Original Article
Extracellular Hsp90 serves as a co-factor for NF-κB activation and cellular pathogenesis induced by an oncogenic herpesvirus

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Abstract: The Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma (KS)—the most common tumor associated with HIV infection and an important cause of morbidity and mortality in this patient population. The majority of patients with KS exhibit little or no clinical response to existing therapies. The nuclear factor-kappaB (NF-κB) family of transcription factors plays a critical role in facilitating cancer pathogenesis associated with oncogenic viruses, and a better understanding of how cellular factors regulate NF-κB activation in the context of KSHV infection may facilitate development of new therapies for KS. Existing data implicate heat shock protein-90 associated with the cell surface (csHsp90) as a co-factor in cancer cell migration and invasion, and we recently reported that csHsp90 serves as a co-factor for mitogen-activated protein kinase (MAPK) activation during *de novo* KSHV infection. However, whether csHsp90 regulates NF-κB activation, or cellular pathogenesis associated with KS, has not been established. We have found that csHsp90 serves as an important co-factor for canonical NF-κB activation by KSHV during *de novo* infection of primary human cells relevant to KS. Furthermore, our correlative functional studies reveal that csHsp90 inhibition suppresses KSHV-induced, NF-κB-dependent secretion of the pro-migratory factors interleukin-8 and vascular endothelial growth factor as well as invasiveness for primary cells following *de novo* infection. These data implicate csHsp90 in KSHV-mediated activation of NF-κB and associated pathogenesis, and support the potential utility of targeting csHsp90 as a therapeutic approach for KS.

Keywords: Cancer, Kaposi's sarcoma, NF-κB, KSHV, heat shock protein, invasion, transcription factors, signal, transduction

Introduction

The Kaposi's sarcoma-associated herpesvirus (KSHV) is one of the most common etiologic agents for cancers arising in the setting of HIV infection, including primary effusion lymphoma (PEL) [1], multicentric Castleman's disease (MCD) [2], and Kaposi's sarcoma (KS) [3]. Although highly active antiretroviral therapy (HAART) has successfully reduced the incidence of HIV-associated KS [4], KS still represents the most common HIV/AIDS-associated malignancy and an important cause of morbidity and mortality in the modern era [5,6]. Moreover, KS has been increasingly recognized in patients exhibiting successful suppression of HIV replication with HAART [7-10]. Notably, KS also represents an important cause of morbidity and mortality in patients receiving solid organ transplants [11].

Standard therapy for KS includes cytotoxic chemotherapeutic agents administered alone or in combination. Unfortunately, the majority of patients exhibit little or no clinical response with this approach, and progression-free intervals are often less than one year [12,13]. Moreover, these agents incur many side effects compounding those caused by HAART [4,14]. During *de novo* infection, KSHV proteins initiate signal transduction resulting in activation of promigratory cytokine responses and induction of cell motility and angiogenesis [15-22]. These data suggest that suppression of KSHV-induced cancer pathogenesis through targeting of signal

transduction initiated by KSHV may offer an appealing therapeutic strategy. However, existing approaches targeting signal transduction pathways activated by KSHV are not widely used in clinical practice due to their inability to achieve remission for many existing tumors, or to their well-characterized toxicities (due in part to non-specificity of drug targets) [23-26]. Identification of cellular co-factors regulating signal transduction initiated during KSHV infection may lead to identification of new and safer therapeutic targets for KS.

NF-κB represents a highly conserved family of transcription factors, including RelA (p65), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2), that share an N-terminal Rel homology domain responsible for DNA binding, dimerization and nuclear translocation [27,28]. Canonical (a.k.a. classical) activation of NF-κB occurs in response to inflammatory mediators and involves IκB kinase (IKKβ) phosphorylation of IκBα, leading to IκBα ubiquitination and degradation and NF-κB nuclear translocation [29]. KSHV-encoded proteins, including the viral G-protein coupled receptor (vGPCR) and viral FLICE inhibitory protein (vFLIP), induce gene transcription, secretion of soluble mediators of cell migration and invasion, and endothelial cell transformation through canonical activation of NF-κB [30-26]. It follows that inhibition of NF-κB may interfere with migration and invasion for KSHV-infected tumor cells. Although under evaluation in clinical trials, small molecule inhibitors of NF-κB also incur toxicities that may limit their clinical utility [37]. Therefore, a better understanding of alternative mechanisms for KSHV activation of NF-κB might facilitate development of novel strategies for targeting this pathway.

Heat shock proteins (Hsp) modulate a wide variety of intracellular processes through the stabilization or regulation of protein folding [38], and Hsp90 plays an important role in the activation of signaling proteins relevant to cell migration and invasion [39]. Furthermore, Hsp90 inhibitors have proven beneficial for reducing solid tumor burden, and their utility is under evaluation in clinical trials for a variety of cancers [40]. Recent identification of Hsp90 on the cell surface (csHsp90) [41] has led to the observation that csHsp90 serves as a co-factor in the activation of specific intracellular signal transduction pathways in a manner distinct from the intracellular form of the protein [42]. Moreover, we have recently demonstrated a role for csHsp90

in KSHV induction of MAPK activation during *de novo* infection [43]. In the present study, we sought to determine whether csHsp90 regulates KSHV-initiated NF-κB activation and whether targeting csHsp90 interferes with NF-κB-associated cell migration and invasion for KSHV-infected cells.

Materials and methods

Cell culture and infection assays

KSHV-infected body cavity-based lymphoma (BCBL-1) cells were maintained in RPMI 1640 media (Mediatech) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.5), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, and 0.02% (wt/vol) sodium bicarbonate. To obtain purified KSHV for infection experiments, BCBL-1 cells were incubated with 0.6 mM valproic acid for 6 days and purified virus concentrated from culture supernatants as described previously [43]. For negative controls using ultraviolet light-inactivated KSHV (UV-KSHV), viral aliquots were exposed to 1200 J/cm^2 UV light for 10 min using a CL-1000 Ultraviolet Crosslinker. Human primary dermal microvascular endothelial cells (pDMVEC) were maintained according to the manufacturer's instructions (Lonza). HeLa and human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% FBS, 10 mM HEPES (pH 7.5), 100 U/mL of penicillin, and 100 μg/mL streptomycin. Human umbilical vein endothelial cells (HUVEC) were maintained in Dulbecco's modified Eagle's medium/Ham's F12 50/50 mix (DMEM F12, Mediatech) supplemented with 1 μg/mL puromycin, 10 mM HEPES (pH 7.5), and 5% FBS. Infectious titers were determined using both pDMVEC and HeLa cells and previously described methods [43].

Hsp90/ NF-κB inhibition

The previously characterized csHsp90 inhibitor DMAG-N-oxide (DNo) [42] and the NF-κB inhibitor Bay11-7082 (Sigma-Aldrich) were dissolved in DMSO and aliquots frozen at -80°C. Cells were incubated with DNo for 16 h at 37°C, Bay11-7082 for 1 h at 37°C or with equivalent volumes of DMSO (vehicle) for negative controls prior to subsequent analyses. Cells were also incubated with 20 ng/mL TNF-α (Cell Signaling)

for 20 minutes to induce canonical NF-κB activation for some experiments. In parallel experiments, cells were incubated for 12-16 h with 15 µg/mL of an anti-Hsp90 monoclonal antibody (Stressgen) or a control rat IgG2A isotype antibody (Invitrogen).

Cell viability assays

Cell viability was assessed using a standard MTT assay as previously described [42]. A total of 5×10^3 cells were incubated in individual wells in a 96-well plate for 24 h. Serial dilutions of DNo or Bay11-7082 were added and cells subsequently incubated in 1 mg/mL MTT solution (Sigma-Aldrich) at 37°C for 3 h followed by 50% DMSO overnight. Optical density was determined thereafter at 570 nm using a spectrophotometer (Thermo Labsystems).

Transfection assays

pFLAG-CMV2-p65 constructs were used in transfection assays to overexpress p65, and pFLAG-CMV2 empty vectors were used as negative controls. For other experiments, cells were transfected with luciferase-based NF-κB reporter constructs for quantification of endogenous NF-κB activation. Cells were transfected in 12-well plates for 24-48 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For luciferase expression assays, cells were lysed with 100 µL of lysis buffer (Promega), and 20 µL aliquots from each lysate were analyzed for luciferase activity using a Berthold FB12 luminometer. Light units were normalized to total protein levels for each sample using the BCA protein assay kit (Pierce) according to the manufacturer's instructions to determine relative luciferase units (RLU). Transfection efficiency was assessed through co-transfection of a lacZ reporter construct, and β-galactosidase activity was determined using a commercially available β-galactosidase enzyme assay system according to the manufacturer's instructions (Promega). Three independent transfections were performed for each experiment, and all samples were analyzed in triplicate for each transfection.

Immunoblotting

Cells were lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM EDTA, 5 mM NaF and 5 mM Na_3VO_4. Lysates were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes. Proteins were identified using antibodies recognizing total and phosphorylated proteins as follows: phospho-NF-κB p65 (Ser536), phospho-IKKα/β (Ser176/180), NF-κB p65 (C22B4), IKKα, IKKβ (L570), IκBα (L35A5), Ref1 (Cell Signaling), α-Tubulin (Sigma), and β-Actin (Sigma) for loading controls. Immunoreactive bands were developed using an enhanced chemiluminescence reaction (Perkin-Elmer). Nuclear protein fractions were isolated using a Nuclear Extract Kit (Active Motif) as previously described [31], and the nuclear origin of these extracts was verified using anti-Ref1 antibodies. Anti-α-tubulin antibodies were also used to exclude contamination of nuclear extracts with extranuclear proteins. Protein concentrations were determined using a bicinchoninic acid reagent (Pierce Biotechnology) and stored at -80°C.

ELISA

Concentrations of IL-8 and VEGF in culture supernatants were determined using human IL-8 (Becton Dickinson) and VEGF (Pierce Biotechnology) ELISA kits according to the manufacturers' instructions.

Transwell invasion assays

After incubation, Matrigel Invasion Chambers (Becton Dickinson) were hydrated for 2 h at 37°C with appropriate media. After hydration, fresh media was added to the bottom of the well, then 5×10^4 HFF or $1\text{-}1.5 \times 10^4$ HUVEC were plated in the top of the chamber. After 24 h, the cells were fixed with 3% paraformaldehyde in PBS for 15 min at room temperature and the chambers were rinsed in PBS and stained with 0.2% crystal violet for 10 minutes. After washing the chambers 5 times with dH_2O, the cells at the top of the Matrigel membrane were removed with cotton swabs. "Invading" cells at the bottom of the membrane were counted using a phase contrast microscope. Relative invasion for cells in experimental groups was calculated as follows: relative invasion = # invading cells in experimental group / # invading cells in control groups.

Statistical analysis

Significance for differences between experimental and control groups was determined using the Student's two-tailed t-test (Excel 8.0). P val-

ues less than 0.05 or 0.01 were considered significant or highly significant, respectively.

Results

Canonical activation of NF-κB by KSHV is csHsp90-dependent

KSHV induces canonical activation of NF-κB during *de novo* infection [30,31,32]. We recently demonstrated that targeting csHsp90 suppressed KSHV activation of MAPK during *de novo* infection [43]. However, ectopic MAPK overexpression during csHsp90 inhibition only partially rescued KSHV gene expression [43], suggesting that multiple signaling pathways initiated by KSHV may be csHsp90-dependent. Therefore, we sought to determine whether KSHV induction of NF-κB during *de novo* infection was csHsp90-dependent using two complimentary methods for specifically inhibiting csHsp90 function that we and others have employed in previous studies [38,39,43]: pharmacologic inhibition of csHsp90 using a membrane non-permeable compound (DMAG-N-oxide, or DNo) targeting the extracellular ATP-binding pocket of the Hsp90-alpha subunit located in the N-terminal region of the protein; and targeting of an N-terminal epitope within the Hsp90-alpha subunit using a monoclonal antibody. First, we confirmed that DNo induced minimal cytotoxicity over a range of concentrations and exposure times for two primary cell types relevant to KS pathogenesis, primary dermal microvascular endothelial cells (pDMVEC) and human foreskin fibroblasts (HFF) (**Figure 1A, B**). KSHV initiates canonical NF-κB activation through phosphorylation of p65 and an increase p65/p50 dimerization [31, 32, 44]. Immunoblotting experiments using whole cell lysates revealed that csHsp90 inhibition suppressed KSHV induction of p65 phosphorylation (**Figure 1C, D**). To confirm these results and to assess the role of csHsp90 in KSHV-induced activation of NF-κB binding to gene promoters, we employed NF-κB reporter assays in which binding of endogenous NF-κB to an ectopically expressed promoter induces luciferase expression. These assays showed that csHsp90 inhibition significantly suppressed KSHV-induced activation NF-κB (**Figure 1E-H**). The degree of NF-κB inhibition observed with csHsp90 targeting in our immunoblotting and reporter assays approximated that observed with direct NF-κB inhibition using a well-characterized NF-κB inhibitor

(**Figure 1C, E-H**).

TNF-α initiation of canonical NF-κB activation is well-characterized [45,46] and may play an important role in facilitating KS pathogenesis, possibly through paracrine upregulation of NF-κB following interactions between TNF-α and cell surface receptors [47-51]. HeLa cells have been used previously to investigate the role of canonical NF-κB activation in KSHV pathogenesis [52,53]. Therefore, we used HeLa cells to determine whether csHsp90 regulates TNF-α and/or KSHV induction of canonical NF-κB activation. TNF-α induced phosphorylation of p65 and IKKα/β and the degradation of IκBα (consistent with canonical NF-κB activation) in HeLa cells as previously demonstrated, and csHsp90 inhibition suppressed these effects to a degree similar to that achieved with direct NF-κB inhibition (**Figure 2A**). Nuclear translocation of p65 is a key step in canonical NF-κB-mediated gene transactivation [28], and we confirmed that csHsp90 inhibition significantly reduced TNF-α-mediated nuclear translocation of p65 (**Figure 2B**). In subsequent *de novo* infection experiments, we observed that KSHV increased phosphorylation of p65 and IKKα/β as well as nuclear translocation of p65 as anticipated, and that csHsp90 inhibition suppressed these effects to an extent similar to that observed with direct NF-κB inhibition (**Figure 2C, D**).

csHsp90 regulates KSHV-initiated/NF-κB-dependent secretion of pro-migratory factors and cell invasiveness

Previous studies have demonstrated that NF-κB initiates expression and secretion of soluble mediators of tumor cell invasion and angiogenesis, including VEGF and IL-8, within a variety of cancer cells [54-56]. Neovascular proliferation and proliferation of KSHV-infected cells are cardinal pathologic features of KS, and VEGF and IL-8 are upregulated by KSHV-induced NF-κB activation during *de novo* infection of endothelial cells [57-60]. In addition, KSHV induces cell migration and invasion following *de novo* infection [61,62]. We found that targeting csHsp90 significantly reduced KSHV-initiated secretion of VEGF and IL-8 during infection of pDMVEC and HFF, approximating the effect seen with direct inhibition of NF-κB (**Figure 3A-F**). Next, we used transwell invasion assays to determine whether csHsp90 facilitates KSHV-mediated invasiveness for human umbilical vein endothelial cells
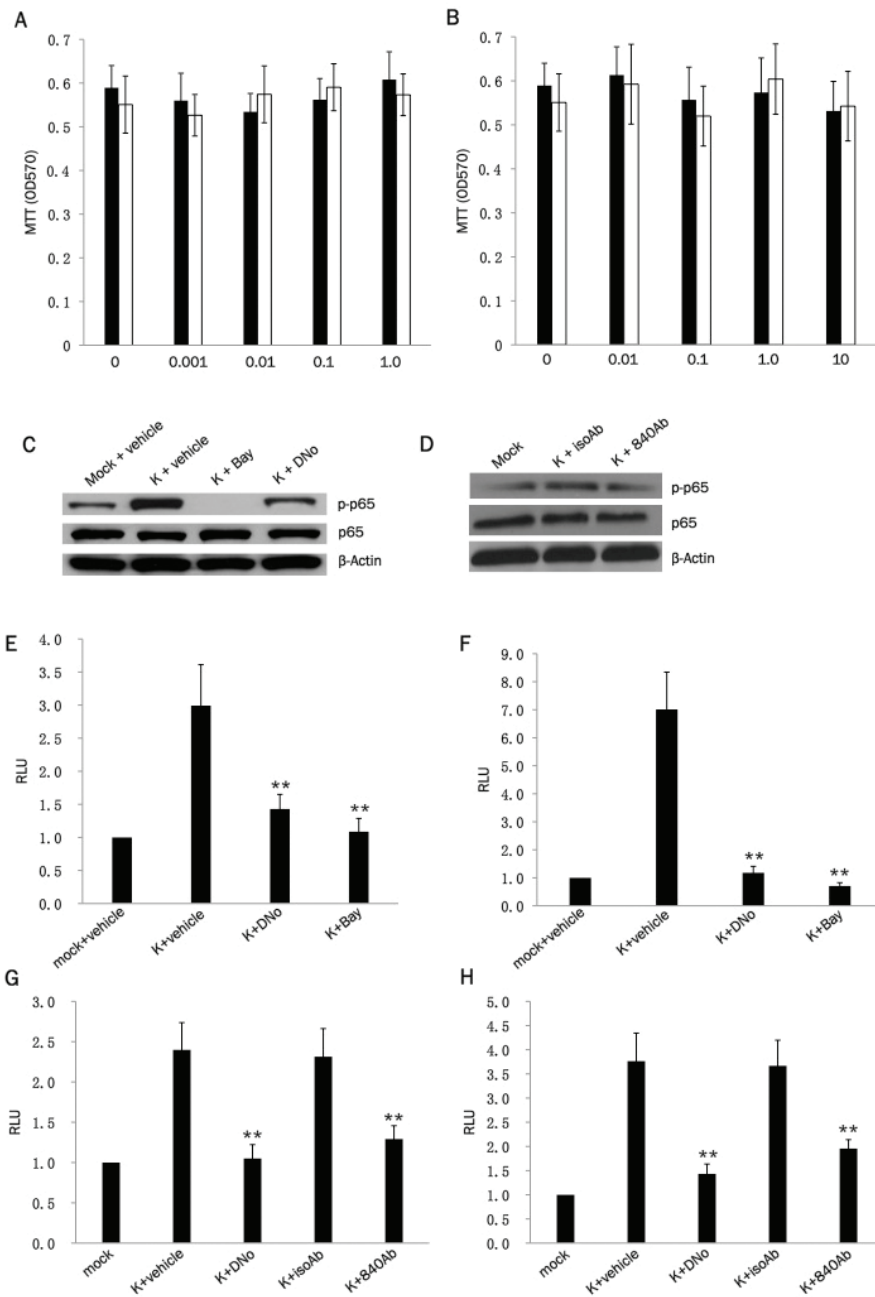
Figure 1. KSHV activation of NF-κB is cs-Hsp90-dependent. (**A, B**) pDMVEC (black bars) and HFF (white bars) were incubated with the indicated concentrations of DNo (μM) for 16 h (A) or Bay11-7082 (Bay) for 1 h (B), and cell viability was determined thereafter using a standard MTT assay according to the manufacturer's instructions. (**C, D**) Following their incubation with KSHV for 2 h, pDMVEC (C) were incubated with DMSO (vehicle control), DNo or Bay as above. HFF (D) were incubated with 15mg/mL monoclonal anti-Hsp90 antibody (840Ab) or 15mg/mL antibody control (isoAb) for 16 h. Immunoblots were performed to quantify total or phosphorylated signaling intermediates and β-actin as an internal loading control. (**E, F**) pDMVEC (E) and HFF (F) were transiently transfected with a luciferase-based NF-κB reporter construct and, 48 h later, incubated with KSHV for 2 h followed by either DMSO, 1 μM DNo for 16 h, or 10μM Bay for 1 h prior to assessment of NF-κB activity (RLU) as described in Methods. (**G, H**) pDMVEC (G) and HFF (H) were transiently transfected as in (E) and (F) and incubated with KSHV for 2 h prior to their incubation with either DMSO, 1 μM DNo, 15mg/mL monoclonal anti-Hsp90 antibody (840Ab) or 15mg/mL antibody control (isoAb) for 16 h prior to assessment of NF-κB activity (RLU) as described in Methods. Error bars represent the S.E.M. for three independent experiments. * = p<0.05, ** = p<0.01.
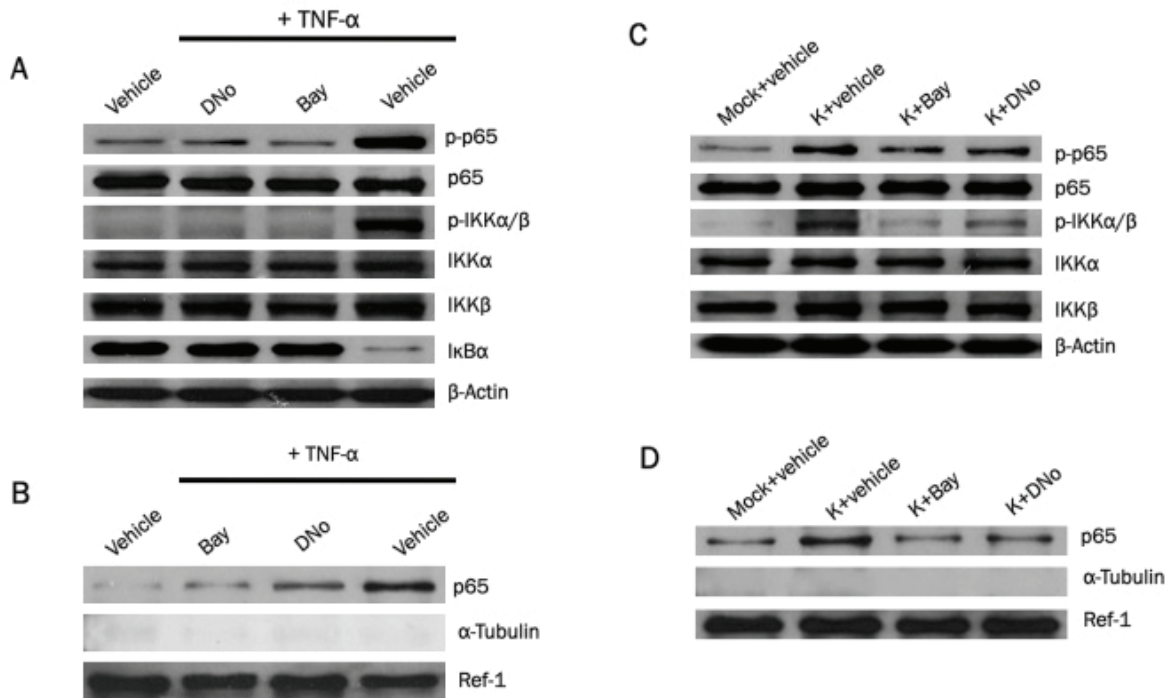
Figure 2. csHsp90 mediates KSHV induction of canonical NF-κB activation. (**A**) HeLa cells were incubated with DMSO (vehicle control), 1.0 μM DNo for 16 h, or 10 μM Bay for 1 h followed by 20 ng/mL TNF-α for 20 minutes. Immunoblots were performed 12 h later to quantify total or phosphorylated signaling intermediates or β-Actin as an internal control. (**B**) HeLa cells were incubated with DNo, Bay and TNF-α as in (A), then nuclear fractions isolated as described in Methods. Immunoblots were performed to detect signaling molecules, Ref-1 as a positive internal control for nuclear protein expression/loading, and α-tubulin to exclude the possibility of contamination of nuclear fractions with extranuclear proteins. (**C**) Following their incubation with KSHV for 2 h, HeLa cells were incubated with DNo or Bay as in (A), then immunoblots performed 12 h after viral incubation to detect expression of signaling molecules as above. (**D**) Following their incubation with KSHV, HeLa cells were incubated with DNo or Bay as in (A), then nuclear fractions isolated after 12 h and immunoblots performed as above.

(HUVEC) and HFF. We used HUVEC rather than pDMVEC since we find that pDMVEC do not efficiently pass through the matrigels used in our transwell assays. Of note, we observed no apparent toxicity for HUVEC incubated with DNo over the range of concentrations and exposure times in our assays (**Figure 4A**). We found that csHsp90 inhibition significantly suppressed KSHV-induced invasiveness for both HUVEC and HFF (Fig. 4B-I). In subsequent experiments, we transfected HUVEC with a construct encoding p65 (**Figure 5A**) and found that NF-κB overexpression restored secretion of VEGF and IL-8, as well as invasiveness, for KSHV-infected cells in the presence of csHsp90 inhibition (**Figure 5B-H**), implicating csHsp90 regulation of NF-κB as a mechanism for KSHV induction of cell migration and invasion following *de novo* infection.

Discussion

We recently demonstrated that csHsp90 serves as a co-factor for KSHV-induced MAPK activation and viral gene expression [43]. In that study, we found that MAPK overexpression only partially restored KSHV gene expression in the presence of csHsp90 inhibition, suggesting that csHsp90 regulates multiple signal transduction pathways activated during KSHV infection. NF-κB transcriptional activation plays a key role in facilitating KSHV gene expression as well as cell proliferation and survival of transformed cells, and several KSHV-encoded proteins initiate canonical activation of NF-κB [30-32, 36, 44, 53, 60, 63]. Existing data support a role for intracellular Hsp90 in the regulation of NF-κB activation and herpesvirus gene expression [64] as well as NF-κB-mediated survival for KSHV-infected lym-
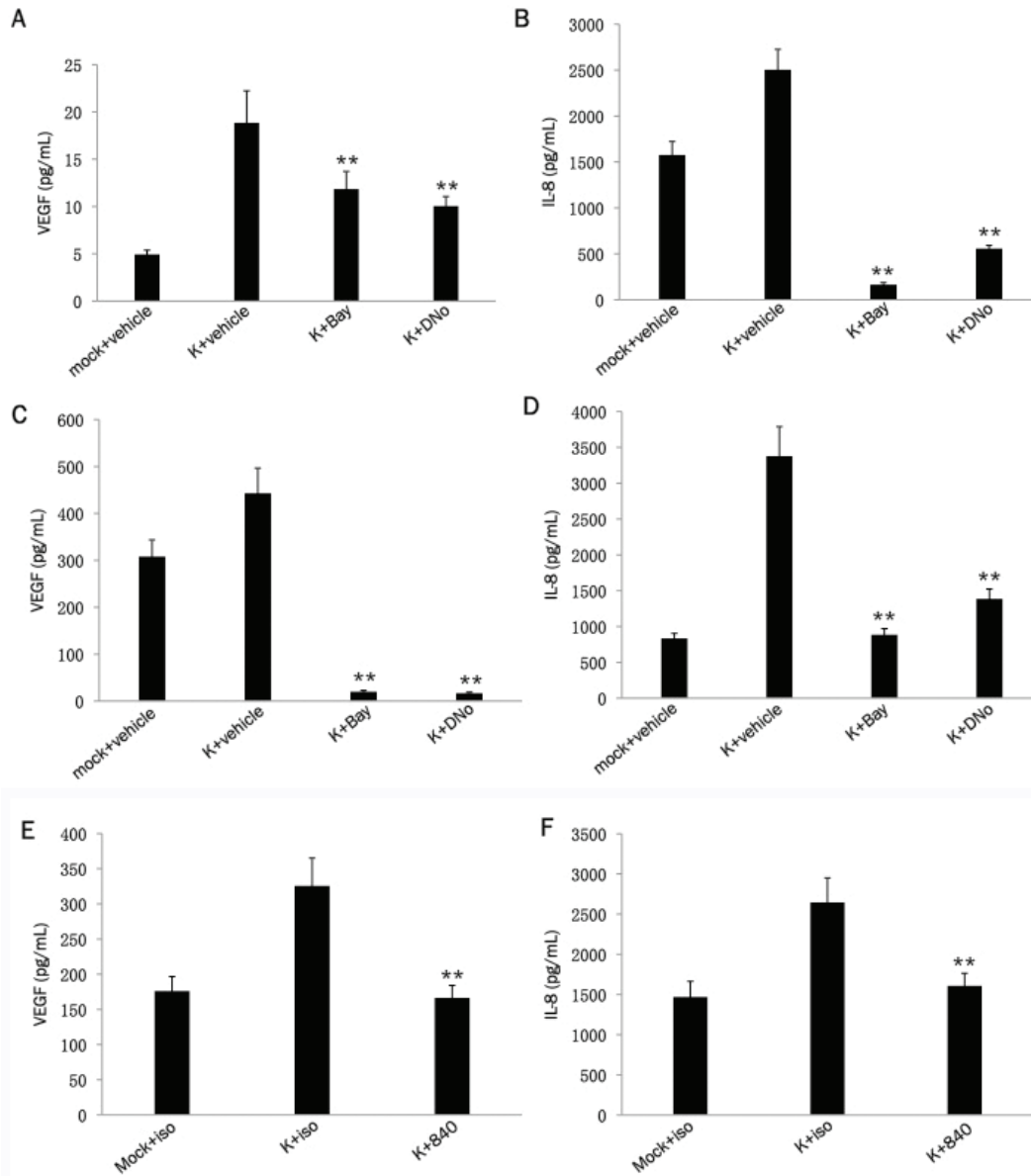
Figure 3. KSHV-induced secretion of pro-migratory factors is csHsp90-dependent. Following their incubation with either media (mock) or KSHV (K) for 2 h, pDMVEC (**A, B**) or HFF (**C, D**) were incubated with DMSO (vehicle control), 10 µM Bay for 1 h, or 1.0 µM DNo for 16 h. In separate experiments, KSHV-infected pDMVEC (**E**) and HFF (**F**) were incubated with 15mg/mL of a monoclonal anti-Hsp90 antibody (840) or 15mg/mL of an isotype control antibody (iso). VEGF and IL-8 were quantified within culture supernatants 20 h later using a commercial ELISA kit as described in Methods. Error bars represent the S.E.M. for three independent experiments. ** = p<0.01 relative to K + vehicle (A-D) or K + iso (E-F).

phoma cells [65]. Other work has demonstrated that intracellular Hsp90 regulates TNF-associated canonical activation of NF-κB [66], and that a cell permeable Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), suppresses IκB kinase-dependent IκBα phosphorylation/degradation, NF-κB activation, and cancer cell invasiveness [67]. Although exhibiting promise for reducing solid tumor burden in phase II clinical trials, cell-permeable Hsp90 inhibitors incur toxicities due to off-target effects that may ultimately limit their efficacy [40].
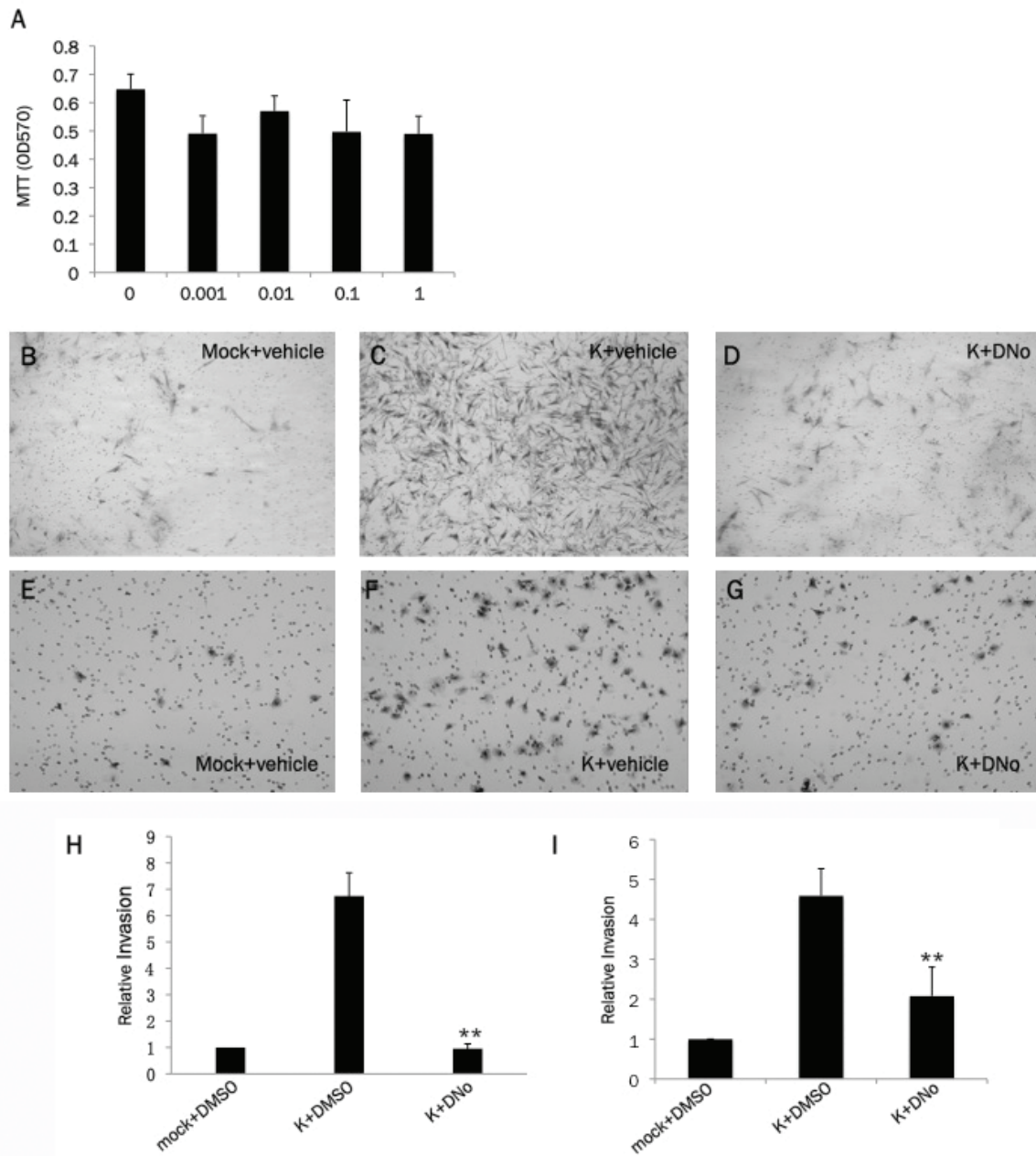
Figure 4. KSHV-induced invasiveness is csHsp90-dependent. (**A**) HUVEC were incubated with the indicated concentrations of DNo (in μM) for 16 h and cell viability determined using a standard MTT assay according to the manufacturer's instructions. (**B-G**) Following a 2 h incubation with either media (mock) or KSHV (K), HFF (B-D) or HUVEC (E-G) were incubated with DMSO (vehicle control) or 1.0 μM DNo prior to assessment of cell invasiveness using matrigel invasion assays as described in Methods. Representative images from one of three independent experiments are shown. (**H, I**) Relative invasiveness for groups B-G was determined as described in Methods. Error bars represent the S.E.M. for three independent experiments. ** = p<0.01.

csHsp90 regulates a more limited array of signal transduction pathways relative to the intracellular protein [42], justifying additional studies to explore the role of csHsp90 in viral cancer pathogenesis. To our knowledge, there are no prior studies addressing whether csHsp90 regulates virus-associated NF-κB activation and related cancer pathogenesis. Therefore, we
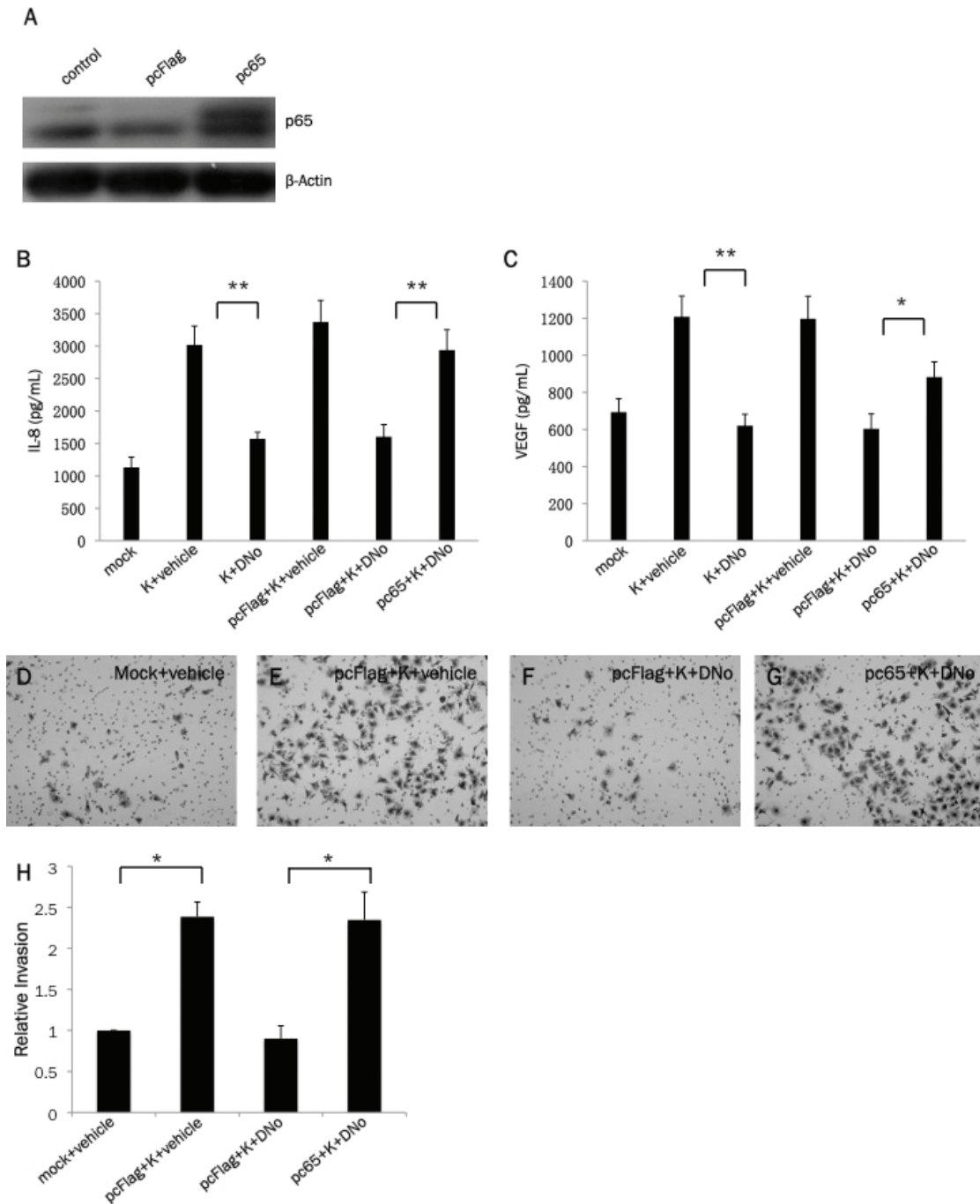
Figure 5. csHsp90 regulation of KSHV-initiated pathogenesis is mediated through csHsp90 regulation of NF-κB. (**A**) HUVEC were transfected with a construct encoding p65 (pc65) or empty control vector (pcFLAG), and 24 h later immunoblots were used to identify expression of p65 and β-Actin for loading controls as described in Methods. (**B, C**) HUVEC were incubated with media (mock) or KSHV (K) for 2 h in the presence or absence of p65 (or control vector) transfection as in (A), then incubated with either DMSO (vehicle control) or 1.0 μM DNo for 16 h. IL-8 and VEGF were then quantified within culture supernatants 20 h later using a commercial ELISA kit as described in Methods. (**D-G**) HUVEC were treated as above, and matrigel invasion assays used to assess invasiveness as described in Methods. Representative images from one of three independent experiments are shown. (**H**) Relative invasiveness for groups D -G was quantified as described in Methods. Error bars represent the S.E.M. for three independent experiments. * = p<0.05, ** = p<0.01.

sought to extend our prior observations regarding the role of csHsp90 in KSHV-initiated signal transduction, and to identify related functional effects relevant to KS cellular pathogenesis, through determination of whether csHsp90 serves as a co-factor for KSHV activation of NF-κB and KSHV-initiated cell invasion.

We found that targeting csHsp90 reduces both TNF-α and KSHV-induced canonical activation of NF-κB. TNF-α is a well-characterized inducer of canonical NF-κB activation [45,46, 66] and may play an important role in facilitating KS pathogenesis, in part through TNFR-mediated canonical activation of NF-κB [47-51]. Therefore, it is interesting to speculate whether targeting csHsp90 inhibits KS pathogenesis indirectly through a reduction in soluble TNF-α-receptor interactions, or other undetermined mechanisms. Furthermore, since NF-κB regulates KSHV gene expression during *de novo* infection [31], and since csHsp90 inhibition suppresses MAPK-dependent and MAPK-independent KSHV gene expression [43], we hypothesize that targeting csHsp90 reduces NF-κB-mediated viral gene expression following *de novo* infection, although this is not addressed in the present studies. Furthermore, our studies do not address whether direct interactions between KSHV and csHsp90 initiate NF-κB activation. This possibility is supported by published data revealing that direct interactions between infectious pathogens and csHsp90 activate NF-κB [68].

NF-κB transactivation of soluble mediators of angiogenesis facilitates pathogenesis related to a wide variety of cancers [69, 70]. Existing data also suggest that NF-κB-dependent induction of angiogenic cytokines and chemokines by KSHV facilitates KS progression [54,57,58,60]. Inhibition of csHsp90 using DNo reduces cell motility *in vitro* and cancer metastasis *in vivo* [41,71,72]. Our experiments including primary human endothelial cells and fibroblasts (two cell types present within KS lesions) revealed that targeting csHsp90 effectively suppresses KSHV-induced secretion of VEGF and IL-8, two well-characterized mediators of cell migration and angiogenesis induced following KSHV activation of NF-κB [55, 56]. These results complimented our functional assays wherein targeting csHsp90 effectively suppressed KSHV-induced migration and invasiveness following *de novo* infection. Furthermore, invasiveness was restored with ectopic p65 expression, suggesting

that suppression of invasion during csHsp90 inhibition is mediated by NF-κB and its downstream effectors. These results are in agreement with published data supporting a role for extracellular Hsp90 in the induction of NF-κB-mediated transactivation of IL-8 [73]. While VEGF and IL-8 likely participate in csHsp90-mediated migration for KSHV-infected cells, additional pro-angiogenic factors not examined in our studies are also secreted following KSHV infection, including matrix metalloproteinases [62], and we cannot exclude the possibility that targeting csHsp90 suppresses secretion of these factors as well. In addition, our studies do not address whether interactions between cell surface proteins and csHsp90 in KSHV-infected cells induce cell migration and invasion through NF-κB-independent mechanisms. For example, we recently demonstrated that KSHV induces cell invasiveness through upregulation of the membrane-associated protein emmprin (Extracellular Matrix MetalloPRoteinase INducer) [61], and emmprin associates with Hsp90 and other proteins within lipid rafts at the cell surface [74]. Future studies are needed to explore the potential relationship between csHsp90, surface-localized proteins, and NF-κB activation.

The data presented in this study provide novel observations implicating csHsp90 as a co-factor in KSHV-mediated cell migration and invasion through its regulation of NF-κB activation. These studies provide rationale for determining more precisely the mechanistic role of csHsp90 in KSHV-host cell interactions and whether drugs targeting extracellular Hsp90 inhibit KSHV-mediated angiogenesis and KS progression *in vivo*.

Acknowledgements

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