HSV-1 Hijacks the Host DNA Damage Response in Corneal Epithelial Cells through ICP4-Mediated Activation of ATM

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Herpes simplex virus type I (HSV-1) causes a wide range of ocular disorders, including infection of the conjunctiva, cornea, uvea, and retina. Herpes keratitis (HK) is a commonly encountered ocular disease that is the leading cause of both cornea-derived and infection-associated blindness in the developed world. HK can lead to corneal scarring, neovascularization, thinning, or even perforation, with severe cases necessitating corneal transplantation to restore vision. Clinical management of herpetic corneal infections relies on topical and systemic nucleoside analogues. Although these are excellent antiviral agents, their limitations include corneal epithelial toxicity and the emergence of drug-resistant HSV-1 strains. Drug resistance is rare and is primarily encountered in the immunocompromised population, where it may become a more significant issue as immunosuppressive and immunomodulatory medications continue to improve. Drug resistance is further compounded by the cross-resistance between nucleoside analogue drugs, most of which, with a few exceptions, share a common mechanism of action. This highlights the need for a novel therapeutic approach to targeting HSV-1 in the corneal epithelium.

We have previously reported our phenomenological studies on the inhibition of the host DNA damage response (DDR) as a means to suppress HSV-1 replication in in vitro, ex vivo, and in vivo models of HK. We had shown that inhibition of the ataxia telangiectasia mutated (ATM) kinase, or its downstream target checkpoint kinase 2 (Chk2), markedly suppresses viral replication in cultured corneal epithelial cells and in explanted rabbit and human corneas. ATM inhibition also had an antiviral effect in a mouse model of HK. However, the molecular mechanisms behind this effect have remained poorly understood, which has motivated this study.

HSV-1 engages in extensive molecular interactions with the host in order to optimize the cellular environment for replication. Interference with the host DDR is one of the most intriguing and least characterized virus-host interactions. Manipulation of the host DDR has been well documented for infections with viruses, bacteria, and even...
parasites. The ATM kinase seems to be a common target for manipulating the DDR, because of its apical position in the pathway. HSVG1 induces robust ATM activation early in the course of infection. Despite numerous investigations, the molecular events underlying ATM activation by HSV-1 remain to be fully characterized, as is the functional purpose of ATM activation. Although it is clear that ATM activity is necessary to maximize viral replication, insufficient evidence exists to firmly implicate specific pathways downstream of ATM. Paradoxically, the other sensor kinases involved in DNA damage processing, ATR and DNA-PK, are selectively counteracted by the virus, indicating a preference for ATM signaling in the host.

This study investigates the causative mechanisms of ATM activation during HSV-1 infection of corneal epithelial cells. We demonstrate that ATM is activated independently of damaged DNA and in a manner dependent on the viral immediate early gene product ICP4. The presence of the viral genome in the nucleus is also necessary for ATM activation, which suggests a genome-ICP4 interaction may underlie this critical step in the viral life cycle. Investigations of the kinetics of this phenomenon point to the existence of a very early ATM-dependent step in the lytic cycle of HSV-1. Our results suggest that pharmacological interference with ATM or ICP4 may provide a promising avenue for the development of novel antiviral agents for the treatment of HK.

METHODS

Cells

All cells were cultured at 37°C and 5% CO2, and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. The hTCEpi14 human corneal epithelial cells, a gift from Dr. James Jester (University of California, Irvine, CA, USA), were cultured in KGM-2 (Lonza, Basel, Switzerland). HCE15 human corneal epithelial cells, a gift from Dr. Peter Reinach (SUNY College of Optometry, New York, NY, USA), were cultured in Dulbecco’s modified Eagle medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS). EPC26 human esophageal epithelial cells, a gift from Dr. Anil Rustgi (University of Pennsylvania School of Medicine), were cultured in KSFM (Carlsbad, CA, USA). OKF637 human oral epithelial cells, a gift from Dr. James Rheinwald (Harvard Medical School, Cambridge, MA, USA), were cultured in KSFM. E5 cells, which are Vero cells stably expressing HSV-1 ICP4 protein, were a gift from Dr. Neal DeLuca (University of Pittsburgh, Pittsburgh, PA, USA) and were cultured in DMEM supplemented with 10% FBS. HEK293 human embryonic kidney epithelial cells, HeLa human cervical adenocarcinoma cells, U2OS human osteosarcoma cells, H1299 human lung carcinoma cells, and SH-SYSY human neuroblastoma cells were all obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS.

Viruses

All HSV-1 virus stocks were prepared and titered on CV-1 monolayers and stored at −80°C. The KOS strain was a gift from Dr. Stephen Jennings (Drexel University College of Medicine, Philadelphia, PA, USA). The 7134 strain, which is an ICP0 double-deletion mutant, was a gift from Dr. Nigel Fraser (University of Pennsylvania School of Medicine, Philadelphia, PA, USA). The tsB7 strain, which is a temperature-sensitive nuclear entry mutant, was a gift from Dr. David Knipe (Harvard Medical School). The d120 strain, which is an ICP4 double-deletion mutant, the i13 strain, which has an inactive DNA-binding domain of ICP4, and the n208 strain, which is a C-terminal ICP4 truncation mutant, were gifts from Dr. Neal DeLuca (University of Pittsburgh).

Infections

Cultured cells were infected by applying the desired viral load in an inoculum volume equal to 10% of the normal volume of growth medium. Multiplicity of infection (MOI) was calculated as a number of infectious viral particles per one cell in the monolayer. During infection, cells were maintained at 37°C and 5% CO2 and rocked every 10 to 15 minutes for one hour. Cells were then rinsed thoroughly with phosphate-buffered saline solution and overlaid with fresh medium. For synchronized infections, virus was allowed to adsorb to cells while rocking at 4°C. After one hour, cells were transferred to 37°C and 5% CO2 to initiate synchronized infection.

Treatments

All chemicals were tissue culture grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise. ATM inhibitor KU-55933 (Batch No. 5, 99.7% purity; Tocris Bioscience, Bristol, UK) was used at 10 μm/mL, cycloheximide (CHX) was used at 5 μg/mL, phosphonoacetic acid (PAA) was used at 400 μg/mL, and H2O2 was used at 150 μg/mL. Viral particles were pre-treated with UV light at 0.2 J/cm2.

Constructs

The fHSVΔpac BAC24 was a gift from Dr. Antonio Chiocca (The Ohio State University Medical Center, Columbus, OH, USA). The pM24 BAC25 was a gift from Dr. Samuel Rabkin (Harvard Medical School). The set of 27 expression constructs (all in the pEYFP-N1 backbone) representing the complete tegument proteome of HSV-1 (RS1, RL1, RL2, UL7, UL11, UL13, UL14, UL16, UL21, UL23, UL36, UL37, UL41, UL46, UL48, UL49, UL50, UL51, UL54, UL55, US1, US1.5, US2, US3, US10, US11, and US12)26 was a gift from Dr. Chunfu Zheng (Wuhan Institute of Virology, Wuhan, China). The pLKO-shPML vector27 and the control pLKOshNT vector were gifts from Dr. Roger Everett (University of Glasgow, Glasgow, Scotland). Lentiviral vectors pLNY-ICP0 and pLNY-ICP4 were generated by cloning the HSV-1 ICP0 and ICP4 genes into a lentiviral pLNY backbone under the control of the HSV-1 glycoprotein D promoter, which was a gift from Dr. Roger Everett (University of Glasgow). The ICP0 coding sequence was cloned from the pCI-ICP0 expression construct, which was a gift from Dr. Patrick Lomonte (Université Claude Bernard Lyon 1, Lyon, France), and the ICP4 coding sequence was cloned from the pK1-2 expression construct,28 which was a gift from Dr. Neal DeLuca (University of Pittsburgh).

Transfections

Transfection of plasmids was performed with GenDrill (BamaGen, Gaithersburg, Maryland, MD, USA), whereas transfection of BACs was accomplished with Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA). All
transfections followed standard protocols and manufacturer's instructions. Medium was changed at 6 hours post-transfection.

**Transductions**

Lentiviral particles were produced in HEK293T packaging cells cotransfected with lentivector (pLNGY-ICP0, pLNGY-ICP4, pLKO-shPML, or pLKO-shNT) and three packaging constructs (pCMV-VSV-G, pRSV-Rev, and pMDLg/pRRE). Virus was collected 48 hours post-transfection, filter-sterilized, supplemented with 8 μg/mL of polybrene, and stored at -80°C. Transductions were performed by adding virus to cells in suspension, plating the mixture, and changing the medium after 12 hours.

**Western Blot**

Standard protocol was followed for Western blot analysis. Cell lysates were collected in Laemmli buffer, vortexed, and boiled at 95°C for 5 minutes. Protein concentrations were measured by reducing agent-compatible bicinchoninic acid (BCA) assay. SDS-PAGE was performed by following a PVDF membrane. Primary antibody staining was performed overnight, and blots were visualized on film or with the Odyssey infrared system (LI-COR, Lincoln, NE, USA). Primary antibodies against the following proteins were used: ICP0 (Virusys Corporation, Taneytown, MD, USA), ICP4, PML, and nucleolin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ICP8 (a gift from Dr. Bill Ruyechan at SUNY-Buffalo, Buffalo, NY, USA), glycoproteins B and C (gifts from Dr. Roselyn Eisenberg at the University of Pennsylvania School of Medicine), ATM and pATM-Ser1981 (Rockland, Gilbertsville, PA, USA), Chk2 and pChk2-Thr68 (Cell Signaling, Danvers, MA, USA).

**Immunofluorescence**

Cells were grown on cover slips, treated as indicated, fixed in 3% paraformaldehyde/2% sucrose solution for 10 min, and permeabilized with 0.5% Triton X-100 for 5 minutes. Indirect immunofluorescence was performed with primary antibodies overnight followed by secondary antibody staining for 2 hours. Primary antibodies were same as those used for Western blotting. Nuclei were counterstained with 10 mg/mL of Hoechst 33258.

**qRT-PCR**

Total DNA was isolated from infected cells using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Real time quantitative PCR was performed with SYBR Green (Bio-Rad, Hercules, CA, USA). Target primers for UL30 (DNA polymerase catalytic subunit) and reference primers for GAPDH were used to measure genome replication. Primer sequences had been published previously.7 Real time PCR data were processed using the ΔΔCt method.

**Comet Assay**

Standard comet assay protocol28 was followed. Briefly, treated cells were suspended by trypsinization, mixed with 1% low-melting temperature agarose, and pipetted onto agarose-covered slides, which were submerged in alkaline lysis solution (1.2 mol/L NaCl, 100 mmol/L Na₂EDTA, 0.1% sarkosyl, 0.26 mol/L NaOH [pH>13]) for 18 to 20 hours at 4°C in the dark. Alkaline rinse solution (0.03 mol/L NaOH, 2 mmol/L Na₂EDTA [pH12.3]) was used to remove traces of salt and detergent. Slides were electrophoresed in fresh rinse solution for 25 minutes at 0.6 V/cm and stained with 2.5 μg/mL of propidium iodide for 20 minutes. Individual comet images were obtained using an inverted fluorescence microscope (Leica DM-IRB, Wetzlar, Germany) and analyzed with CometScore software (TriTek, Sumeduck, VA, USA).

**Statistical Analysis**

Statistical significance was determined using Student's two-tailed t-test and is indicated with ns (P > 0.05), * (P < 0.05), ** (P < 0.01), or *** (P < 0.001).

**RESULTS**

**HSV-1 Activates ATM in the Absence of DNA Damage**

HSV-1 infection elicits robust activation of ATM in corneal epithelial cells (Fig. 1A), as measured by phosphorylation of Chk2 on Thr 68, a residue phosphorylated exclusively by ATM. This phenomenon is well recognized,7,11,20–31 yet the underlying events responsible for ATM activation remain unknown. The incoming HSV-1 genome is a linear double-stranded DNA molecule known to contain single-stranded nicks and gaps in the sugar-phosphate backbone.32 These features, along with the ends of the linear genome, may be detected as DNA damage and trigger ATM activation. To test this hypothesis, we used fHSVΔpac, a bacterial artificial chromosome (BAC) that contains the full HSV-1 genome with a deletion of both pac sequences. The pac sequences serve as cleavage sites in the process of viral replication; thus their absence renders the viral genome conformation constitutively circular without free DNA ends. Transfection of fHSVΔpac BAC into HEK293 cells successfully activated ATM despite the absence of linear ends and single-stranded damage. Notably, activated ATM colocalized to the viral replication compartments, resulting in a pattern similar to that seen in wild type (WT) HSV-1 infection (Fig. 1B). Thus, this result excludes the endogenous defects and linear ends of the HSV-1 genome as being required for ATM activation.

Nuclear injection of the viral genome and the initiation of stressful events, such as chromatin remodeling, induction of apoptosis, and dysregulation of repair pathways, could potentially activate ATM through the induction of cellular DNA damage. To address this hypothesis, we compared the amount of nuclear DNA damage induced in response to HSV-1 infection or hydrogen peroxide treatment, under the conditions where these two stimuli produce equivalent levels of ATM activation (Fig. 1C). OKF6 cells were processed by alkaline comet assay for detection of nuclear DNA damage. Olive moment measurements revealed far greater nuclear DNA fragmentation in the peroxide-treated cells compared to HSV-1-infected cells, whose DNA damage levels were similar to those of untreated controls (Fig. 1D). Therefore, the level of ATM activation in HSV-1 infection is disproportional to the amount of DNA damage in the host cell.

Taken together, these experiments show that ATM is activated during HSV-1 infection independently of the presence of DNA damage, whether in the host or in the viral genome. These data point to a DNA damage-independent mechanism that may be used by the virus to activate ATM.
ATM Activation Requires Nuclear Entry of the Genome and Is Only Partial in the Absence of De Novo Protein Synthesis

We chose to undertake a reductionist approach to identifying the factors involved in ATM activation by HSV-1. Since the process of viral genome replication generates complex concatameric and branched DNA structures and is known to experience replication fork collapse,33 we hypothesized that ATM activation occurs in a replication-dependent manner. However, infection of hTCEpi cells in the presence of a viral DNA polymerase inhibitor, PAA, had no effect on ATM activation (Fig. 2A). To confirm this result, we transfected purified HSV-1 genome into HEK293 cells that were cultured in the presence or absence of PAA. As expected, untreated cells exhibited strong ATM activation that colocalized to the replication compartments. In line with the Western blot data, PAA-treated cells were not hindered in ATM activation, despite the absence of proper replication compartments (Fig. 1B). Together, these experiments demonstrate that ATM activation occurs in HSV-1-infected cells independently of the viral replication processes.

Posttranslational modifications of host factors by viral proteins are diverse and well documented for many viruses, including HSV-1. To address the possibility that a specific virally-encoded protein is involved in ATM activation, we infected hTCEpi cells in the presence or absence of CHX, an inhibitor of the ribosome. Western blot staining for pChk2, a direct target of ATM, revealed a partial inhibitory effect of CHX on ATM activation. This partial inhibition was highly consistent and replicable and held true for all MOIs tested (Fig. 2B). To rule out the possibility that the CHX effect is simply due to the inhibition of a host protein, we pretreated viral particles with ultraviolet (UV) light to specifically inhibit viral protein synthesis. Infection of hTCEpi cells with UV-pretreated virus produced the same partial inhibitory effect on ATM activation (Fig. 2B), demonstrating the involvement of a viral protein. Combination treatment with CHX and UV produced no additional reduction of ATM activation, further supporting the activating role of...
a viral protein. Since total inhibition of de novo viral protein synthesis produced only a partial reduction of ATM activation, we hypothesized that the responsible protein has a dual source—from de novo synthesis and from the tegument. Thus, CHX and UV would only inhibit ATM activation de novo synthesized protein but not de novo synthesized protein. Because both of these components are essential, it is likely that ATM activation is achieved as a consequence of an interplay between the viral genome and the responsible viral protein in the host nucleus.

ICP0 Is Neither Sufficient Nor Necessary for ATM Activation

To gain further insight into the identity of the ATM-activating protein, we performed a synchronized HSV-1 infection in hTCEpi cells, which revealed a surprisingly early onset of ATM activation, with pChk2 staining detectible as early as 20 minutes post infection (Fig. 2D), consistent with partial activation by a tegument protein and strongly suggesting immediate early (IE) expression kinetics of the protein in question. Of the six IE proteins of HSV-1, only ICP0 and ICP4 are both present in the tegument and known to interact with viral DNA.

ICP0 interacts with DNA indirectly by influencing the packaging state of the genome through the dispersal of PML bodies, antiviral structures that assemble on the incoming viral genome early during infection. There is conflicting evidence regarding whether ICP0 alone is sufficient to activate ATM. Studies in HeLa cells have suggested ICP0 to be necessary for ATM activation at low MOI but dispensable at high MOI. Since tumorigenic cell lines often have abnormal or dysregulated DDR processes, we chose to investigate the role of ICP0 in a nontumorigenic cell line, hTCEpi. In our hands, exogenous expression of ICP0 in hTCEpi cells failed to activate ATM (Fig. 3B). In addition, cells infected with 7134, an ICP0-null strain of HSV-1, or a WT parental strain showed no difference in ATM activation (Fig. 3A, D). Because PML bodies serve as nuclear depots of numerous DDR proteins, we sought to test the possibility that ICP0 modulates ATM activation through the dispersal of these structures. Cycloheximide treatment of PML-depleted hTCEpi cells produced the same partial reduction of pChk2 staining as seen in WT hTCEpi cells (Fig. 3C), indicating that PML bodies do not have a role in ATM activation by HSV-1.

Overall, these findings argue that ICP0 is neither sufficient nor necessary for HSV-1-induced ATM activation.

HSV-1 Activates ATM in an ICP4-Dependent Manner

Having eliminated ICP0 as a potential ATM-activator, we focused on the remaining candidate protein, ICP4. To
FIGURE 3. HSV-1 activates ATM in an ICP4-dependent and ICP0-independent manner. (A) Confluent monolayers of hTCEpi cells were infected with ICP0-null or WT HSV-1 at low MOI and overlaid with methocellulose-containing medium. Once plaques developed, cells were fixed and stained for pATM (Ser1981). ICP8 served as a marker of infected cells. (B) The hTCEpi cells were transduced with lentiviral vectors harboring ICP0 or ICP4 expression sequences or empty lentiviral backbone, or infected with WT HSV-1 at indicated MOIs. Protein lysates were collected at 20 hours post transduction and 4 hpi, respectively, and analyzed by Western blot with the indicated antibodies. (C) The hTCEpi cells were lentivirally transduced with shRNA against all isoforms of PML, or nontargeting shRNA as a control. After selection, knockdown was verified by Western blot staining with antibody recognizing all PML isoforms. PML-depleted cells were infected with HSV-1 at a range of MOIs in the absence or presence CHX (5 μg/mL). Protein lysates were collected at 1 hpi and analyzed by Western blot with the indicated antibodies. (D) The hTCEpi cells were infected with WT HSV-1 or with mutant ICP4 viruses i13 or n208 at MOI 10. Protein lysates were collected at 1 hpi and analyzed by Western blot with the indicated antibodies. n ≥ 2 independent experiments for all.

address the hypothesis that ICP4 is required for ATM activation, we used pM24, a BAC that contains the full HSV-1 genome with a deletion of both ICP4 coding sequences and constitutively expresses GFP from a CMV promoter. Consistent with our hypothesis, transfection of this BAC into HEK293 cells failed to produce any detectible ATM activation (Fig. 1B). To confirm this finding, we infected hTCEpi cells with d120, an ICP4-null strain of HSV-1.
Compared to WT HSV-1, infection with d120 only achieved the partial level of ATM activation (Fig. 3E), which is consistent with ICP4 being present in the tegument, derived from the supporting cells during viral stock production. Importantly, ATM activation by d120 was not affected by CHX, consistent with the hypothesis that the partial inhibition effect is due to the absence of de novo ICP4 synthesis. However, exogenous expression of ICP4 alone in hTCEpi cells failed to activate ATM (Fig. 3B). Taken together, these experiments demonstrate that ICP4 is necessary but not sufficient for HSV-1-induced activation of ATM.

Given that ICP4 is a potent transactivator of viral gene expression, we sought to determine whether this plays a role in ATM activation. The viral genome contains well-characterized consensus binding sequences, which ICP4 binds as an oligomer or in complex with host proteins\(^\text{37}\) to serve as a transactivator. The ability of ICP4 to bind viral promoters is strictly dependent on its DNA-binding domain (DBD)\(^\text{38}\) and further enhanced by its C-terminal multimerization domain.\(^\text{39}\) We infected hTCEpi cells in the presence or absence of CHX with mutant HSV-1 strains lacking these functionalities. The i13 strain harbors an inactivating mutation in the ICP4 DBD, rendering it unable to bind transcriptional consensus sequences, whereas the n208 strain produces an ICP4 truncation mutant that lacks the C-terminal multimerization domain, thereby reducing its affinity to DNA. Cycloheximide treatment of hTCEpi cells infected with i13 and n208 viral strains produced the same partial reduction of pChk2 staining as seen in WT HSV-1 infection (Fig. 3F). In contrast, the ICP4-null strain d120 failed to reproduce this phenotype, as infection with this virus induced only low levels of pChk2 that were equivalent between CHX-treated and untreated cells (Fig. 3F). This indicates that ATM activation in HSV-1 infection is directly a function of ICP4, as opposed to being mediated by another gene product transactivated by ICP4.

**ATM Activity Is Critical to HSV-1 Replication Early in the Progress of Infection**

Although the antiviral effect of ATM inhibition on HSV-1 is well described,\(^\text{7,11,35}\) the mechanism whereby ATM activity promotes HSV-1 infection remains unknown. In order to gain insight into this phenomenon, we infected hTCEpi cells with HSV-1 in the presence of KU-55933, a small molecule inhibitor of ATM. The drug was added to cells at various time points with respect to the start of infection (−1, 0, +1, +2, +3, and +4 hpi), and the experiment was terminated at 8 hpi. Western blot analysis for glycoprotein C (Fig. 4A) and qRT-PCR measurement of viral genome replication (Fig. 4B) showed that KU-55933 treatments administered prior to the 1 hpi timepoint achieved notable reduction in viral replication, whereas treatments administered at 1 hpi and later produced only a marginal effect. This result clearly demonstrates the presence of a very early ATM-dependent event in the lytic cycle of HSV-1. Following this event, ATM activity seems to be largely dispensable to the progress of infection.

**DISCUSSION**

The presence of nicks and gaps inherent in the HSV-1 DNA genome,\(^\text{32}\) along with its linear ends and environmentally-derived damage experienced by the virion, have been posited as the factors responsible for host DDR activation. Our studies demonstrate that HSV-1 activates ATM in a manner that is disproportional to the extent of DNA damage...
incurred by the host during infection, and that the elimination of DNA ends from the viral genome has no effect on its ability to activate ATM. These findings provide direct evidence for a noncanonical mechanism of ATM activation, whereby the virus induces rapid and robust DDR activation independently of the presence of DNA lesions. Specialized mechanisms for DDR manipulation by many other viruses provide precedent for the evolutionary development of such functionality.

Identification of the viral factor responsible for ATM activation has presented an experimental challenge. The complicated DNA structures that arise in the process of viral genome replication and require resolution by the cellular repair machinery have been proposed to be responsible. The very early timing of ATM activation observed in our experiments, along with its independence of viral DNA replication, exclude replication processes as the causative agent for DDR activation. However, it is possible that these structures contribute to sustained ATM activity later during the course of infection, when the nucleus becomes overwhelmed with viral genome copies.

The involvement of the IE protein ICP0 in ATM activation has been studied with conflicting outcomes. Li et al. have observed strict ICP0 dependency of ATM activation. Interestingly, exogenously expressed ICP0 alone was sufficient to activate ATM in their studies. This finding could not be confirmed by Lilley et al., who failed to detect ATM activation with exogenous ICP0 expression alone. However, their experiments with an ICP0-null mutant strain suggested that this protein is necessary for ATM activation specifically in the setting of a low MOI infection, but dispensable in high MOI infection. By contrast, our studies using exogenous ICP0 expression and ICP0-null virus have shown ICP0 to be neither necessary nor sufficient for the activation of ATM. The differences in experimental outcomes may be explained by the choice of the cell line model. HCT116 and HeLa cells, used by Li et al. and Lilley et al., respectively, are both highly transformed cancer-derived cell lines. Since DDR-associated processes are often profoundly altered in cancer cells, our studies used nontumorigenic, highly differentiated, and disease-relevant human corneal (and other) epithelial cell lines to provide a more physiological model of epithelial infection. It is important to note that in our experiments, KU-55933 potently suppressed HSV-1 replication in all non-tumorigenic cell types tested (hTCEpi, HCE, OKF6, EPC2), yet had less pronounced effect in known transformed or tumorigenic cell types tested (hTCEpi, HCE, OKF6, EPC2). This functionality, which is used at the very onset of infection, is in line with our demonstration of both the rapid induction of ICP4-dependent ATM activation and the early dependence of viral replication on ATM signaling. The use of the host DDR for viral DNA recombination purposes is not without biological precedent, as ATM activation during human immunodeficiency virus infection is required for chromosomal integration of the viral genome.

Further support for this hypothesis is lent by the previously discussed lack of inhibitory activity of KU-55933 in cancer cell lines. Since DNA recombination activities are generally hyperactive in cancer cells, it is plausible that additional upregulation of these pathways is not necessary for genome circularization in cancer cells, as opposed to normal cells; thus, preventing ATM activation would have no effect on viral replication. It is worth noting that while genome circularization might be the relevant downstream function of ATM activation at the early time points, its sustained activity throughout the course of infection may have additional utility, such as in the resolution of branched genome replication intermediates and the facilitation of genome inversion and recombination events.

Taken together, this study serves to further expand our knowledge of HSV-1 virus-host interactions in corneal epithelial cells. A detailed understanding of the molecular mechanisms behind viral hijacking of the host DDR will inform the design of precise new antiviral agents to treat resistant HK cases, or to be used as combination therapy with nucleoside analogues to reduce epithelial toxicity and potentially suppress the emergence of antiviral resistance. The development of new highly potent and specific clinical-grade ATM inhibitors is currently underway and holds promise for advanced future studies and clinical trials. Alternatively, identification of the relevant critical mediators upstream and downstream of ATM may provide a more targeted means of interfering with the DDR than inhibition of the apical ATM kinase.
Our results ascribe yet another essential function to the already complex and crucial ICP4 protein. ICP4 drives the pathogenesis of epithelial HK by orchestrating the viral transcriptional program and activating the host DNA damage response. Furthermore, ICP4 facilitates the transition to the more clinically challenging stromal HK by breaking down the corneal immune privilege. ICP4 acts as a lymphangiogenesis factor by binding the Sp1 transcription sites in the vascular endothelial growth factor A (VEGF-A) promoter and thereby driving its expression. Neovascularization of the cornea allows for the influx of immune cells and subsequent development of stromal keratitis. These activities of ICP4 are all critical to the pathogenesis of HSV-1. Development of novel therapies targeting ICP4 directly or functionally, as in the case of ATM inhibition, may hold promise for improved clinical management of patients with refractory and advanced herpetic eye disease.

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