Differential Recruitment of Sphingosine Kinases by Old World and New World Alphaviruses

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Article

Differential Recruitment of Sphingosine Kinases by Old World and New World Alphaviruses

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Abstract

Alphaviruses have recently re-emerged as important disease-causing agents resulting in debilitating disease in humans and animals. To develop therapeutic strategies, it is important to identify host factors co-opted by viruses during infection. Sphingosine kinases (SKs) have been identified as important host factors for viruses. In this study, we extend on our previous findings and demonstrate a pattern to re-localization of sphingosine kinases during alphavirus infection. Specifically, we observed that encephalitic alphaviruses preferentially re-localize SK1, while arthritogenic alphaviruses re-localize SK2, during infection. Inhibition of SK1 specifically resulted in decreased encephalitic alphavirus infection. Thus, sphingosine kinases are differentially recruited by alphaviruses and represent a viable therapeutic target.

Keywords: sphingosine kinase; SK1; SK2; alphavirus; Old World; New World

1. Introduction

The genus *Alphavirus*, a member of the family *Togaviridae* is responsible for human and animal diseases whose symptoms can range from fever, rashes, headaches, and either arthralgia or encephalitis. Alphaviruses can be classified as Old World (OW) or New World (NW) based on the regions of the world where they are found (Kuhn 2013). OW alphaviruses include species such as Chikungunya virus (CHIKV), Ross River virus (RRV), Semliki Forest virus (SFV), Sindbis virus (SINV), O'Nyong Nyong virus (ONNV), and Barmah Forest virus (BFV) and are endemic to parts of Africa and Asia. Venezuelan Equine Encephalitis virus (VEEV), Western Equine Encephalitis virus (WEEV), and Eastern Equine Encephalitis virus (EEEV) are New World (NW) alphaviruses which are found in the Americas (Griffin 2013). These viruses differ in the symptoms they manifest following infection, with OW alphaviruses causing rashes, fever, and arthralgia, sometimes leading to chronic arthritis. In contrast, NW alphavirus infections are characterized by rash, fever and encephalitis.

Alphaviruses contain a positive-sense RNA genome that encodes four nonstructural proteins (nsP1-nsP4) and five structural proteins (capsid, E3, E2, 6K/TF and E1). The nsPs drive replication and transcription of the viral RNA; nsP1 has genome-capping and membrane-binding activity, nsP2 has helicase and protease activity, while nsP4 is the RNA-dependent RNA polymerase (Kaariainen and Ahola 2002). In contrast, relatively little is known about the function of nsP3 during infection.

Organizationally, nsP3 is a modular protein, with three distinct domains – the macro domain, alphavirus unique domain and the hypervariable domain (HVD) (Figure 1A). The hypervariable domain is of particular interest as this domain shows the most divergence in sequence between OW and NW alphaviruses. Also, the domain is disordered, and this disorder has been shown to be useful in host-virus interactions in a number of viruses. Thus, this region of the protein could be important in the differential interactions of the viruses with host proteins during infection. Indeed, the HVD of

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OW and NW alphaviruses have been demonstrated to differentially recruit host factors to the viral replication complex (VRC) during infection (Foy, Akhrymuk et al. 2013, Foy, Akhrymuk et al. 2013, Kim, Reynaud et al. 2016, Frolov, Kim et al. 2017). During CHIKV infection, nsP3 has been shown to associate with the lipid kinase, sphingosine kinase (SK) 2. This association is likely critical for CHIKV infection since targeted inhibition of the kinase resulted in impaired viral replication (Reid, Tritsch et al. 2015).

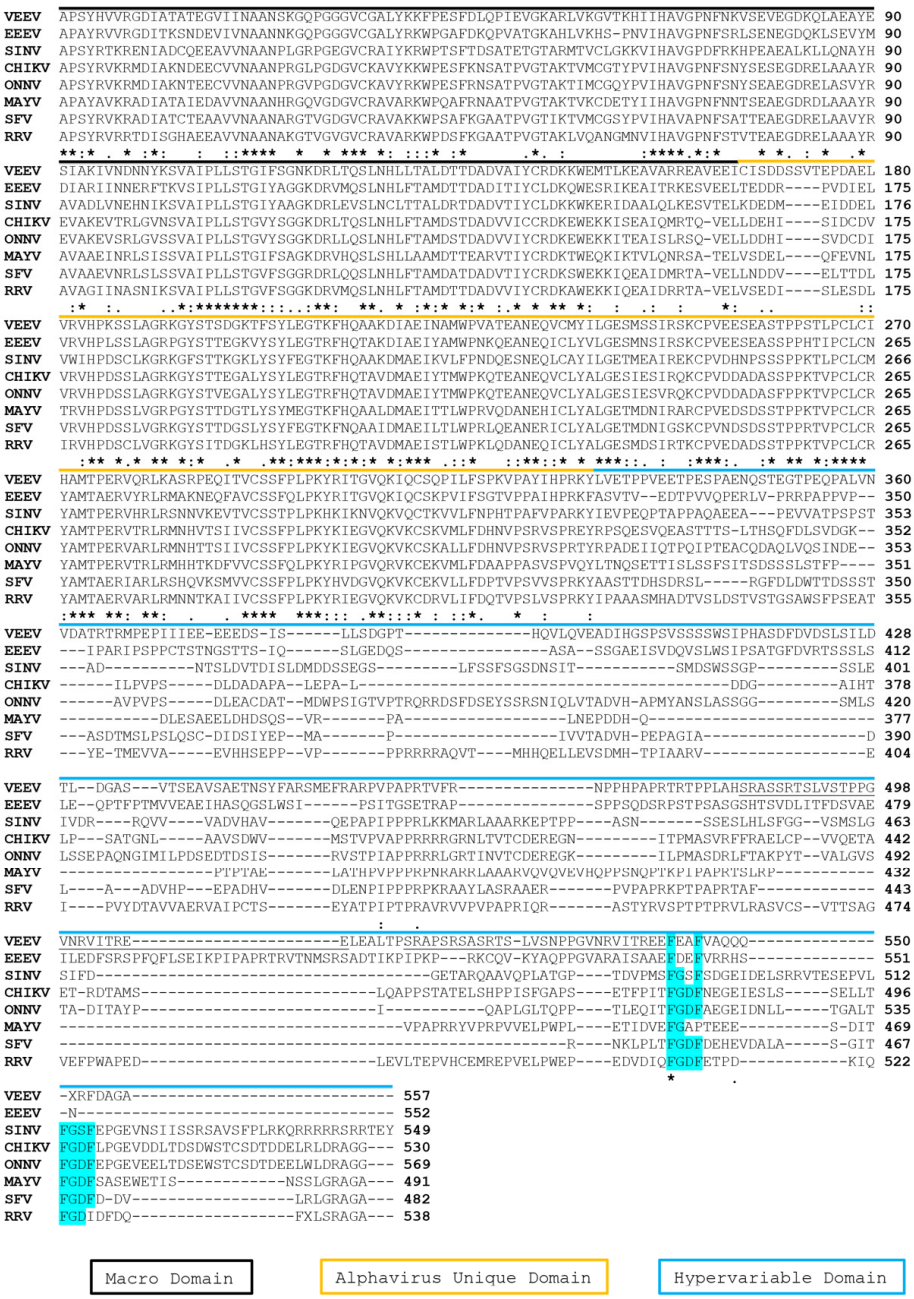

Figure 1. Alignment of OW and NW alphaviruses showing differences in HVD sequence. The amino acid sequence of OW and NW alphaviruses were aligned to show sequence similarities and differences using Clustal Omega. Sequences were obtained from VIPRBC and NCBI databases. Key: * = 100% homology, : = homology between groups of strongly similar properties; . = homology between groups of weakly similar properties.

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The SKs, SK1 and SK2, are isozymes that phosphorylate sphingosine to produce sphingosine-1-phosphate (S1P). S1P can act both as a first messenger, binding to and signaling through five G protein-coupled receptors (GPCRs) $S1PR_{1-5}$, and as a second messenger, activating calcium signaling and regulating DNA transcription (Olivera and Spiegel 1993, Lee, Van Brocklyn et al. 1998). S1P is involved in a variety of cell processes, including cell survival, proliferation, differentiation and inflammation (Zhang, Desai et al. 1991, Spiegel and Milstien 2003). SKs display different developmental expression, tissue distribution and subcellular localization patterns (Taha, Hannun et al. 2006, Pitson 2011). The kinases have also been shown to play a role in viral infection. SK1 was demonstrated to be a pro-viral factor during Influenza A virus (IAV) infection, promote measles virus (MV) replication, enhance respiratory syncytial virus (RSV)-induced cell survival and human cytomegalovirus (HCMV) infectious virion production (Monick, Cameron et al. 2004, Machesky, Zhang et al. 2008, Carr, Mahalingam et al. 2013, Seo, Pritzl et al. 2013, Vijayan, Seo et al. 2014). SK1 has also been shown to play a role as an anti-viral factor during bovine diarrhea viral disease (BDVD) and dengue virus (DENV) infections (Carr, Kua et al. 2013, Ammari, McCarthy et al. 2014). In contrast, a role for SK2 during viral infection is only recently being revealed. A recent report demonstrated that SK2 promotes IAV infection (Xia, Seo et al. 2018). Additionally, SK2 has been shown to maintain viral latency during Kaposi's sarcoma-associated herpesvirus (KSHV) infection and enhance hepatitis C virus (HCV) replication (Dai, Plaisance-Bonstaff et al. 2014, Yamane, McGivern et al. 2014). We reported that SK2 was re-localized to the VRC early during CHIKV infection (Reid, Tritsch et al. 2015). Moreover, genetic or chemical targeting of SK2 significantly impaired infection, providing strong evidence that it is a critical host factor during CHIKV infection (Reid, Tritsch et al. 2015). In contrast, targeting of SK1 had no effect on CHIKV infection. Preliminary evidence indicates SK2 interacts with nsP3 through the HVD (unpublished observations). The involvement of nsP3 HVD in the recruitment of SK2 and the absence of SK1 effect during CHIKV infection prompted the hypothesis that the interaction of alphaviruses with SKs would differ based on their geographical classification as OW or NW, due to the differences in the HVD domain. In the current study, we show that OW alphaviruses preferentially induce SK2 re-localization during infection, while NW alphaviruses induce SK1 re-localization. Furthermore, targeted inhibition of SK1 by siRNA or chemical inhibitors significantly impaired NW alphavirus infection. Taken together, we identify SK1 as a NW alphavirus host factor that is important for infection.

2. Materials and Methods

2.1. Cell Culture, Plasmids, Compounds and Viruses

Baby Hamster Kidney (BHK)-21 and HeLa cells were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% inactivated Fetal Bovine Serum (FBS) (GE Healthcare). For chemical inhibition of sphingosine kinase, the following inhibitors were used: SKI-II (Tocris Bioscience), ABC294640 (Cayman Chemical), and SKI-178 (Tocris Bioscience). For genetic inhibition studies, siRNA targeting SK1 and non-targeting siRNA were used (Ambion).

CHIKV (181/25), VEEV (TC-83) and SINV (Ar-339) were propagated from the USAMRIID collection of viruses. MAYV (TRVL 15537) was purchased from ATCC.

2.2. Immunofluorescence Experiments

Infected BHK21 cells were fixed in ice-cold methanol and subsequently blocked in PBS containing 3% bovine serum albumin (3% BSA-PBS) for 1 h then stained with the indicated antibodies. For viral infection experiments, viral double-stranded RNA (dsRNA) was detected using the mouse monoclonal antibody J2 (1:500, Scicons) along with the rabbit anti-SK2 antibody (1:1000, ECM Bioscience). Secondary antibodies Alexa 488-conjugated donkey anti-mouse and Alexa 568-conjugated donkey anti-rabbit antibody (1:2000, Life Technologies) were used to visualize primary

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antibodies. Confocal images were collected with the Leica TCS-SP5 confocal/multiphoton microscope.

CHIKV/MAYV-SK1 localization studies were done in HeLa cells. Cells were infected with CHIKV/MAYV at MOI 5 for 24 h. Cells were then fixed in 4% PFA-PBS for 15 minutes and permeabilized with 0.1% Triton-X-PBS solution for 10 minutes at room temperature. Blocking was done with 3% BSA-PBS for 1 h. Primary antibodies corresponding to dsRNA and SK1 (1:1000, ECM Biosciences) were used to stain the cells overnight at 4 ºC. Secondary antibodies including Alexa 568-conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit were used to visualize the primary antibodies. Confocal images were taken using the Zeiss LSM 800 confocal microscope.

VEEV-SK1/SK2 localization studies were carried out in HeLa cells. Twenty-four hours after plating, cells were either mock-infected or infected with VEEV at a MOI of 0.01 for 20 h. The cells were then fixed with 10% formalin for 1 h, followed by permeabilization with 0.2% Triton-X for 15 minutes. Cells were then blocked in 3% BSA-PBS for 1 h. Cells were incubated for 1 h with primary antibodies corresponding to virus glycoprotein, anti-TC83 1A4A (mouse hybridoma, USAMRIID) and SK1 (ECM Biosciences) were used. Secondary antibodies used to visualize the primary antibodies include Alexa 568-conjugated goat anti-mouse and DyLight 488-conjugated goat anti-rabbit. Images were collected using Leica TCS-SP5 confocal/multiphoton microscope.

2.3. Sphingosine Kinase Inhibitor Studies

For the inhibitor experiments, HeLa cells were pre-treated with the indicated concentrations of inhibitors for 1 h prior to infection. Inhibitors were aspirated from cells which were then infected with VEEV (MOI = 0.01) or EEEV (MOI = 1) for 20 h. For the siRNA experiment, HeLa cells were transfected with 18 nM of either control or SK1 siRNA (Ambion) for 48 h prior to infection with VEEV (MOI = 0.006). The cells were fixed in formalin and subsequently processed as previously described. The primary anti-TC83 1A4A antibody was used followed by Alexa 488–conjugated goat anti-mouse antibody. Cell nuclei and cytoplasm were labeled with Hoechst 33342 and HCS CellMask Red (Life Technologies), respectively. High-content quantitative imaging data were acquired and analyzed on an Opera Confocal Plate-reader Model 3842 (Quadruple Excitation High Sensitivity (QEHS), PerkinElmer). Image analysis was accomplished using standard Acapella scripts. Percentage inhibition was calculated using mean percentage infection from infected and untreated cells as a positive control (100% inhibition).

3. Results

3.1. OW Alphaviruses Preferentially Re-Localize SK2

We previously showed that CHIKV requires SK2 and not SK1 during infection (Reid, Tritsch et al. 2015). CHIKV infection induced a dramatic re-localization of SK2 which served a pro-viral role. Furthermore, preliminary studies indicate SK2 associates with nsP3 via the HVD. In this study, we investigated whether re-localization of SK2 is preserved in other members of the alphavirus genus. Alignment of the nsP3 amino acid sequence highlighted differences in the alphavirus HVD which have been previously noted to be instrumental in differential nsP3 interactions with host proteins (Schulte, Liu et al. 2016, Goertz, Lingemann et al. 2018, Gotte, Panas et al. 2019). Despite these differences, there are similarities between viruses in the same geographical classification, such as the FGDF motifs for the OW alphaviruses (Figure 1). These differences as well as CHIKV re-localization of SK2 during infection led us to investigate whether there would also be a pattern in the interactions of alphaviruses with the kinases, based on alphavirus classification as OW or NW.

To test this, we infected cells with OW alphaviruses - SINV and Mayaro virus (MAYV) and evaluated localization of SK2. Notably, although MAYV is classified as a NW alphavirus based on its discovery in South America, it is known to manifest with OW symptoms upon infection (Tesh, Watts et al. 1999). We observed that SINV, a classic OW alphavirus, as well as MAYV, caused re-localization of SK2 from the nucleus to the cytoplasm following infection (Figure 2B & C). This observation was

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similar to previously published findings for CHIKV (Reid, Tritsch et al. 2015). In contrast, infection with VEEV did not result in SK2 re-localization, suggesting VEEV does not recruit SK2 during infection (Figure 2D).

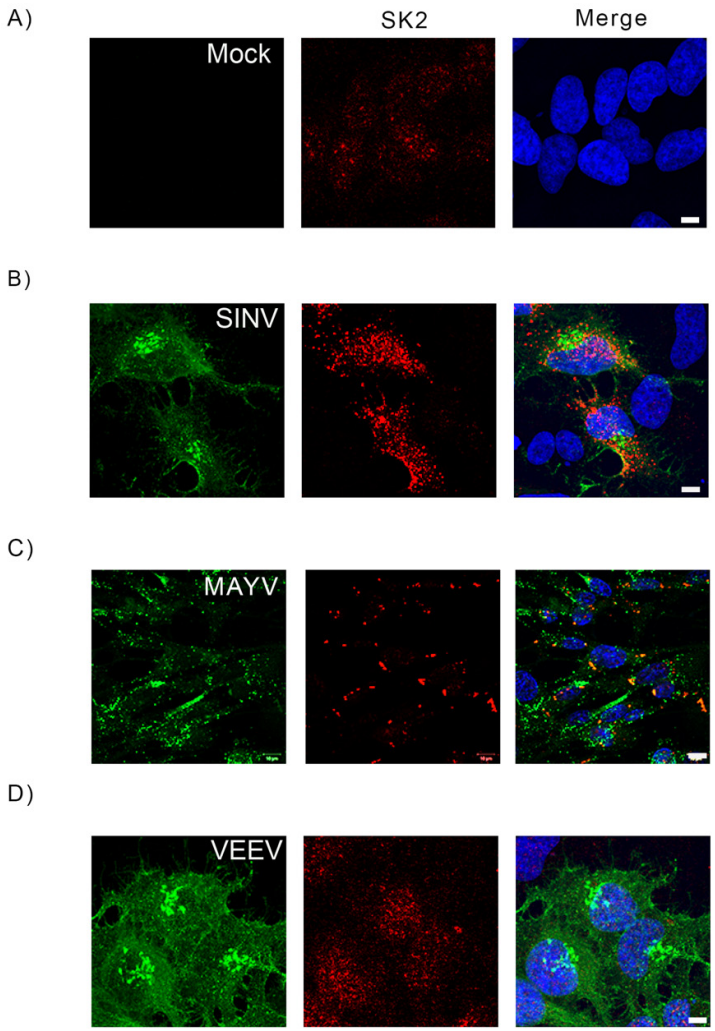

Figure 2. SK2 re-localizes during infection with arthritogenic, but not encephalitic alphaviruses. A) BHK21 cells were A) mock infected or infected with B) SINV (MOI = 1) or C) MAYV (MOI = 5) or D) VEEV (MOI = 0.01) for 24 h. The cells were then fixed and stained for viral antigen (green), SK2 (red) and Hoechst nuclear stain (blue). Scale bars = 5 μm (A, B, D), 10 μm (C).

3.2. New World Alphaviruses Preferentially Re-Localize SK1

In contrast to SK2, which is primarily localized in the nucleus, SK1 is typically localized in the cytoplasm and upon activation moves to the plasma membrane where it phosphorylates sphingosine. To test whether SK1 is re-localized during NW alphavirus infection, cells were infected with VEEV and SK1 localization was evaluated. VEEV infection caused a dramatic re-organization of SK1 in the cytoplasm (Figure 3A). In contrast, CHIKV and MAYV had no effect on SK1 localization (Figure 3B), thus confirming previous observations that targeting SK1 does not affect CHIKV infection. Our results show that VEEV induces SK1 re-localization, indicating that SK1 is likely utilized by NW alphaviruses.

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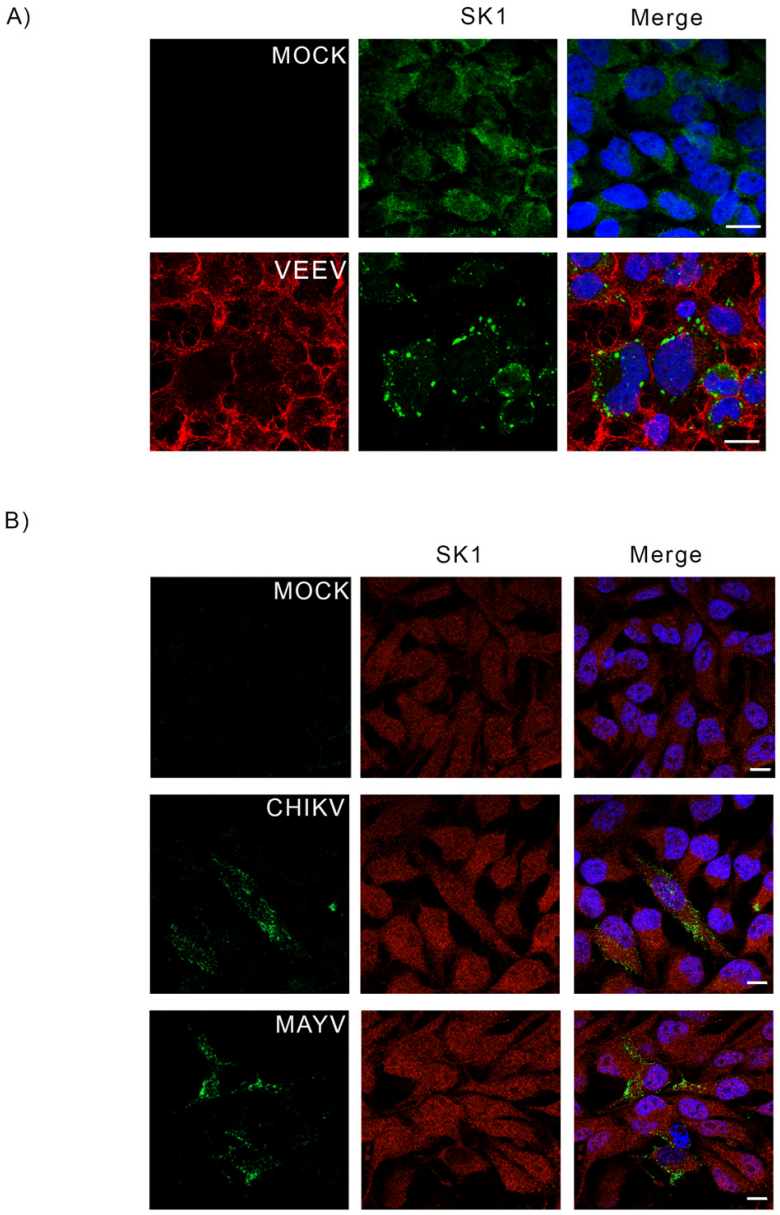

Figure 3. SK1 re-localizes during infection with VEEV, but not arthritogenic alphaviruses. A) HeLa cells were either mock infected or infected with VEEV (MOI = 0.01) for 24 h. Cells were then fixed and stained for viral glycoprotein using VEEV TC83 1A4A antibody (red), SK1 (green) and Hoechst nuclear stain (blue) and then visualized with the Leica TCS SP5 confocal/multiphoton microscope. Scale bars = 25 µm. B) HeLa cells were either mock infected or infected with CHIKV (MOI = 1) or MAYV (MOI = 1) for 24 h. The cells were then fixed, stained for dsRNA (green), SK1 (red) and Hoechst nuclear stain (blue) and visualized with the Zeiss LSM800 confocal microscope. Scale bars = 10 µm.

3.3. SK1 Inhibition Decreases NW Alphavirus Infection

To confirm the functional requirement of SK1 during NW alphavirus infection, we performed genetic and chemical inhibition of SK1. Cells were pre-treated with the dual SK1/2 inhibitor (SKI-II), SK1 inhibitor (SKI-178), and SK2-specific inhibitor (ABC294640) (Gao, Peterson et al. 2012, Pitman, Costabile et al. 2016). Pre-treatment with SKI-II and SKI-178 significantly inhibited VEEV infection (Figure 4A & C). In contrast, the SK2-inhibitor ABC294640, displayed no activity against VEEV (Figure 4B). Similarly, siRNA-mediated knockdown of SK1 significantly impaired VEEV infection

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(Figure 4D). Furthermore, SKI-II pre-treatment prior to infection with EEEV, another NW alphavirus, significantly impaired infection while ABC294640 had very little effect on infection (Supp Figure S1A & B). These data demonstrate that SK1 and not SK2, is required during NW alphavirus infection.

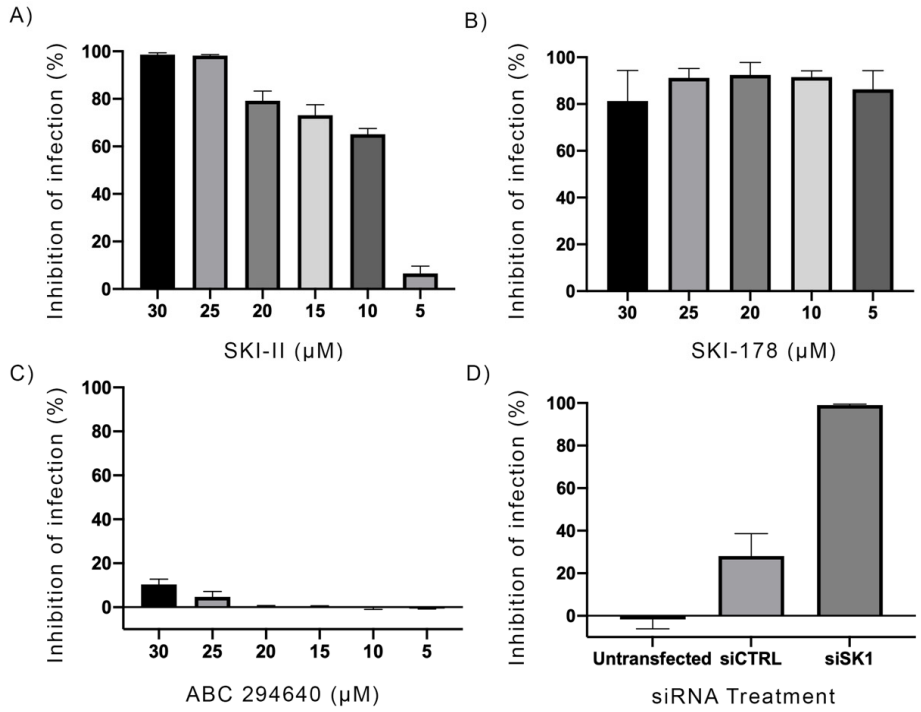

Figure 4. NW alphavirus infection requires SK1. (A) HeLa cells were pretreated for 1 h with 5, 10, 15, 20, 25 and 30 μM of A) SKI-II, B) SKI-178, C) ABC294640, or D) transfected with control siRNA or siSK1 for 48 hours and then infected with VEEV at (A and C) MOI = 0.01 or (B and D) MOI = 0.006. 20 h post infection, cells were fixed and analyzed for infection by high-content confocal microscopy. Percent inhibition of infection was calculated using mean percentage infection from infected, untreated cells as a positive control.

4. Discussion

The re-emergence of alphaviruses as causative agents of severe disease in humans has highlighted this group of viruses as important targets for therapy. In order to efficiently develop targeted therapies, it is essential to understand virus interaction with host cells during infection. To this end, we studied the interaction of both OW and NW alphaviruses with sphingosine kinases, lipid kinases that have been shown to be important during infection of Dengue virus, Measles virus, and several other viruses previously highlighted. Our results strongly suggest that sphingosine kinases are differentially utilized by OW and NW alphaviruses during infection. We have observed that CHIKV interacts with SK2 via the HVD of nsP3 (unpublished observations). However, it is unknown whether nsP3 mediates NW alphavirus interaction with SK1. It was previously shown that unique repeat domains present in VEEV HVD are important for interaction with FXR proteins during virus replication (Kim, Reynaud et al. 2016). In that study, EEEV was shown to interact with both FXR and G3BP proteins via regions in its HVD, mimicking both OW alphaviruses and VEEV. SK1 appears to be selectively important for both VEEV and EEEV, thus, it would be of interest to further understand what viral protein(s) are involved in SK1 interaction. The role of the kinase in the context of viral entry, replication, translation and exit would be useful determining its utility as a target for therapy. Understanding the interaction between alphaviruses and sphingosine kinases represents an important strategy in designing therapies to treat diseases caused by alphaviruses.

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Supplementary Materials: The following are available online at Preprints.org, Figure S1: EEEV infection is not affected by SK2 inhibitors.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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