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Kaposi's Sarcoma-Associated Herpesvirus-Encoded Latency-Associated Nuclear Antigen Reduces Interleukin-8 Expression in Endothelial Cells and Impairs Neutrophil Chemotaxis by Degrading Nuclear p65▽

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Latency-associated nuclear antigen 1 (LANA-1) of Kaposi's sarcoma-associated herpesvirus (KSHV) is the major viral latent protein and functions as a multifaceted protein. Here, we report that LANA-1 attenuates the endothelial response to tumor necrosis factor alpha (TNF-α) stimulation and inhibits consequent neutrophil chemotaxis. Reporter assays showed that LANA-1 constantly repressed nuclear factor (NF)-κB transactivity upon TNF-α stimulation. We also found that LANA-1 decreased nuclear p65 protein levels through enhancement of polyubiquitylation-mediated p65 degradation and that an elongin B/elongin C–cullin 5–LANA-1–p65 complex assembled by LANA-1 was responsible for this enhanced p65 degradation. In telomerase-immortalized human umbilical vein endothelial cells, LANA-1 was demonstrated to repress interleukin-8 expression, which was involved in neutrophil recruitment to the inflammatory site. Through an *in vitro* transmigration assay, we determined a suppressive effect of LANA-1 on neutrophil chemotaxis. Our work suggests that KSHV LANA-1 is a negative modulator of acute inflammation and sheds light on a new mechanism by which KSHV during the latent life cycle evades the host innate immune response.

Kaposi's sarcoma-associated herpesvirus (KSHV) is a recently discovered DNA tumor virus (15, 16), which has been identified as the etiological agent of three malignancies: Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma (14, 89). Like other herpesviruses, KSHV displays dual-phase, lytic and latent life cycles. During the lytic life cycle, a large number of KSHV genes are expressed, host cells are broken down, and KSHV progeny virions are released (79, 85, 90). During the latent life cycle, the KSHV genome is maintained as episomes tethered to host chromosomes and replicates along with infected cells (78). To evade host immune surveillance, only a few KSHV genes are expressed during latency (26, 85).

Latency-associated nuclear antigen 1 (LANA-1), which is encoded by KSHV open reading frame 73 (ORF73), is one of several latently expressed genes and is highly expressed in KSHV latently infected cells (85, 90). Previous studies have demonstrated that LANA-1 is a multifunctional protein. LANA-1 is required for long-term maintenance of KSHV episomes in host cells (101). LANA-1 acts as a transcriptional modulator for KSHV and host cell genes. Among KSHV transcriptomes, LANA-1 represses ORF50, the master molecule that controls the KSHV latent/lytic switch, which helps maintain the latent state (54). Likewise, LANA-1 can activate and repress cellular genes by interacting with and altering transcriptional specificity of several transcription factors such as Daxx, CREB-binding protein, RING3, ATF4, and c-Jun (5, 58, 59, 67, 74, 98). As the major oncogenic protein of KSHV, LANA-1 binds to and antagonizes a series of cellular tumor suppressor proteins. LANA-1 binds to glycogen synthase kinase-3 and Sel-10 to stabilize their respective substrates c-Myc, β-catenin, and intracellular Notch, which are involved in host cell proliferation (11, 33, 34, 55, 61, 62). LANA-1 also inhibits retinoblastoma protein-mediated cell cycle progression arrest and p53-induced apoptosis (32, 76). Remarkably, a new mechanism by which LANA-1 counteracts p53 proapoptotic effect has been recently reported, in which LANA-1 degrades p53 protein by recruiting it to elongin B/C-cullin 5 ubiquitin ligase and enhancing its polyubiquitylation-mediated degradation (12). This indicates that LANA-1 could utilize cellular ubiquitin-proteasome machinery to facilitate KSHV survival and propagation.

Acute inflammation is a major part of the host innate immune response. It commences within vascular tissues and eradicates invading foreign microbes and injured host tissues. During acute inflammation, neutrophils migrate from flowing blood to the site of inflammation and destroy invading foreign microorganisms (3, 8, 25, 66, 72). Recruitment of neutrophils to the endothelium and subsequent transendothelial migration depend on activation of vascular endothelial cells by proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (23, 66, 75). Upon stimulation of TNF-α or IL-1, endothelial cells upregulate E-selectin and intercellular adhesion molecule 1 (ICAM-1) expression on

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the luminal surface and increase several chemokines, mainly IL-8 (CXCL-8), secretion. E-selectin is responsible for the initial tethering of neutrophils to the endothelium; released IL-8 is captured and presented on the endothelium by heparin sulfate proteoglycans, the bound IL-8 triggers integrin activation on neutrophils, and subsequent ICAM-1–integrin interaction mediates firm adhesion of endothelium and neutrophils. Established IL-8 gradients outside endothelial cells are thought to guide neutrophil transmigration over the endothelium into inflammatory tissue (27, 50, 53, 64, 65, 88).

In mammalian cells, the nuclear factor (NF)-κB transcription factor family includes five members: RelA (p65), NF-κB1 (p50/p105), RelB, NF-κB2 (p52/p100), and c-Rel. They form homo- or heterodimers and bind to DNA sequences, known as κB elements, to induce and repress gene expression (35, 39, 95). It is well established that activation of the NF-κB dimer p65-p50 is responsible for endothelial cell activation and proinflammatory gene expression upon stimulation with TNF-α (7, 38, 93). TNF-α engagement with TNF-α receptor 1 (TNFR1) causes recruitment of adaptor proteins, such as TNFR1-associated via death domain protein (TRADD), to the cytoplasmic domain of TNFR1. TRADD further recruits receptor interacting protein 1 and TNFR-associated factor 2, and this complex activates the trimeric IκB kinase (IKK) complex. Phosphorylation of inhibitor of NF-κBα (IκBα) by IKK and subsequent ubiquitin-proteasome-mediated degradation of IκBα result in release and translocation of NF-κB dimer (p65-p50) to the nucleus. The NF-κB dimer along with activated AP-1 transactivates a series of inflammatory genes, such as ICAM-1, vascular cell adhesion molecule 1 (VCAM-1), IL-6, and IL-8 (7, 38, 93).

Neutrophils eradicate pathogens at the infected site by releasing microbicidal but also normal tissue-destructive proteases and reactive oxygen intermediates in an acute inflammatory response (8, 20, 41, 68, 72). How latently infected KSHV survives this cytotoxic pressure in acute inflammation triggered by a secondary infection is not known. Here, we reported that KSHV LANA-1 decreased nuclear p65 protein levels and repressed p65 transactivation upon stimulation of TNF-α. We found that, similar to the mechanism by which LANA-1 degrades p53, LANA-1 enhanced p65 polyubiquitylation by recruiting p65 to the elongin B/C-cullin 5 ubiquitin ligase complex. LANA-1 reduced IL-8 expression in telomerase-immortalized human umbilical *v*ein endothelial (TIVE) cells upon stimulation of TNF-α. LANA-1 significantly impaired neutrophil chemotaxis. This is believed to be the first study to demonstrate that LANA-1, a KSHV latent gene product, interferes with neutrophil recruitment. Given that neutrophils are the main executor in acute inflammation, our findings provide clues to understanding how latently infected KSHV survives during host acute inflammation.

MATERIALS AND METHODS

Antibodies and plasmids. A 9E10 hybridoma cell line, which produces c-Myc monoclonal antibody, was obtained from the University of Michigan Hybridoma Core Facility (Ann Arbor, MI). Anti-hemagglutinin (HA) and anti-Flag (H9658 and F3165) monoclonal antibodies and rabbit anti-β-actin polyclonal antibody (A2066) were purchased from Sigma (St. Louis, MO). Rabbit anti-p65 polyclonal antibody (PC137) was obtained from Merck (Darmstadt, Germany). Anti-lamin B1 monoclonal antibody (332000) was purchased from Invitrogen Corporation (Carlsbad, CA). Anti-ubiquitin, anti-elongin B, anti-elongin C, and anti-cullin 5 monoclonal antibodies (sc-130410, sc-11447, sc-28245, and sc-13014, respectively) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

pA3M-LANA-1, pA3F-LANA-1, and pEGFPC1-LANA-1-3Flag, which encode full-length LANA-1, have been described previously (96). The 3κB-luc NF-κB reporter plasmid was a kind gift from Chen Wang (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) and has been described previously (24). Human p65 was amplified from a 293T cell cDNA library and cloned into a pCDNA3.1-HA (Invitrogen) expression vector at BamHI and XhoI sites. A lentivector packaging p65 was constructed by inserting subcloned p65 sequence into pCDH-CMV-MCS-EF1-CoGFP (where CMV is cytomegalovirus and GFP is green fluorescent protein) (System Biosciences, Mountain View, CA) at BamHI and NotI sites. Human ubiquitin was also amplified from a 293T cell cDNA library and cloned into pCMV-myc (Clontech, Mountain View, CA) expression vector at EcoRI and SalI sites. An enhanced GFP (EGFP)-LANA-1-3Flag fragment cut from pEGFPC1-LANA-1-3Flag with NheI and XbaI was ligated into pCDH-CMV-MCS-EF1-Puro lentivector (System Biosciences), which was designated pCDH-G3F-LANA-1, and enhanced green fluorescent protein was ligated into the same lentivector as the controls. An oligonucleotide that was complementary to the C-terminal (GCTAGGCCACAACACATCT) fragment of LANA as described before (34) was inserted into the PLL3.7 vector (a gift from Bing Sun, Institute Pasteur of Shanghai, Shanghai, China) to construct a small interfering RNA directed against LANA-1 (si-LANA-1). The oligonucleotide 5′-AGATTCCTGGCGTAAAAGCTT-3′ as described before (9) was inserted into PLL3.7 vector to construct si-cullin 5. The oligonucleotide 5′-CTTACGCTGAGTACTTCGA-3′ as described before (47) was inserted into PLL3.7 vector to construct si-Luc.

Cell lines and reagents. 293T cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine growth serum (HyClone, Logan, UT), 2 mM L-glutamine, 25 U/ml penicillin, and 25 μg/ml streptomycin. TIVE cells and KSHV long-term-infected TIVE cells (LTC) were kindly provided by Rolf Renne (University of Florida, Gainesville, FL) and characterized as described previously (4). TIVE cells and LTC were cultured in EGM-2 endothelia growth medium (Lonza, Cologne, Germany). TNF-α (300-01A) was purchased from Peprotech, Inc. (Rocky Hill, NJ), and reconstituted in deionized water. MG132 (C2211) was purchased from Sigma and diluted in dimethyl sulfoxide (DMSO).

Transfection and transduction by lentivirus. 293T cells were transiently transfected using the calcium phosphate method as described previously (70), with minor modifications. Briefly, 40 to 50% confluent 293T cells were used for transfection. One hour prior to transfection, cell culture medium was replaced, and a DNA-$CaCl_2$ mixture was prepared by diluting 2.5 M $CaCl_2$ in DNA solution at a ratio of 1:10. The DNA-$CaCl_2$ mixture was carefully added dropwise into equal volumes of HEBS solution (140 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 50 mM HEPES, pH 7.05). This mixture was incubated at room temperature for 15 min and then added to the cell culture medium.

Twenty-seven micrograms of lentivirus packaging plasmids (12 μg of lentivector, 9 μg of pCMV8.9 that contained different accessory genes, and 6 μg of vesicular stomatitis virus G protein that encodes lentivirus envelope protein) was used to transfect one 100-mm dish of 293T cells. At 48 h posttransfection, packaging cell culture medium was collected, filtered with 0.45-μm-pore-size filters, and ultracentrifuged at 25,000 rpm for 2.5 h using an L-80XP Beckman ultracentrifuge (Beckman Coulter, Inc., Brea, CA). The virus pellet was resuspended in EGM-2. To determine the viral titer, serial dilutions of viral suspension were used to transduce 293T cells, and the number of GFP-positive cells was measured by fluorescence-activated cell sorter analysis. TIVE cells or KSHV long-term-infected TIVE cells were transduced with lentivirus at a multiplicity of infection (MOI) of 20.

Dual-luciferase reporter assay. A dual-luciferase reporter assay system (E1910) was purchased from Promega (Madison, WI), and the assay was performed according to the manufacturer's protocol. pRL-SV40 (SV40 is simian virus 40) (Promega) that constitutively expresses *Renilla* luciferase in 293T cells was used to normalize firefly luciferase activity. Light emission of each sample was quantified in a luminometer (Veritas; Turner Biosystems). Results are shown as the relative fold difference compared to the unstimulated control cells.

Immunoprecipitation and immunoblotting. For immunoprecipitation, cells were lysed in radioimmunoprecipitation assay (RIPA) cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate plus protease inhibitor cocktail [sc-29131; Santa Cruz Biotechnology]) for 15 min, with vortexing every 5 min. Five percent of the cell lysate was kept as input, and the remainder was preincubated with protein A- and G-coupled Sepharose (Invitrogen) overnight at 4°C and then immunoprecipitated with antibodies indicated on the figures plus Sepharose for at least 4 h. Immunoprecipitates were

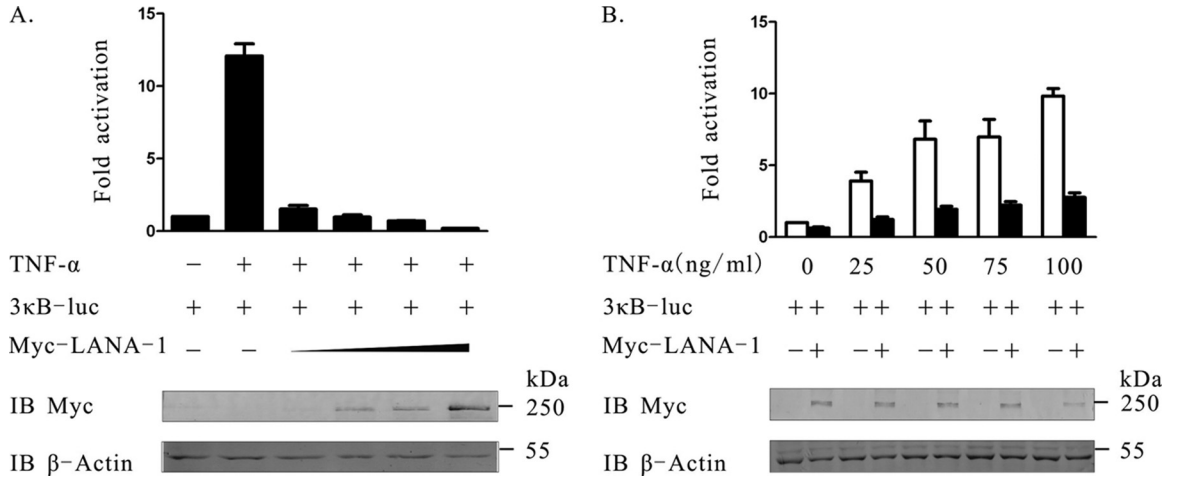

FIG. 1. KSHV LANA-1 represses NF-κB transcriptional activity induced by TNF-α in a luciferase reporter assay. (A) 293T cells were cotransfected with 1 μg of 3κB-luc reporter plasmid, 20 ng of pRL-SV40 normalizing plasmid, and either 30, 100, or 300 ng or 1 μg of pA3M-LANA-1 plasmid. Twenty-four hours after transfection, cells were stimulated with 50 ng/ml TNF-α for 45 min and harvested in another 24 h for luciferase assay. (B) 293T cells were transfected with 50 ng of pA3M vector, 500 ng of 3κB-luc reporter plasmid, and 10 ng of pRL-SV40 normalizing plasmid (open bars) or transfected with 50 ng of pA3M-LANA-1 plasmid, 500 ng of 3κB-luc reporter plasmid, and 10 ng of pRL-SV40 normalizing plasmid (filled bars). Then, cells were stimulated with TNF-α at increasing concentrations for 45 min, and cells were harvested after 24 h for luciferase assay. Means and standard deviations shown in the graphs were obtained from four independent experiments. IB, immunoblotting.

further washed with RIPA buffer at least three times and then boiled in SDS-PAGE loading buffer for immunoblotting analysis.

For immunoblotting analysis, protein samples were resolved by SDS-PAGE and transferred onto nitrocellulose membranes, which were blocked in 5% skim milk in TBST solution (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and probed with indicated antibodies for detection.

Immunofluorescence analysis. 293T or TIVE cells were cultured on poly-L-lysine precoated glass coverslips and transiently transfected or transduced with lentivirus. After stimulation with TNF-α or no stimulation, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 15 min, blocked with 5% normal goat serum (Invitrogen) in phosphate-buffered saline (PBS), and incubated with p65 and c-Myc antibodies as indicated in the figure legends. Cells were washed with phosphate-buffered saline–Triton X-100 (PBST) three times and further stained with appropriate secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen). Cell nuclei were indicated by staining with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma). Following staining, coverslips were mounted onto an inverted fluorescence microscope (Leica DM IRB; Leica, Solms, Germany) and photographed by using a digital camera and software (Leica).

Quantitative real-time PCR. Total RNA was extracted using TRIzol (Invitrogen), following the manufacturer's instructions. Five micrograms of RNA was used for reverse transcription with a First Strand cDNA Synthesis Kit (Fermentas UAB; Fermentas International, Inc., Burlington, Ontario, Canada). Quantitative real-time PCR (qRT-PCR) was performed in 96-well plates using a SYBR Real-Time PCR Master Mix Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Primers used were designed with software Primer Premier, version 5.0. The data represent fold changes compared to untreated control cells.

Enzyme-linked immunosorbent assay (ELISA). A human IL-8 Instant ELISA Kit (BMS204/3INST) was purchased from eBioscience (San Diego, CA). IL-8 in cell culture supernatant was quantified according to the manufacturer's instructions. Briefly, 50 μl of sample was added in duplicate to designated wells. The plate was incubated for 3 h at room temperature, emptied, and washed six times with wash buffer. Each well was incubated with 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution for 10 min at room temperature, 100 μl of stop buffer was added, and the color intensity was measured at 450 nm.

Neutrophil isolation. Peripheral blood neutrophils from healthy volunteers were isolated according to previously described protocols (51, 77), with minor modifications. Briefly, 5 ml of whole blood was diluted with an equal volume of RPMI 1640 medium (HyClone) and incubated with 10 ml of 3% dextran T-500 (Pharmacia) in a CO_2 incubator at 37°C for 30 min to allow sedimentation of red blood cells. The remaining leukocyte-rich plasma was layered on top of Ficoll Paque Plus (Amersham Pharmacia Biotech) and centrifuged at 500 × *g* for 40 min at 20°C. Neutrophils were enriched in the bottom layer, and remaining erythrocytes were lysed with ammonium chloride buffer [155 mM NH_4Cl, 2.96 mM $KHCO_2$, 3.72 mM $EDTA(Na)_2$]. After neutrophils were washed in PBS, they were resuspended in serum-free RPMI 1640 medium at a density of 10^6/ml for chemotaxis assay.

Neutrophil chemotaxis assay. A neutrophil chemotaxis assay was performed using a CytoSelect 96-Well Cell Migration Assay with 3-μm pores (CBA-104; Cell Biolabs). The assay was conducted following the product manual. The conditioned medium, along with negative control (PBS) and positive control (fetal bovine serum [FBS]), was added to the lower chamber, while neutrophil suspensions were added to the upper chamber. The plate was transferred to a cell incubator for 2 h, neutrophil lysis buffer mixed with CyQuant dye was added to the lower chamber, and fluorescence emitted from each well was recorded by a fluorescence reader at 480 nm. The data represent fold changes of transmigrated neutrophils compared to the wells filled with conditioned medium of untreated control endothelial cells.

Statistical analysis. Data are shown as mean values with standard errors of the means (SEM). The *P* value was determined by a Student *t* test with GraphPad Prism, version 5, software ($n = 4$ for each group).

RESULTS

LANA-1 represses NF-κB transcriptional activity induced by TNF-α. LANA-1 is the predominant protein expressed in KSHV latency and brings about profound cellular changes. We investigated whether LANA-1 interfered with NF-κB transactivator-mediated inflammation. An NF-κB reporter plasmid that contained three copies of κB consensus sequences, which drive the expression of firefly luciferase, was cotransfected with a LANA-1 expression plasmid or control vector into 293T cells. Proinflammatory cytokine TNF-α was chosen as the stimulus for transfected 293T cells. We found that LANA-1 significantly repressed NF-κB transcriptional activity upon TNF-α induction in a dose-dependent manner (Fig. 1A). Also, 293T cells transfected with an NF-κB reporter plasmid and control vector responded to TNF-α sensitively and in a TNF-α dose-dependent manner (Fig. 1B, open bars), whereas LANA-1-express-

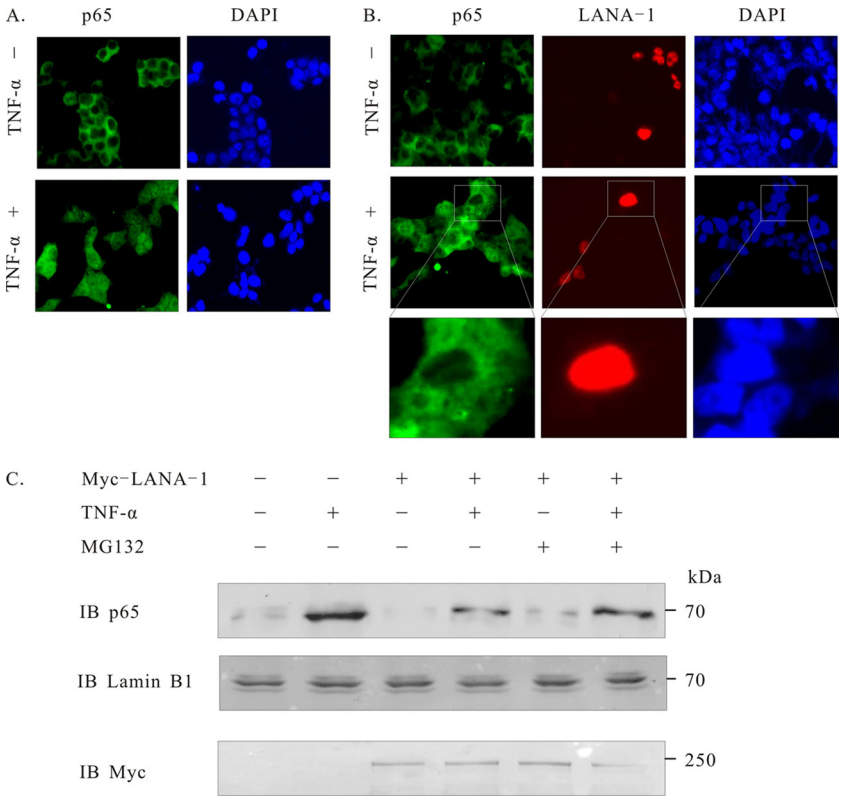

FIG. 2. LANA-1 decreases nuclear p65 protein level in 293T cells. (A) 293T cells were transfected with pA3M control vector. At 24 h posttransfection, cells were rested or stimulated with TNF-α at 50 ng/ml for 1 h and harvested for immunofluorescence analysis with p65 antibody. (B) 293T cells were transfected with pA3M-LANA-1 expression plasmid. At 24 h posttransfection, cells were left unstimulated or stimulated with TNF-α at 50 ng/ml for 1 h and harvested for immunofluorescence analysis with p65 and c-Myc antibodies. (C) pA3M- or pA3M-LANA-1-transfected 293T cells were stimulated with TNF-α at 50 ng/ml for 1 h or left unstimulated. MG132 at 20 μM was thereafter used to treat pA3M-LANA-1-transfected 293T cells for 2 h, or cells were left untreated. Then, cell nuclear extracts were subjected to SDS-PAGE and immunoblotting with antibodies.

ing 293T cells were unable to respond effectively (Fig. 1B, filled bars). These results indicated that LANA-1 acted as an inhibitor of NF-κB transactivation.

LANA-1 decreases nuclear p65 protein level. p65 is predominantly arrested in cytoplasm in rested cells and translocates into the nucleus upon activation. Given that KSHV LANA-1 exclusively resides in the nucleus, we tried to determine whether p65 nuclear behavior was disturbed by LANA-1. By immunofluorescence assay we found in control vector-transfected 293T cells that there was robust translocation of p65 from the cytoplasm to nucleus upon induction of TNF-α (Fig. 2A). However, in 293T cells that expressed LANA-1, there was a significant decrease in the level of nuclear p65 after TNF-α stimulation (Fig. 2B). These findings were confirmed by immunoblotting analysis of nuclear protein samples from control and LANA-1-expressing 293T cells (Fig. 2C). Similarly, through immunofluorescence analysis, we observed that nuclear p65 was significantly diminished upon stimulation in TIVE cells transduced with EGFP–LANA-1 compared to control TIVE cells (Fig. 3A and B). This was confirmed by immunoblotting analysis (Fig. 3C). It was noticeable that there was no evident concomitant accumulation of p65 in the cytoplasm (Fig. 2B and 3B). This suggests that LANA-1 promotes p65 degradation in the nucleus. Through degradation of translocated p65, LANA-1 attenuates its transactivation effect.

LANA-1 enhances nuclear p65 polyubiquitylation by recruiting it to elongin B/C-cullin 5 complex. The ubiquitin-proteasome pathway is the major mechanism by which cells target proteins for degradation (42, 48, 73, 86, 99). Several E3 ubiquitin ligases, including PDLIM2 (PDZ and LIM domain), SOCS-1 (suppressor of cytokine signaling), and COMMD1 (COMM domain-containing), have recently been shown to degrade nuclear p65 through the ubiquitin-26S proteasome pathway (63, 82, 92). By degrading nuclear p65, the NF-κB response is strongly terminated.

KSHV LANA-1 is a large nuclear protein that comprises 1,162 amino acids that form the molecular basis of interaction with various cellular and viral proteins. Recently, LANA-1 has been reported to act as an E3 component that is responsible for substrate recognition and targeting of p53 for degradation by recruiting it to the elongin B/C-cullin 5 complex (12). We found that nuclear p65 protein was significantly restored by the proteasome inhibitor MG132 in LANA-1-expressing 293T cells (Fig. 2C). This indicated that LANA-1 decreased the nuclear p65 protein level through the ubiquitin-26S proteasome pathway. We therefore hypothesized that LANA-1 pro-

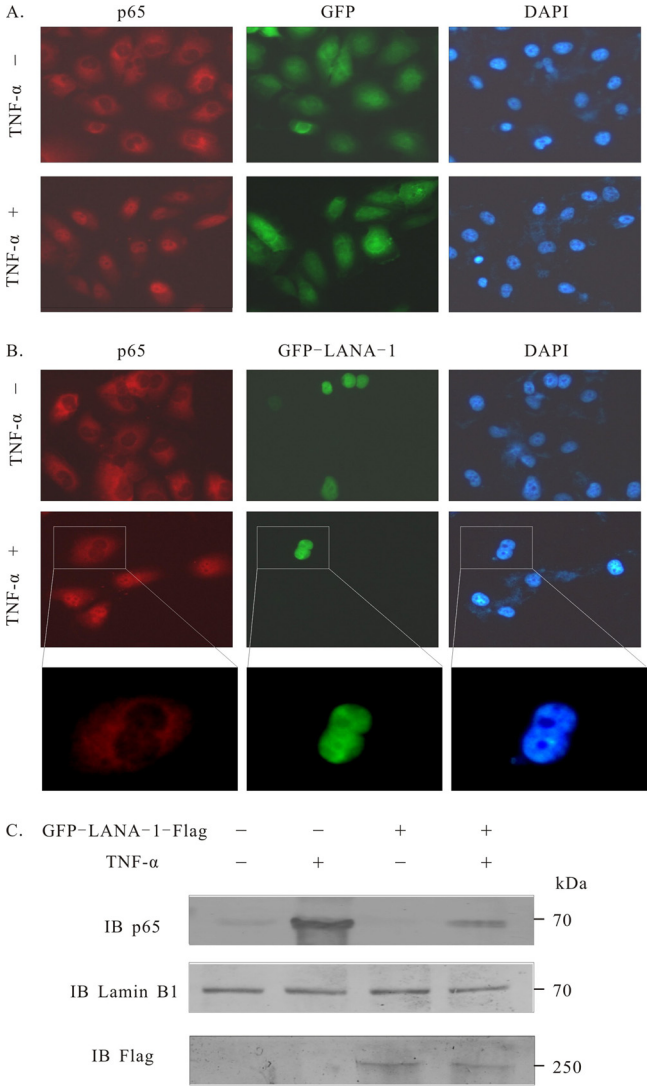

FIG. 3. LANA-1 decreases nuclear p65 protein levels in TIVE cells. (A) TIVE cells transduced with the EGFP gene were stimulated with TNF-α at 50 ng/ml for 15 min or left unstimulated and harvested for immunofluorescence analysis with p65 antibody. (B) TIVE cells were transduced with EGFP–LANA-1-3Flag sequence, and expression of the LANA-1 gene was monitored by the green fluorescence of EGFP–LANA-1-3Flag fusion protein. The transduced TIVE cells were stimulated with TNF-α at 50 ng/ml for 15 min or left unstimulated and harvested for immunofluorescence analysis with p65 antibody. (C) Control lentivirus or LANA-1-delivering lentivirus-transduced TIVE cells were stimulated with TNF-α for 15 min or were unstimulated. Then, their nuclear extracts were subjected to SDS-PAGE and immunoblotting with indicated antibodies.

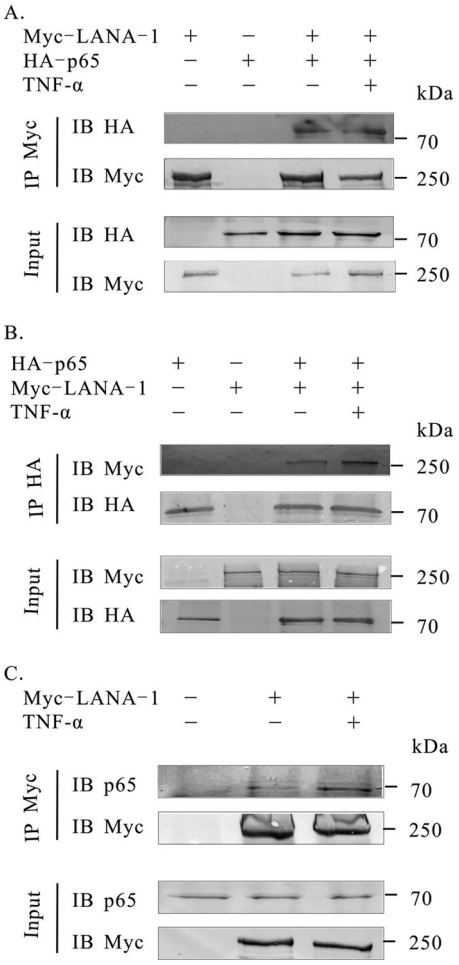

FIG. 4. LANA-1 binds to p65 *in vivo*. (A and B) 293T cells were transfected with the indicated expression plasmid combinations, cell lysates were immunoprecipitated using c-Myc antibody and HA antibody, respectively, and the immunoprecipitates and 5% of whole-cell lysates were assessed by immunoblotting with indicated antibodies. (C) 293T cells transfected with pA3M-LANA-1 or pA3M were lysed and subjected to immunoprecipitation with c-Myc antibody, and the immunoprecipitates and 5% of whole-cell lysates were assessed by immunoblotting with p65 antibody. IP, immunoprecipitation.

motes polyubiquitylation-mediated degradation of nuclear p65 by recruiting it to the elongin B/C-cullin 5 complex.

First, we performed an immunoprecipitation analysis in 293T cells to determine if LANA-1 bound to p65. We found that overexpressed p65 was coimmunoprecipitated with cotransfected LANA-1 in 293T cells and vice versa (Fig. 4A and B). Also, binding of p65 and LANA-1 was confirmed by coimmunoprecipitation of endogenous p65 with LANA-1 in 293T cells (Fig. 4C). To determine whether LANA-1 enhanced p65 polyubiquitylation, we cotransfected 293T cells with p65 plus LANA-1 or empty vector. TNF-α was added to facilitate colocalization of overexpressed LANA-1 and p65. MG132 was used to stabilize potential polyubiquitylated proteins. Immunoprecipitated nuclear p65 was subjected to immunoblotting with ubiquitin antibody, and typical polyubiquitin-conjugated protein smears were observed. As expected, ubiquitylation of p65 was dramatically increased in the presence of LANA-1 (Fig. 5A). To determine if the complex of LANA-1/cullin 5/elongin B/C was responsible for polyubiquitylation of p65, cullin 5 knockdown 293T cells were created using an siRNA construct that targeted cullin 5 (Fig. 5B). p65, ubiquitin along with LANA-1, or a control vector was cotransfected into cullin 5 knockdown and control 293T cells, and immunoprecipitated nuclear p65 was subjected to immunoblotting with tag antibody detecting transfected ubiquitin. We found that promotion of polyubiquitylation of nuclear p65 triggered by LANA-1 in con-

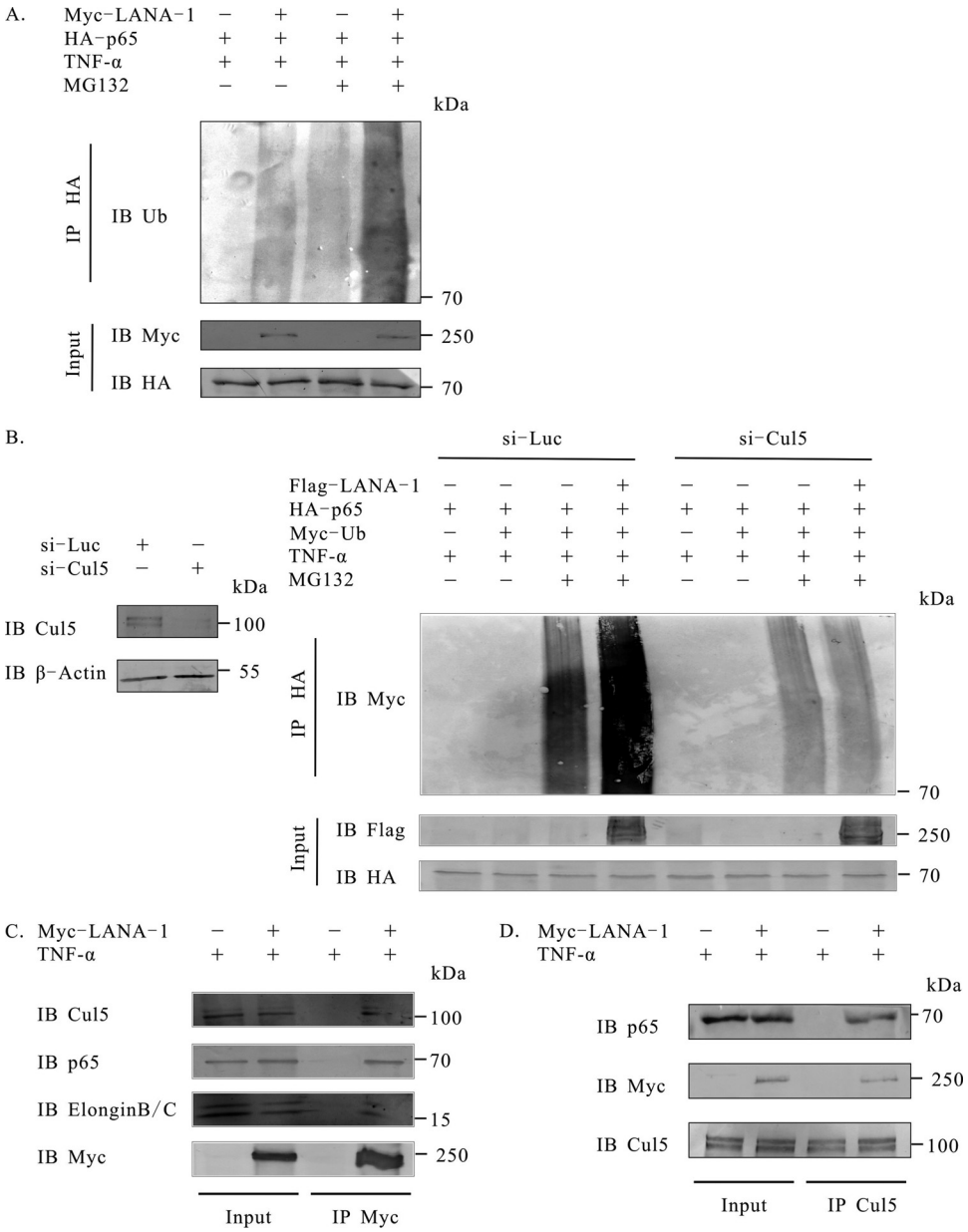

FIG. 5. LANA-1 enhances nuclear p65 polyubiquitylation by recruiting it to elongin B/C-cullin 5 complex. (A) 293T cells were transfected with HA-p65 together with pA3M or pA3M-LANA-1. At 24 h posttransfection, cells were stimulated with 50 ng/ml TNF-α for 30 min and treated with DMSO (mock) or MG132 for 4 h. Nuclear extracts were subjected to immunoprecipitation with HA antibody and immunoblotting with human ubiquitin antibody to detect polyubiquitylation-modified nuclear HA-p65. (B) Cullin 5 knockdown and control 293T cells were cotransfected with the indicated plasmid combinations. At 24 h after transfection, cells were incubated with 50 ng/ml TNF-α for 30 min and treated with DMSO (mock) or MG132 for 4 h. Nuclear extracts were prepared for immunoprecipitation with HA antibody and immunoblotting with c-Myc antibody to assess polyubiquitylation of nuclear HA-p65. (C) pA3M- or pA3M-LANA-1-transfected 293T cells were stimulated with TNF-α for 30 min. Cell nuclear extracts were prepared and subjected to immunoprecipitation with c-Myc antibody, and the immunoprecipitates and 5% nuclear extracts were further assessed by immunoblotting with the indicated antibodies. (D) Control and LANA-1-expressing 293T cells were treated with TNF-α for 30 min. Cell nuclear extracts were subjected to immunoprecipitation with Cul5 antibody, and the immunoprecipitates and inputs for immunoprecipitation were further assessed by immunoblotting with the indicated antibodies.

trol 293T cells was diminished in cullin 5 knockdown 293T cells (Fig. 5B). This suggested that enhancement of p65 polyubiquitylation by LANA-1 was a cullin 5-dependent event. Next, we tried to determine if p65 translocated from the cytoplasm to the nucleus upon induction was recruited into elongin B/C-cullin 5 E3 ligase by LANA-1. After induction by TNF-α, nuclear extracts from Myc–LANA-1-expressing and control 293T cells were subjected to immunoprecipitation with c-Myc antibody and Cul5 antibody, respectively, and immunoprecipitates were detected by p65, cullin 5, elongin B, and elongin C antibodies. The result showed that immunoprecipitated nuclear p65 was associated with endogenous cullin 5, elongin B,

and elongin C only in LANA-1-expressing 293T cells (Fig. 5C and D) and suggested that LANA-1 imposed its repressive effect on NF-κB activation by assembling a multiunit complex that contained p65, elongin B/C, and cullin 5.

LANA-1 represses IL-8 expression in endothelial cell lines. The above studies revealed that LANA-1 impaired the NF-κB signaling pathway by promoting polyubiquitylation-mediated p65 degradation. We explored the effects of LANA-1 repression of TNF-α-stimulated NF-κB activation in endothelial cells. TIVE cells, which not only maintain the endothelial cell phenotype but also are capable of long-term propagation, were used as a cell model. Lentivirus that packaged EGFP or EGFP–LANA-1 was used to transduce TIVE cells, and postransduction TIVE cells were stimulated with TNF-α or were left unstimulated. The NF-κB target gene IL-8, which was upregulated by TNF-α induction in control TIVE cells (about 33-fold), was found to be silenced in LANA-1-expressing TIVE cells (about 18-fold), using a quantitative real-time PCR (Fig. 6A). It was also discovered that under stimulation, the IL-8 concentration in LANA-1-expressing TIVE cell supernatant was reduced compared to that in the control supernatant (500 pg/ml versus 1,200 pg/ml) (Fig. 6B). To test if LANA-1 exerted its suppressive effect on endothelial IL-8 expression via targeting p65, we cotransduced p65 and LANA-1 into TIVE cells. We found that overexpressed p65 restored the IL-8 mRNA level (Fig. 6A) and release (Fig. 6B) to levels comparable to those in control TIVE cells. This observation confirmed that LANA-1 targeted p65 for deregulation of IL-8 in endothelial cells. IL-8 is one of the best-characterized chemokines and is predominantly chemotactic for neutrophils (45, 57). Our findings suggested that LANA-1 expression restricted endothelial participation in neutrophil chemotaxis.

KSHV infection or LANA-1 expression impairs neutrophil chemotaxis. The above studies demonstrated that KSHV LANA-1 degraded nuclear p65 and repressed endothelial IL-8 expression upon TNF-α stimulation. This suggests that endothelial cells latently infected with KSHV might be inhibited in their ability to induce neutrophil chemotaxis. Therefore, we tested the effects of KSHV infection or LANA-1 expression on neutrophil chemotaxis using a modified Boyden chamber assay (17). Neutrophils were placed in the top wells of the chamber to migrate into bottom wells. A positive control (10% FBS) and negative control (PBS), along with the conditioned cell culture medium, were placed into bottom wells. We found that migration was significantly downregulated when neutrophils were exposed to culture medium TNF-α treated from KSHV long-term-infected TIVE cells compared to treated TIVE cell culture medium (Fig. 6C, $TKsC^+$ and TG^+). Conditioned medium of TNF-α-treated, LANA-1-expressing TIVE cells also had a smaller chemotactic effect on neutrophil migration (Fig. 6C, TGL^+ and TG^+). To determine if LANA-1 contributed to neutrophil chemotaxis impairment caused by KSHV latent infection, LANA-1 was knocked down using siRNA in KSHV long-term-infected TIVE cells (LTC). We observed that conditioned medium of TNF-α-treated, si-LANA-1 LTC was more chemotactic for neutrophils than TNF-α-treated control LTC conditioned medium (Fig. 6C, $TKsL^+$ and $TKsC^+$). Taken together, these results indicated that ectopic expression of LANA-1 in endothelial cells or endogenous LANA-1 in KSHV-infected endothelial cells impaired neutrophil chemotaxis.

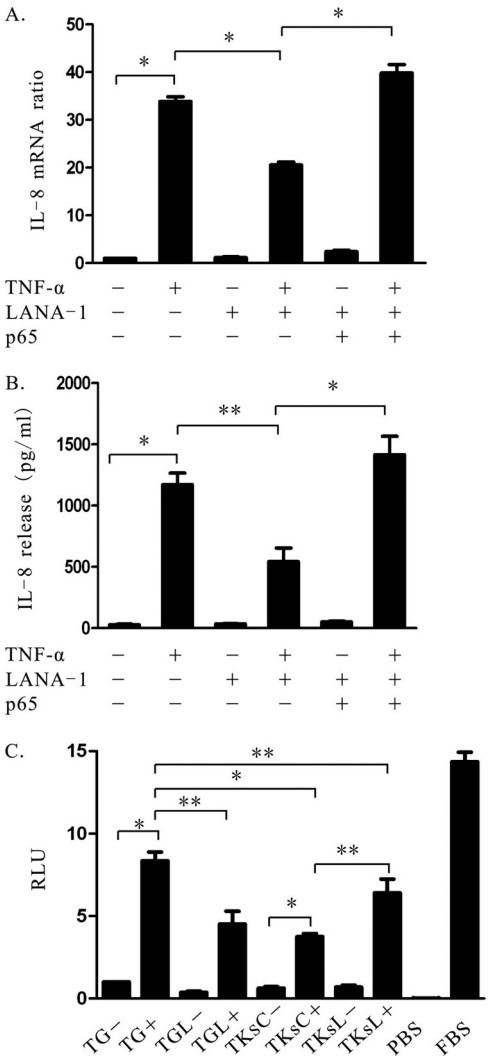

FIG. 6. LANA-1 inhibits IL-8 expression in endothelial cells upon induction of TNF-α and impairs the ability of endothelial cells to guide neutrophil migration. (A) TIVE cells were transduced with mock or LANA-1 or cotransduced with LANA-1 and p65. Transduced TIVE cells were induced with 50 ng/ml TNF-α for 1 h or left unstimulated. At 6 h after induction, total RNA extracts were obtained, and IL-8 mRNA was determined by quantitative real-time PCR. (B) Control transduced, LANA-1 transduced, and LANA-1/p65 cotransduced TIVE cells were stimulated with TNF-α for 1 h or unstimulated. At 16 h postinduction, the cell culture supernatant was collected for ELISA. (C) TIVE cells were transduced with control lentivirus (TG) or LANA-1-packaged lentivirus (TGL). KSHV long-term-infected TIVE cells (LTC) were transduced with mock lentivirus (TKsC) or lentivirus delivering siRNA that targeted endogenously expressed LANA-1 (TKsL). Transduced TIVE cells and LTC were induced with TNF-α for 1 h or not. At 16 h after induction, cell culture medium was collected for transwell assay. PBS and 10% FBS were used as negative and positive controls, respectively. +, induction with TNF-α; −, no induction with TNF-α; *, $P < 0.01$; **, $P < 0.05$.

DISCUSSION

The innate immune response is the first line of defense against invading pathogens, which is initiated with recognition of foreign microbes by a series of evolutionarily conserved receptors called pattern recognition receptors (PRRs) (10, 56,

91). Upon activation of PRRs, host sentinel cells such as macrophages and dendritic cells (DCs) increase type I interferon and proinflammatory cytokine expression (1, 2, 56). Proinflammatory cytokines stimulate endothelial cells to increase endothelium-leukocyte adhesion molecule expression on their surfaces and to release chemokines that facilitate leukocyte migration from the blood to subvascular tissues. Neutrophils are mainly responsible for clearance of microorganisms in acute inflammation, and these phagocytes carry an array of cytotoxic agents and migrate to infected tissue, where they destroy the pathogens.

Several groups have reported that neutrophil motility is regulated by viruses. Work aimed at identifying the agents responsible for equine herpesvirus type 1 (EHV-1) inhibition of IL-8-mediated equine neutrophil chemotaxis has found that EHV-1-encoded glycoprotein G can directly bind to IL-8 and inhibit its chemotactic function (94). Lentivirus also exhibits a negative effect on neutrophil recruitment. Neutrophils isolated from human immunodeficiency virus-1-infected patients are defective in chemotaxis to IL-8, and in a feline infection model, feline immunodeficiency virus (FIV) has been shown to reduce chemotactic receptor expression on neutrophils (40, 52).

KSHV is a sophisticated virus that encodes a series of immunomodulatory genes and successfully evades host immune surveillance. To date, a number of gene products of KSHV have been characterized for their ability to modulate the host immune response at multiple levels (6, 21, 80). Two KSHV viral gene products, ORF K2 that encodes viral IL-6 (vIL-6) and ORF K14 that encodes vOX2, have been reported to inhibit neutrophil migration (30, 81), but, notably, these two genes are expressed mainly during lytic replication of KSHV. Little is known about which strategies KSHV adopts during latent infection to prevent being indiscriminately eradicated by neutrophils in acute inflammation triggered by a secondary infection.

LANA-1 is encoded by KSHV ORF73 and is latently expressed at a high level via auto-activation of its own promoter (46, 85, 90). In this study, we investigated if LANA-1 affected neutrophil chemotaxis. We found that endothelial activation upon TNF-α stimulation was impaired by LANA-1, and a series of inflammatory genes, including ICAM-1, VCAM-1 (data not shown), and IL-8, was significantly reduced in endothelial cells upon induction. A transmigration assay revealed that LANA-1 expression dramatically impaired the endothelial chemotactic effect on neutrophils, the extent of which was comparable to that in KSHV infection. A series of biochemical experiments revealed that LANA-1 dramatically attenuated NF-κB activation upon TNF-α induction by degrading nuclear p65 protein through the ubiquitin-26S proteasome pathway.

Degradation of nuclear p65 is thought to be one of the cellular strategies for terminating the inflammatory response to prevent chronic inflammation associated with various diseases such as rheumatoid arthritis and inflammatory bowel disease (31, 100). To date, at least three cellular E3 ligases have been reported to degrade nuclear p65 protein: PDLIM2 (PDZ and LIM domain), SOCS-1 (suppressor of cytokine signaling) and COMMD1 (COMM domain-containing) (63, 82, 92). This degradation of p65 depends on the manner of DNA binding (83), such that the degradation not only eliminates p65 protein but also strongly terminates p65 transactivation and affects specific gene expression. Ubiquitin-26S proteasome-mediated degradation of nuclear DNA-bound p65 provides another level for modulation of NF-κB-transactivated genes. Given that posttranslational modifications of nuclear p65, particularly acetylation and phosphorylation, are frequently detected (18, 19, 29, 49, 97, 102), and that κB sequences disperse discretely among the genome, it is reasonable to presume that, besides the three E3 ligases mentioned above, other E3 ligases are involved in modulation of NF-κB transactivation by degradation of nuclear p65. We reported that LANA-1 enhanced nuclear p65 degradation through the ubiquitin-proteasome pathway by recruiting p65 to an elongin B/C-cullin 5 E3 ligase. The scaffold protein cullin 5 is responsible for binding to specific E2 ubiquitin-conjugating enzyme and elongin B/C binds to the SOCS domain of different adaptor proteins that are responsible for substrate targeting (19, 43, 71). LANA-1 has been reported to contain a SOCS domain and acts as a virus-encoded adaptor protein for elongin B/C-cullin 5 E3 ligase (12). Our results revealed that KSHV LANA-1 endowed elongin B/C-cullin 5 E3 ligase with the ability to recognize nuclear p65 as a substrate (Fig. 5C and D). Polyubiquitinylation-mediated degradation of protein is tightly regulated by the specificity of E3 ligase, and our findings indicated that a KSHV-encoded latent protein, LANA-1, modulated the substrate selectivity of the E3 ligase elongin B/C-cullin 5 complex to facilitate its survival and propagation. Our results deepen our understanding of the molecular characteristics of LANA-1 and its multifunctional role.

Upon infection, KSHV successfully establishes a life-long latent infection until the induction of some stimulus, such as treatment with phorbol esters or sodium butyrate. LANA-1 contributes to maintenance of KSHV latency. On the one hand, LANA-1 structurally tethers KSHV episomes to host chromatin and maintains KSHV latent replication by binding KSHV terminal repeat (TR) sequence (22, 36, 44, 60, 87). On the other hand, LANA-1 has been shown to modulate several viral or cellular signaling pathways; LANA-1 represses Rta (54), the master molecular switcher to lytic replication of KSHV, interferes with cell differentiation (61), and also antagonizes some tumor suppressor proteins such as p53 and retinoblastoma protein (32, 76). These effects could be interpreted as creating a favorable intracellular environment for KSHV latent replication. Consistent with the observation that neutrophils are rarely found in KS lesions (28, 69), we demonstrated that LANA-1 decreased IL-8 secretion of endothelial cells and impaired the consequent neutrophil chemotaxis in an *in vitro* acute inflammation model. By shattering the chemotactic effect on neutrophils, the risk that KSHV latently infected cells are indiscriminately eradicated by neutrophils was reduced, and a favorable intercellular environment for KSHV latent replication was maintained.

Of note, we found that knockdown of virally expressed LANA-1 in KSHV long-term-infected endothelial cells restored chemotaxis of neutrophils but not to the same extent as in uninfected endothelial cells (Fig. 6C). This indicated that other KSHV latent gene products, perhaps including recently discovered KSHV microRNAs (miRNAs) (13, 37, 84), also contributed to inhibition of neutrophil recruitment. ICAM-1 and VCAM-1, which are responsible for firm adhesion of neutrophils to the endothelium, were also found to be silenced by

LANA-1. This suggests that there is a more comprehensive interaction between KSHV latency and neutrophil action in acute inflammation.

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