sodium azide, 1% paraformaldehyde in PBS) and analyzed for activation. Data were analyzed using the Summit v4.3 program.

Viral transcription. In order to determine the amount of viral transcripts present in infected cells, RNAs were isolated from infected pDCs by use of an RNeasy Micro kit (Qiagen) per the manufacturer's instructions and as previously described (35). In order to generate cDNA, 10 μl of RNA was reverse tran-

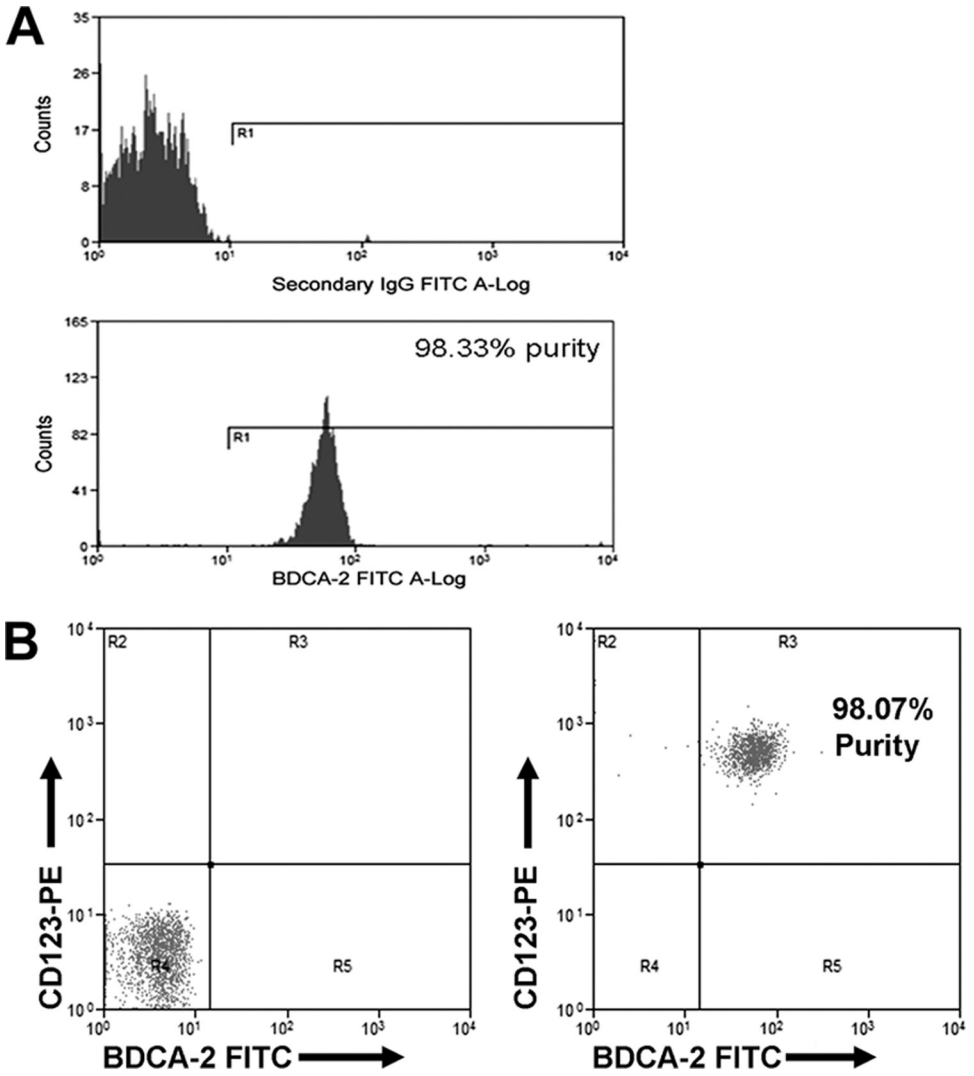

FIG. 1. Purity of pDCs isolated from human blood. We show a representative data set on the purities we were able to achieve upon isolation of pDCs from human donors. Purification was carried out as described in Materials and Methods, using magnetic bead-based separation. Purified pDCs were stained with BDCA-2–FITC (CD303) either alone (A) or in combination with CD123-PE (B) to determine cell purity. Data analysis was performed using Summit v4.3.

scribed, using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen). Reverse transcription-PCR (RT-PCR) was then performed, using the cDNA as a template and ORF57 primers (13) or ORF73 (LANA) primers (20) in a Sybr green PCR master mix (Applied Biosystems). Primers for either glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin were used as controls. RT-PCRs were run on 1.5% agarose gels.

RESULTS

Purification and infection of pDCs. We wanted to determine whether pDCs were involved in the initial recognition of KSHV following infection, so we investigated whether primary KSHV infection could activate human pDCs. pDCs were isolated from the blood of healthy human donors. Briefly, buffy coats were centrifuged over Ficoll-Paque gradients to isolate PBMCs. pDCs were first isolated via negative selection of the PBMC fraction, followed by purification using positive selection with an anti-BDCA-4 antibody, as BCDA-4 is a unique marker of pDCs. The isolated cells were subsequently stained with anti-BDCA-2–FITC and/or anti-CD123–PE (Miltenyi Biotec) antibody to assess the purity of the pDC population. We typically obtained a 95 to 98% pure pDC population. Figure 1A depicts the purity of a representative pDC sample, as analyzed by flow cytometry using only anti-BDCA-2–FITC antibody staining. Figure 1B shows the purity of a representative sample obtained using both anti-BDCA-2–FITC and anti-CD123–PE antibody staining. The purified pDCs were then subjected to KSHV infection, using recombinant GFP-KSHV (rKSHV.219) (33).

KSHV infection leads to activation of pDC surface markers CD86 and CD83. pDCs have previously been shown to elicit a T-cell response to viral infection (7, 11). Both CD83 and CD86 are critical in T-cell-mediated immune responses (22, 28). CD86 can facilitate the activation of T cells and, consequently, the adaptive immune response. CD86 can bind to two different

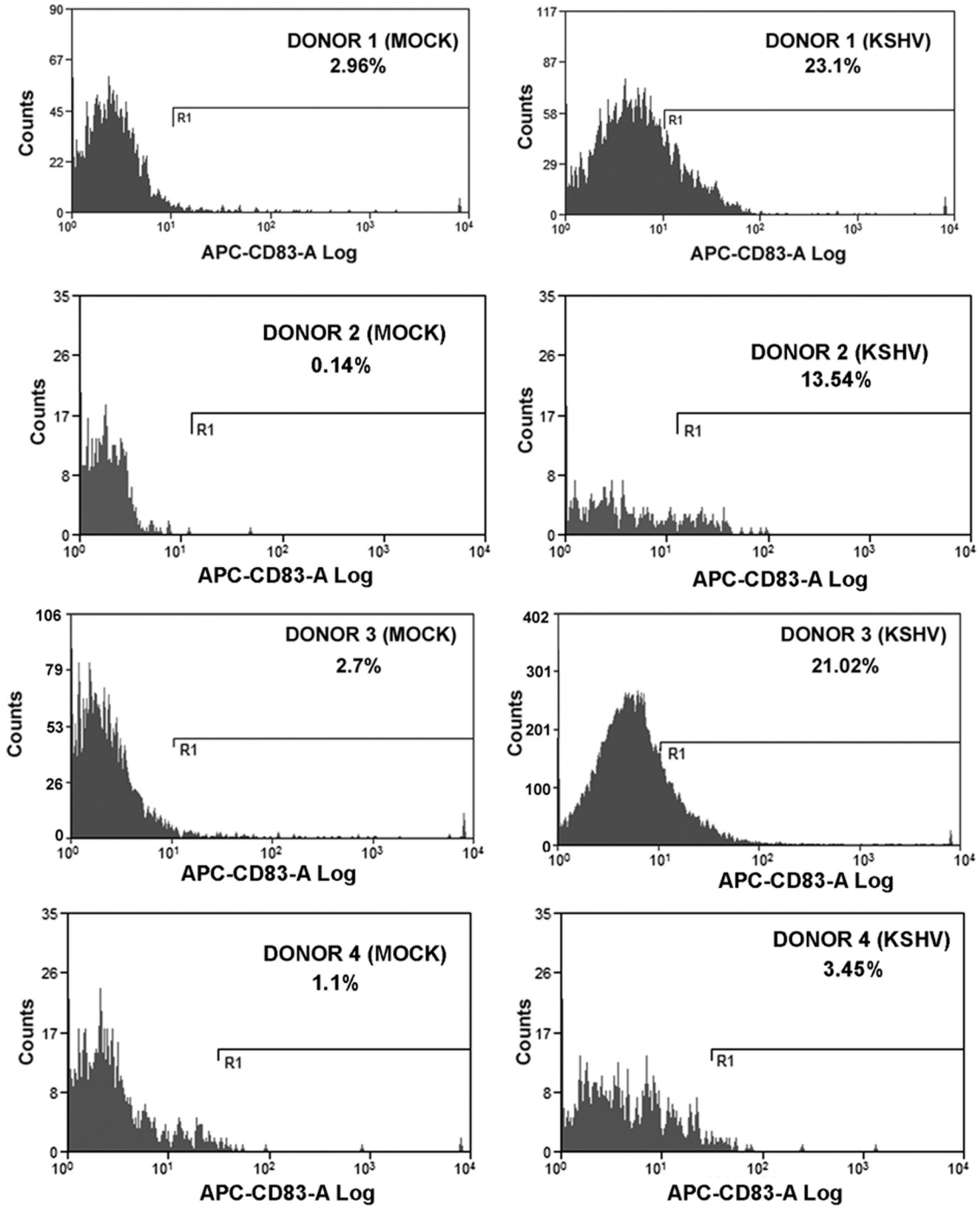

FIG. 2. CD83 activation at 16 h in KSHV-infected pDCs. pDCs were isolated from four different donors, and cells were either mock infected or infected with KSHV. Cells were harvested at 16 h postinfection and stained using an anti-CD83–APC antibody. Flow cytometry was performed on a Miltenyi MACSQuant analyzer. The data were analyzed using Summit v4.3.

receptors on the surfaces of T cells, namely, CD28 and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) (22). Binding of CD86 to CD28 on naïve T cells can lead to increased IL-12 transcription and to upregulation of CD25, pushing the cells into the cell cycle (22). Conversely, binding of CD86 to CTLA-4 can result in inhibition of T-cell receptor and CD28 signaling, resulting in shutdown of the T-cell response (22). CD83 is known primarily for being a marker of maturation of DCs (reviewed in reference 23).

In order to determine whether CD83 and CD86 were upregulated in pDCs following KSHV infection, we infected, on average, between 1×10^5 and 2×10^5 freshly isolated pDCs from different donors with concentrated KSHV (approximately 2×10^7 genomes/ml), as described in Materials and

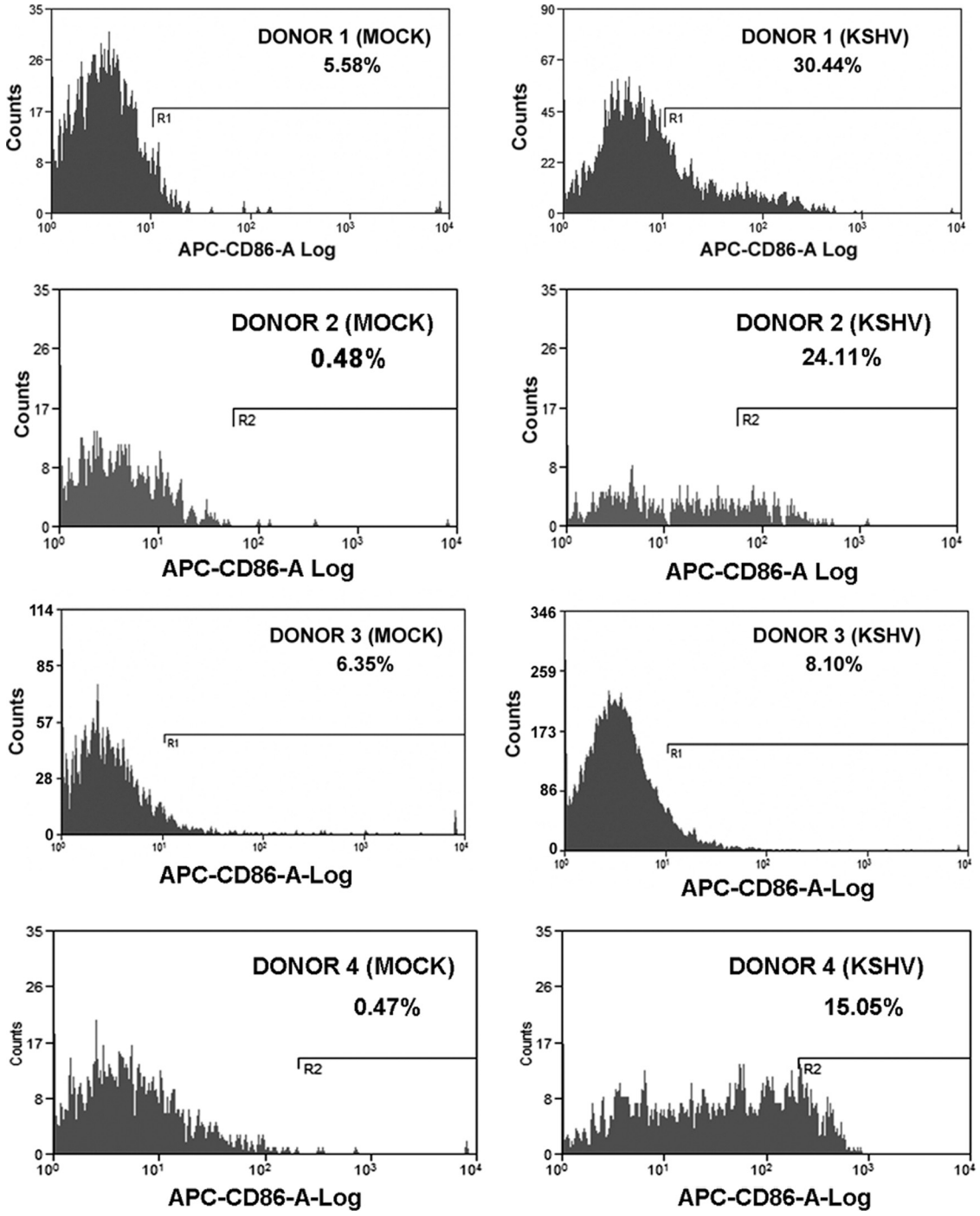

FIG. 3. CD86 activation at 16 h in KSHV-infected pDCs. pDCs were isolated from four different donors. Cells were infected with KSHV or mock infected. Cells were harvested at 16 h postinfection and stained using an anti-CD86–APC antibody. Flow cytometry was performed on a Miltenyi MACSQuant analyzer. The data were analyzed using Summit v4.3.

Methods. At 16 h postinfection, the cells were harvested, washed, and stained with anti-CD86–APC or anti-CD83–APC antibody. The stained cells were then fixed and analyzed by flow cytometry. At 16 h post-KSHV infection, there was an upregulation of both CD83 (Fig. 2) and CD86 (Fig. 3) in the virus-infected pDCs compared to the mock-infected cells. For each donor, KSHV infection upregulated the surface expression of CD83 and CD86, although the percent activation differed by donor. We observed an average increase (across 4 donors) in CD83 expression of 28.9-fold and an average increase in CD86 expression of 22.2-fold in KSHV-infected pDCs compared to mock-infected pDCs. We also analyzed CD83 and CD86 expression following infection with UV-inactivated KSHV (UV-KSHV) to determine whether infectious

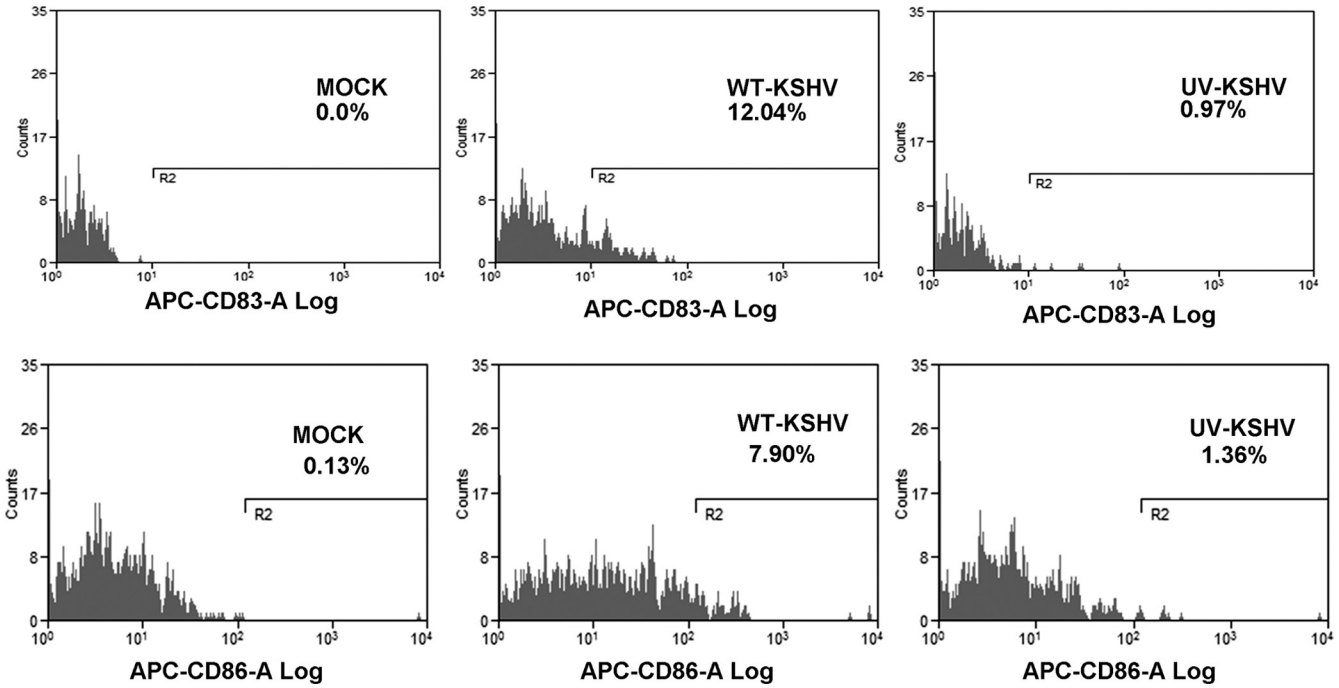

FIG. 4. CD83 and CD86 activation 16 h after infection of pDCs with KSHV. Cells were either mock infected, infected with wild-type (WT) KSHV, or infected with UV-inactivated KSHV (UV-KSHV). Cells were harvested at 16 h postinfection and stained with either CD83-APC or CD86-APC antibody. Flow cytometry was performed on a Miltenyi MACSQuant analyzer. The data were analyzed using Summit v4.3.

virus is required for the upregulation of these two costimulatory molecules. In Fig. 4, we show that pDCs infected with UV-KSHV did not display increased levels of either CD83 or CD86, indicating that infection with live virus is required for upregulation of CD83 and CD86.

KSHV can enter plasmacytoid dendritic cells. In order to corroborate these findings, we infected pDCs with GFP-expressing KSHV and monitored GFP expression by confocal microscopy. The recombinant KSHV strain that we used for infection expresses GFP under the control of a CMV promoter (33). As shown in Fig. 5A, we found that a small percentage of pDCs actively expressed GFP following infection with the recombinant virus, while no GFP expression was detected in the mock-infected cells. We also monitored GFP expression by flow cytometry at 16 h postinfection (Fig. 5B). We found that approximately 23% of pDCs were GFP positive at 16 h postinfection. These data suggest that KSHV can actively infect pDCs.

Viral gene transcription is detected in KSHV-infected pDCs. We next investigated whether viral gene expression could be detected in KSHV-infected pDCs. Freshly isolated pDCs were infected as described above, and at 48 and 72 h postinfection, the cells were harvested and washed. Total RNA was isolated and reverse transcribed to cDNA as described in Materials and Methods. RT-PCR was performed with primers specific for the lytic early gene ORF57. As shown in Fig. 5C, we were able to detect ORF57 gene products via RT-PCR at 48 h postinfection. This indicates that lytic viral transcripts are made in pDCs following KSHV infection, which might represent abortive or complete lytic replication. We also detected expression of the LANA transcript at 72 h post-KSHV infection (Fig. 5D). These data, combined with the observation of GFP expression in virus-infected cells, suggest that pDCs can be infected by KSHV and that both lytic and latent viral transcripts are made following infection.

pDCs produce and secrete IFN-α in response to KSHV infection. The hallmark of pDC activation following viral infection is increased production and secretion of IFN-α (24). We analyzed KSHV-infected pDCs for increased IFN-α production by ELISA (PBL Interferon Source). pDCs and supernatants were harvested at various time points post-KSHV infection, and the supernatants were analyzed for IFN-α levels. As shown in Fig. 6A, IFN-α secretion from KSHV-infected pDCs significantly increased at each time point compared to mock-infected pDCs. Taken together with the CD83 and CD86 activation described above, our data suggest that KSHV can activate pDCs and stimulate the production of large amounts of type 1 IFN.

In order to determine whether pDCs produced IFN in response to live virus infection by KSHV or if the IFN response was a result of surface exposure to KSHV proteins, we infected pDCs with either live GFP-expressing KSHV or UV-inactivated KSHV. Viral infections with UV-inactivated virus were carried out side by side with live virus infections in pDCs freshly isolated from human blood. As shown in Fig. 6B, we did not observe the same induction of IFN-α in the pDCs infected with UV-inactivated KSHV as in those infected with live virus. This indicates that the increased production of IFN-α, as well as upregulation of CD83 and CD86 on the pDC surface, is dependent on uncoating of the virus in the endosome and/or on productive KSHV infection.

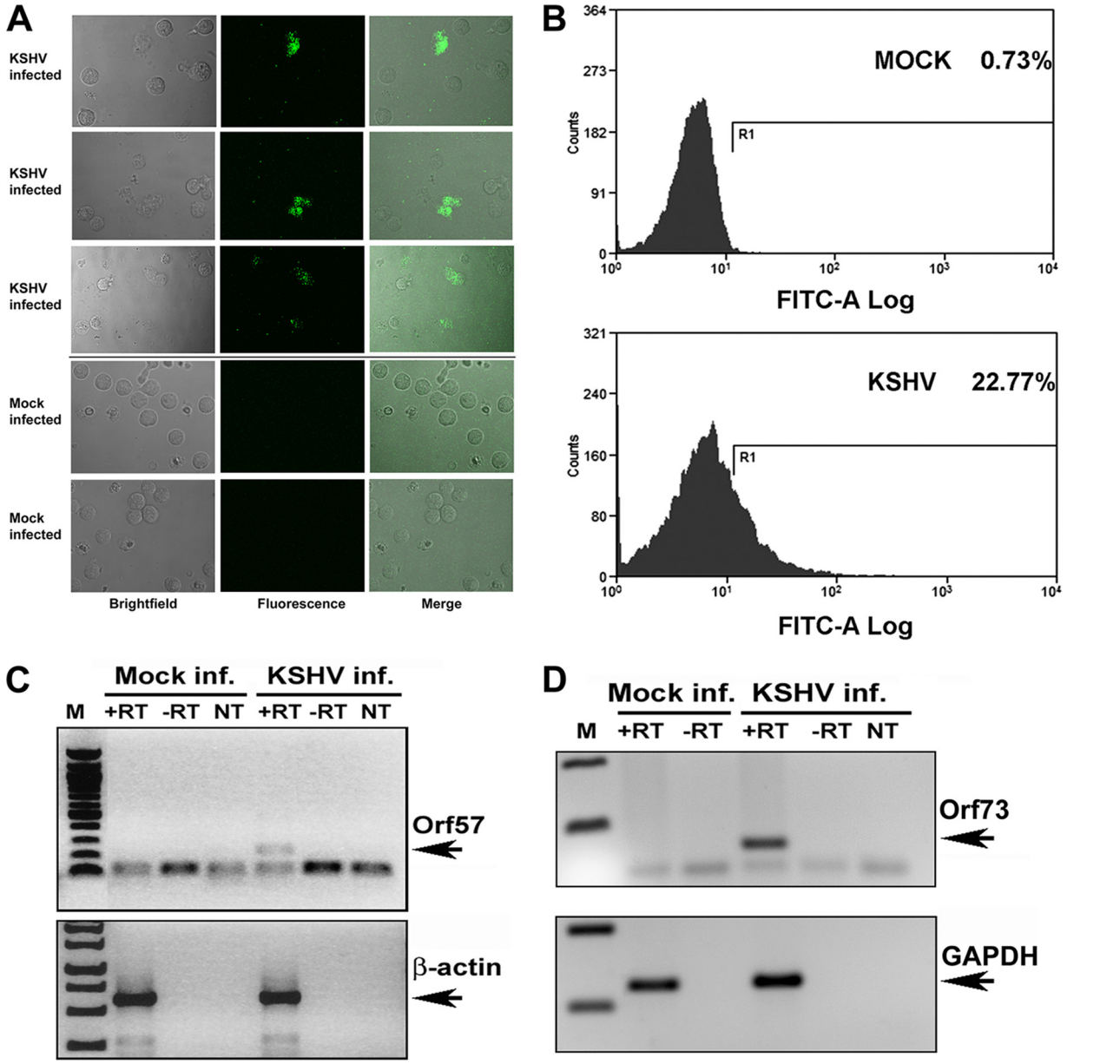

FIG. 5. KSHV infection of pDCs. (A) Mock- or KSHV-infected live pDCs were imaged using confocal microscopy to identify KSHV-infected cells. Cells expressing GFP were detected only in KSHV-infected pDCs, whereas no GFP was detected in the mock-infected cells. Images were taken on a Zeiss LSM5 Pa laser scanning microscope and processed using the Zeiss LSM Image browser. (B) Percent GFP expression in mock-infected pDCs versus KSHV-infected pDCs at 16 h postinfection. Flow cytometry of mock- and KSHV-infected cells. (C) Orf57 transcription in KSHV-infected pDCs. Cell pellets were processed for RNA and reverse transcribed to cDNA. RT-PCR was performed using primers for the viral early lytic gene Orf57. KSHV Orf57 transcription was detected only in KSHV-infected cells, not in mock-infected cells. Controls included RT-PCR of β-actin transcript levels. +RT, reverse transcription of the isolated RNA; −RT, isolated RNA was added to the RT-PCR mix to serve as a control for genomic DNA contamination; NT, no template was added to the RT-PCR mix to serve as a negative control for the primer sets. (D) Orf73 transcription in KSHV-infected pDCs. Analysis was performed as described for panel B. Controls included RT-PCR of GAPDH transcript levels.

Cytokine secretion following KSHV infection. In order to determine the profiles of any additional cytokines that were induced following KSHV infection of pDCs, we analyzed supernatants from mock-infected and KSHV-infected cells using cytokine antibody arrays. We were able to identify several proteins that were induced upon KSHV infection of pDCs. Table 1 shows a list of the cytokines that were consistently upregulated across 3 donors. IL-6, IL-8, and three members of the CC family of chemokines, MIP-1α, MIP-1β, and macrophage-derived chemokine (MDC/CCL22), were all upregulated in KSHV-infected pDCs from three different donors compared to mock-infected pDCs. We did observe other upregulated cytokines, e.g., TNF-α, in some of the donors, but this was not consistent across all donors.

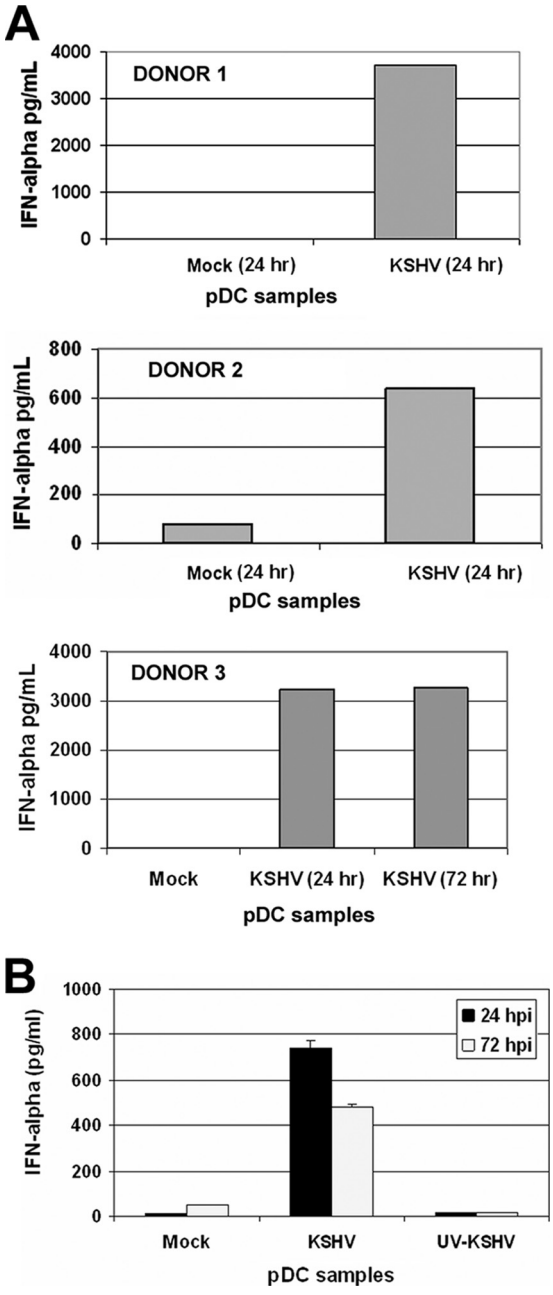

FIG. 6. KSHV infection of pDCs induces secretion of IFN-α. (A) IFN-α secretion was measured at different time points post-KSHV infection. Supernatants from infected pDCs were collected at the indicated times postinfection and analyzed for the presence of IFN-α by ELISA. Mock-infected pDCs were used as the control. (B) Isolated pDCs were subjected to mock infection, WT KSHV infection, or infection with UV-KSHV at the indicated time points. Supernatants were analyzed for the presence of IFN-α by ELISA.

TLR9 inhibition blocks IFN-α secretion following KSHV infection. In order to determine a possible mechanism for pDC activation and IFN-α secretion upon KSHV infection, we tested the ability of pDCs to respond to KSHV infection in the presence of a TLR9 inhibitor, G-ODN. The inhibitory oligonucleotide was added simultaneously with KSHV (as described in Materials and Methods) or with CpG DNA, which served as a positive control. Supernatants from infected pDCs were harvested at 16 h postinfection and were analyzed by ELISA for IFN-α secretion. As shown in Fig. 7A, the IFN-α response in the pDCs treated with CpG alone or virus alone was increased significantly over that in mock-treated cells. However, in samples treated in combination with either KSHV and TLR9 inhibitor or CpG and TLR9 inhibitor, the IFN-α response was significantly inhibited (Fig. 7A). These data suggest that the IFN-α response of pDCs stimulated by KSHV infection is mediated partly through TLR9. We also analyzed two additional donors for IFN-α production at both 16 and 45 h post-KSHV infection in the presence of the TLR9 inhibitor. As shown in Fig. 7B, both donors showed significant inhibition of IFN-α secretion at both 16 and 45 h postinfection in the presence of the inhibitor.

In order to further confirm that infection of pDCs and the subsequent activation of IFN-α via TLR9 were specific to live virus infection, not a result of contaminating DNA present in the concentrated KSHV stock, purified and concentrated virus was treated with 0.1 U of DNase for 10 min at 37°C prior to pDC infection. Following pDC infection with DNase-treated virions, supernatants were collected at 16 and 45 h postinfection and analyzed for IFN-α secretion by ELISA. There was no difference in IFN-α secretion levels following infection of pDCs with DNase-treated KSHV or untreated KSHV (Fig. 7C).

DISCUSSION

Virus infection almost always results in the production of type 1 IFN, one of the primary defenses employed by the innate immune system to control virus infection. Plasmacytoid dendritic cells have been shown to be the major producers of type 1 IFN in response to stimuli in the blood cell population, indicating that they play a vital role in the host response to infection. Interestingly, in patients with hyaline-vascular Castleman's disease, elevated levels of pDCs have been observed; however, the significance of this finding is not currently known (7). The role of pDCs in KSHV pathogenesis is unclear. However, secretion of cytokines and chemokines from virus-infected pDCs may play a role in T-cell recruitment and in promoting T-cell survival, along with T-cell polarization and production of IFN-γ. Recruitment of $CD4^+$ and $CD8^+$ T cells to sites of infection is also a consequence of pDC infection/activation and secretion of chemokines (for a review, see reference 31). In addition, due to the migratory nature of pDCs, it is possible that infection of these cells may aid in the dissemination of KSHV throughout the body (16).

KSHV is known to establish a life-long latent infection in the human host. Hence, KSHV is able to survive the host innate

TABLE 1. Fold increases in cytokines upregulated upon KSHV infection compared to mock infection of human pDCs

Donor	Fold increase in cytokine				
	MDC	MIP-1α	MIP-1β	IL-6	IL-8
1	1.34	3.27	3.98	2.99	2.43
2	1.40	1.71	1.16	3.43	1.59
3	1.57	2.45	2.86	2.07	2.87

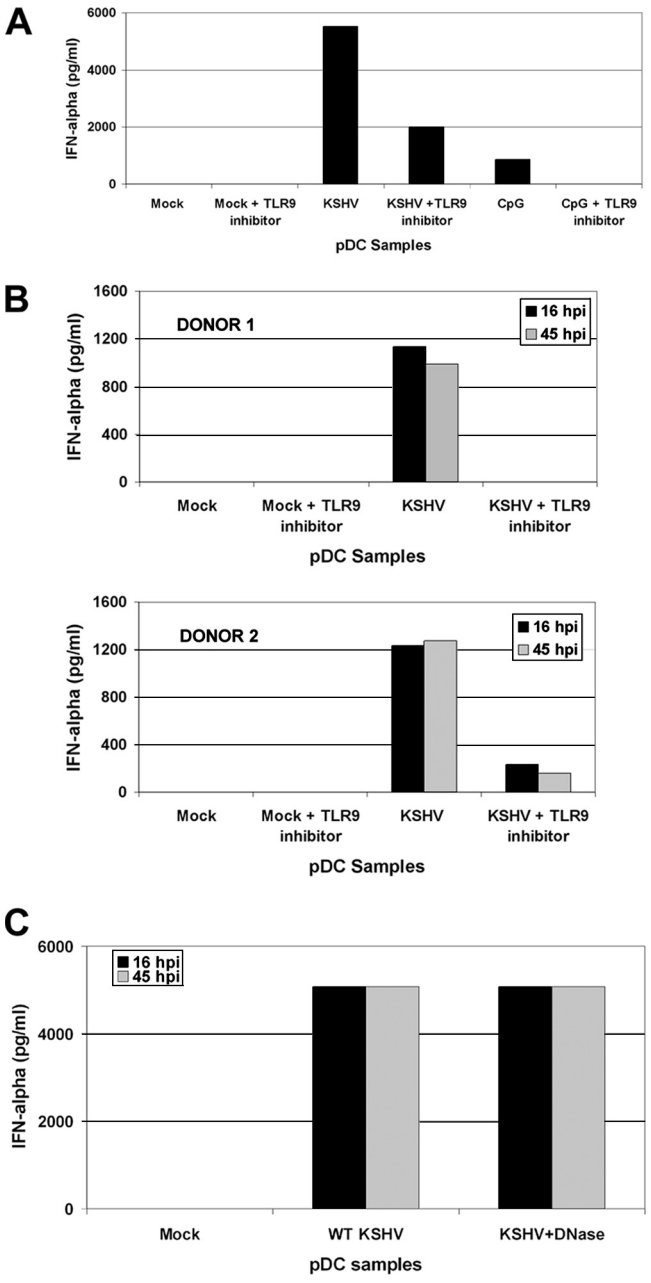

FIG. 7. TLR9 inhibition blocks IFN-α secretion following KSHV infection. (A) pDCs were treated with a TLR9-inhibitory oligonucleotide (G-ODN) at a 25 μM final concentration simultaneously with either KSHV or 10 μM CpG DNA (as a positive control). Infections were carried out as described in Materials and Methods. Supernatants were harvested at 16 h postinfection and were analyzed for IFN-α production by ELISA. IFN-α secretion is shown for cells that were either mock infected, infected with KSHV, or treated with CpG (TLR9 agonist) in the presence or absence of the TLR9 inhibitor G-ODN. (B) pDCs were obtained from 2 different donors and were either mock infected or infected with KSHV in the presence or absence of the TLR9 inhibitor. IFN-α secretion was measured at 16 and 45 h post-KSHV infection. (C) DNase treatment of purified KSHV intact virions does not reduce IFN-α levels following infection. Purified KSHV was treated for 10 min at 37°C with 0.1 U of DNase I. pDCs were infected with either WT or DNase-treated KSHV, and supernatants were collected and analyzed for the presence of IFN-α.

immune response mounted against it during primary infection. We wanted to determine whether the primary blood cell type responsible for type I IFN production, pDCs, were involved in the initial recognition of KSHV during primary infection and whether KSHV could infect or stimulate a response from human pDCs. Additionally, since pDCs are not known to express TLR3, a TLR we previously reported as being important for KSHV activation in human monocytes (35), we wanted to determine if a different TLR was responsible for KSHV recognition in pDCs, since pDCs express exclusively TLR7 and TLR9.

We found that KSHV can infect pDCs and that KSHV transcripts can be detected at 48 and 72 h postinfection. Additionally, we report that pDCs secrete significant amounts of IFN-α in response to KSHV infection. The levels of IFN-α that we observed were similar to those observed when pDCs are infected with HCMV (32) and slightly higher, on average, than the levels observed when pDCs are infected with EBV (29). Following KSHV infection of pDCs, we also observed upregulation of the costimulatory molecules CD83 and CD86. These have been shown to be activated by other herpesviruses, including HSV-1 and HCMV (19, 32). Intriguingly, one of the downstream effects of CD86 activation is the binding of CTLA-4, which can modulate host T-cell responses (22). Downregulation of the host T-cell response would provide a great advantage to KSHV during its mission to establish latency and avoid detection by the adaptive immune response. Thus, inhibition of the T-cell response would greatly increase the chances of KSHV survival and persistence in the host.

It is currently not known whether KSHV can establish latency in pDCs, since under most tissue culture conditions pDCs exhibit a very short life span of a few days (reviewed in reference 21). Additionally, these cells also display short life spans *in vivo* (21). However, it is plausible that KSHV-infected pDCs could serve as transmitters of virus to other cells, since they migrate to lymphatic tissues such as the spleen, thymus, and lymph nodes and also migrate to sites of local infection throughout the host (21).

We found that TLR9 is the primary mediator of the IFN-α response following KSHV infection of pDCs. Pretreatment of pDCs with a TLR9-inhibitory oligonucleotide blocked IFN-α production. We cannot rule out a contribution from TLR7 signaling to activation and IFN production by pDCs, as there are no commercially available TLR7-specific antagonists. However, based on the data in Fig. 7, we can conclude that TLR9 is the primary responder to KSHV infection in pDCs, since inhibition of TLR9 led to an average reduction of 86% in IFN-α secretion from KSHV-infected pDCs compared to that from untreated pDCs.

In summary, we report that pDCs are activated by KSHV infection and respond via upregulation of CD83 and CD86 and secretion of IFN-α. KSHV infection of pDCs results in activation of the TLR9 signaling pathway, leading to increased production and secretion of IFN-α from infected cells.

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REFERENCES

1. **Akira, S., and H. Hemmi.** 2003. Recognition of pathogen-associated molecular patterns by TLR family. Immunol. Lett. **85:**85–95.
2. **Akira, S., and S. Sato.** 2003. Toll-like receptors and their signaling mechanisms. Scand. J. Infect. Dis. **35:**555–562.
3. **Akira, S., and K. Takeda.** 2004. Toll-like receptor signalling. Nat. Rev. Immunol. **4:**499–511.
4. **Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford.** 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U. S. A. **98:**9237–9242.
5. **Blasig, C., C. Zietz, B. Haar, F. Neipel, S. Esser, N. H. Brockmeyer, E. Tschachler, S. Colombini, B. Ensoli, and M. Sturzl.** 1997. Monocytes in Kaposi's sarcoma lesions are productively infected by human herpesvirus 8. J. Virol. **71:**7963–7968.
6. **Boshoff, C., T. F. Schulz, M. M. Kennedy, A. K. Graham, C. Fisher, A. Thomas, J. O. McGee, R. A. Weiss, and J. J. O'Leary.** 1995. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat. Med. **1:**1274–1278.
7. **Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, and M. Colonna.** 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat. Med. **5:**919–923.
8. **Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N. Engl. J. Med. **332:**1186–1191.
9. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865–1869.
10. **Ferbas, J. J., J. F. Toso, A. J. Logar, J. S. Navratil, and C. R. Rinaldo, Jr.** 1994. CD4+ blood dendritic cells are potent producers of IFN-alpha in response to in vitro HIV-1 infection. J. Immunol. **152:**4649–4662.
11. **Fonteneau, J. F., M. Gilliet, M. Larsson, I. Dasilva, C. Munz, Y. J. Liu, and N. Bhardwaj.** 2003. Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity. Blood **101:**3520–3526.
12. **Gilliet, M., W. Cao, and Y. J. Liu.** 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat. Rev. Immunol. **8:**594–606.
13. **Gregory, S. M., J. A. West, P. J. Dillon, C. Hilscher, D. P. Dittmer, and B. Damania.** 2009. Toll-like receptor signaling controls reactivation of KSHV from latency. Proc. Natl. Acad. Sci. U. S. A. **106:**11725–11730.
14. **Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740–745.
15. **Ito, T., R. Amakawa, T. Kaisho, H. Hemmi, K. Tajima, K. Uehira, Y. Ozaki, H. Tomizawa, S. Akira, and S. Fukuhara.** 2002. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. J. Exp. Med. **195:**1507–1512.
16. **Jones, K. S., C. Petrow-Sadowski, Y. K. Huang, D. C. Bertolette, and F. W. Ruscetti.** 2008. Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4(+) T cells. Nat. Med. **14:**429–436.
17. **Kadowaki, N., S. Antonenko, J. Y. Lau, and Y. J. Liu.** 2000. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J. Exp. Med. **192:**219–226.
18. **Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu.** 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. J. Exp. Med. **194:**863–869.
19. **Kittan, N. A., A. Bergua, S. Haupt, N. Donhauser, P. Schuster, K. Korn, T. Harrer, and B. Schmidt.** 2007. Impaired plasmacytoid dendritic cell innate immune responses in patients with herpes virus-associated acute retinal necrosis. J. Immunol. **179:**4219–4230.
20. **Krishnan, H. H., P. P. Naranatt, M. S. Smith, L. Zeng, C. Bloomer, and B. Chandran.** 2004. Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. J. Virol. **78:**3601–3620.
21. **Krug, A., A. R. French, W. Barchet, J. A. Fischer, A. Dzionek, J. T. Pingel, M. M. Orihuela, S. Akira, W. M. Yokoyama, and M. Colonna.** 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity **21:**107–119.
22. **Kurt-Jones, E. A., M. Chan, S. Zhou, J. Wang, G. Reed, R. Bronson, M. M. Arnold, D. M. Knipe, and R. W. Finberg.** 2004. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc. Natl. Acad. Sci. U. S. A. **101:**1315–1320.
23. **Lechmann, M., S. Berchtold, J. Hauber, and A. Steinkasserer.** 2002. CD83 on dendritic cells: more than just a marker for maturation. Trends Immunol. **23:**273–275.
24. **Liu, Y. J.** 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu. Rev. Immunol. **23:**275–306.
25. **Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki.** 2003. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. J. Exp. Med. **198:**513–520.
26. **McKenna, K., A. S. Beignon, and N. Bhardwaj.** 2005. Plasmacytoid dendritic cells: linking innate and adaptive immunity. J. Virol. **79:**17–27.
27. **Monini, P., S. Colombini, M. Sturzl, D. Goletti, A. Cafaro, C. Sgadari, S. Butto, M. Franco, P. Leone, S. Fais, G. Melucci-Vigo, C. Chiozzini, F. Carlini, G. Ascherl, E. Cornali, C. Zietz, E. Ramazzotti, F. Ensoli, M. Andreoni, P. Pezzotti, G. Rezza, R. Yarchoan, R. C. Gallo, and B. Ensoli.** 1999. Reactivation and persistence of human herpesvirus-8 infection in B cells and monocytes by Th-1 cytokines increased in Kaposi's sarcoma. Blood **93:**4044–4058.
28. **Prazma, C. M., N. Yazawa, Y. Fujimoto, M. Fujimoto, and T. F. Tedder.** 2007. CD83 expression is a sensitive marker of activation required for B cell and CD4+ T cell longevity in vivo. J. Immunol. **179:**4550–4562.
29. **Quan, T. E., R. M. Roman, B. J. Rudenga, V. M. Holers, and J. Craft.** 2010. Epstein-Barr virus promotes interferon-alpha production by plasmacytoid dendritic cells. Arthritis Rheum. **62:**1693–1701.
30. **Rappocciolo, G., F. J. Jenkins, H. R. Hensler, P. Piazza, M. Jais, L. Borowski, S. C. Watkins, and C. R. Rinaldo, Jr.** 2006. DC-SIGN is a receptor for human herpesvirus 8 on dendritic cells and macrophages. J. Immunol. **176:**1741–1749.
31. **Swiecki, M., and M. Colonna.** 2010. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. Immunol. Rev. **234:**142–162.
32. **Varani, S., M. Cederarv, S. Feld, C. Tammik, G. Frascaroli, M. P. Landini, and C. Soderberg-Naucler.** 2007. Human cytomegalovirus differentially controls B cell and T cell responses through effects on plasmacytoid dendritic cells. J. Immunol. **179:**7767–7776.
33. **Vieira, J., and P. M. O'Hearn.** 2004. Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. Virology **325:**225–240.
34. **Weslow-Schmidt, J. L., N. A. Jewell, S. E. Mertz, J. P. Simas, J. E. Durbin, and E. Flano.** 2007. Type I interferon inhibition and dendritic cell activation during gammaherpesvirus respiratory infection. J. Virol. **81:**9778–9789.
35. **West, J., and B. Damania.** 2008. Upregulation of the TLR3 pathway by Kaposi's sarcoma-associated herpesvirus. J. Virol. **82:**5440–5449.
36. **Wu, W., J. Vieira, N. Fiore, P. Banerjee, M. Sieburg, R. Rochford, W. Harrington, Jr., and G. Feuer.** 2006. KSHV/HHV-8 infection of human hematopoietic progenitor (CD34+) cells: persistence of infection during hematopoiesis in vitro and in vivo. Blood **108:**141–151.
37. **Yonkers, N. L., B. Rodriguez, K. A. Milkovich, R. Asaad, M. M. Lederman, P. S. Heeger, and D. D. Anthony.** 2007. TLR ligand-dependent activation of naive CD4 T cells by plasmacytoid dendritic cells is impaired in hepatitis C virus infection. J. Immunol. **178:**4436–4444.