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Sphingosine kinase 1 regulates measles virus replication

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ABSTRACT

Measles virus (MV) manipulates host factors to facilitate virus replication. Sphingosine kinase (SK) is an enzyme catalyzing the formation of sphingosine 1-phosphate and modulates multiple cellular processes including the host defense system. Here, we determined the role of SK1 in MV replication. Over-expression of SK1 enhanced MV replication. In contrast, inhibition of SK impaired viral protein expression and infectious virus production from cells expressing MV receptor, SLAM or Nectin-4. The inhibition of virus replication was observed when the cells were infected by vaccine strain or wild type MV or V/C gene-deficient MV. Importantly, SK inhibition suppressed MV-induced activation of NF-κB. The inhibitors specific to NF-κB signal pathway repressed the synthesis of MV proteins, revealing the importance of NF-κB activation for efficient MV replication. Therefore, SK inhibition restricts MV replication and modulates the NF-κB signal pathway, demonstrating that SK is a cellular factor critical for MV replication.

Introduction

Measles is a highly contagious disease that remains as one of the leading causes of pediatric morbidity and mortality worldwide (CDC, 2013). Despite the availability of a safe vaccine, there were approximately 158,000 measles-related deaths globally in 2011 (CDC, 2012). In the USA, 222 measles cases, which were associated with travel/importations, were reported in 2011 (CDC, 2012).

Measles virus (MV), the causative agent for measles, is an enveloped, negative stranded RNA virus that belongs to the order *Mononegavirales*, family *Paramyxoviridae,* and genus *Morbillivirus* (Griffin, 2001). Wild type MV uses the signaling lymphocyte activation molecule (SLAM)/CD150 (Tatsuo et al., 2000) and Nectin-4/PVRL4 as cellular receptors (Muhlebach et al., 2011; Noyce et al., 2011), while the attenuated vaccine strains of MV can interact with CD46 to enter cells in addition to being able to use SLAM and Nectin-4 (Dorig et al., 1993; Naniche et al., 1993). A profound immunosuppression is a hallmark characteristic of MV infection, however the exact mechanisms of this process are not clearly understood (Avota et al., 2010; Hahm, 2009). Transgenic mice bearing human CD46 (Oldstone et al., 1999; Rall et al., 1997; Sellin and Horvat, 2009) or human SLAM (Hahm et al., 2003; Hahm et al., 2004; Ohno et al., 2007; Welstead et al., 2005) have been generated to study MV-induced immune suppression and measles pathogenesis. These animal models have increased our understanding of measles biology (Oldstone et al., 2005), although they did not fully support MV replication to cause clinical symptoms of measles in the presence of the host immune system. However, transgenic mice harboring human Nectin-4 have not yet been established. Furthermore, there are no specific antivirals for treating measles (Moss and Griffin, 2012). Thus, it is important to identify cellular factors that are critically involved in MV replication and to define regulatory pathways of MV–host interaction.

MV is known to modulate host machinery and its signaling pathways to facilitate its own replication (Gerlier and Valentin, 2009; Kerdiles et al., 2006; Rima and Duprex, 2011). For example, MV proteins such as the non-structural V and C proteins inhibit type I interferon (IFN)-mediated anti-viral activity (Ramachandran and Horvath, 2009; Shaffer et al., 2003). Further, although MV was shown to induce the activation of NF-κB signaling (Helin et al., 2001), viral proteins suppress strong activation of NF-κB signaling pathway (Pfaller and Conzelmann, 2008; Schuhmann et al., 2011; Yokota et al., 2008).

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid mediator and its level is tightly regulated by cellular enzymes (Gandy and Obeid, 2013; Rosen et al., 2013). Sphingosine kinase (SK) converts sphingosine to S1P via its kinase activity. SK/S1P pathway mediates a variety of crucial cellular processes such as cell growth/survival/differentiation, lymphocyte trafficking, and host immunity (Maceyka et al., 2012; Spiegel and Milstien, 2011). Intracellular S1P and SK1 bind TNF receptor-associated

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factor 2 (TRAF2) to activate TNF-α-induced NF-κB signaling (Alvarez et al., 2010), which could be important for regulation of the inflammatory responses. Recently, SK was reported to affect virus replication. Bovine viral diarrhea virus inhibited SK1 for efficient viral replication (Yamane et al., 2009), whereas SK1 increased the propagation of influenza virus (Seo et al., 2010; Seo et al., 2013) and human cytomegalovirus (Machesky et al., 2008). Yet, the precise role of the sphingolipid system during virus replication has not been defined.

In this study, we determined if SK1 regulates MV replication. Our data demonstrate that SK1 exhibits a pro-viral function to enhance MV amplification. Further, MV activates NF-κB in an SK-dependent manner to promote virus replication.

Results

Overexpression of SK1, but not exogenous S1P addition, enhances MV replication

In order to investigate whether SK1 affects the replication of MV, we used HEK 293 cells (HEK cells) that were engineered to over-express SK1 (SK1 cells) (Min et al., 2007). SK1 cells or HEK cells were infected with the Edmonston strain of MV (MV-Ed) and at 1 day post-infection (dpi), the expression levels of measles viral nucleoprotein (N) and matrix (M) protein were compared between SK1 cells and HEK cells. As indicated by the Western blot result in Fig. 1A, the amounts of both N and M proteins were clearly increased in SK1 cells compared to HEK cells at both 0.1 and 0.5 multiplicity of infection (MOI) conditions, indicating that SK1 overexpression promotes the expression of MV proteins upon infection. This result directly correlated with the extent of viral cytopathic effect (CPE) that was observed on these cells by visual inspection under a phase contrast microscope, i.e., MV-infected SK1 cells exhibited more CPE compared to the infected HEK cells (data not shown). Since the enzymatic function of SK1 is to catalyze the formation of S1P, we determined if exogenously supplied S1P enhances MV protein synthesis in a manner similar to the effect of SK1 overexpression. However, exogenous addition of S1P did not alter the level of MV N protein (Fig. 1B). Collectively, these results indicate that overexpression of intracellular SK1 enhances MV replication, whereas exogenously added S1P that is known to trigger S1P receptor signaling (Rosen et al., 2013) does not alter MV replication.

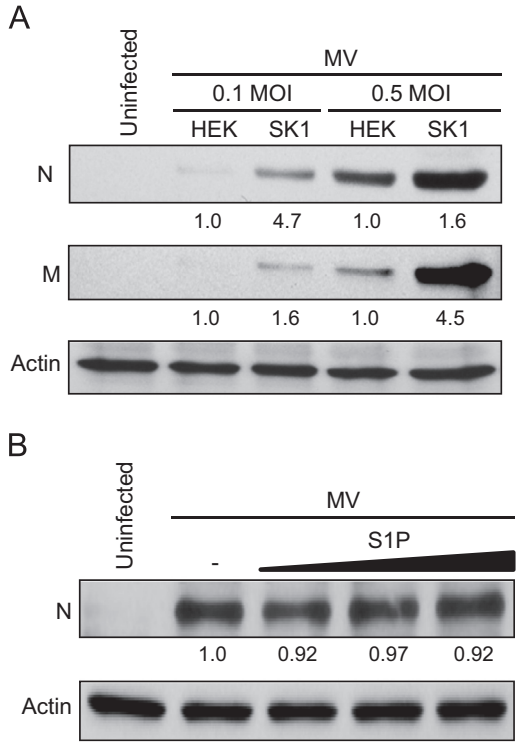

Fig. 1. Overexpression of SK1, but not exogenous S1P addition, enhances MV replication. (A) HEK 293 (HEK) cells or sphingosine kinase 1 (SK1) overexpressing HEK cells were infected with the Edmonston strain of measles virus (MV) either at 0.1 MOI or 0.5 MOI. At 1 day post-infection (dpi) Western blotting was performed to detect the nucleoprotein (N) or the matrix (M) protein of MV. The level of viral protein expressed by the control HEK cells was set to 1.0 at both MOIs. (B) NCI-H358 cells (H358) were pre-treated with 50, 100, or 500 nM of S1P for 10 min followed by infection with MV at 0.1 MOI. At 1 dpi, cell lysates were harvested for Western blot analysis to detect N protein of MV. The relative intensities for each band of N protein are shown.

Inhibition of SK impairs the replication of MV

To further investigate the role of SK in MV replication, we employed a pharmacological approach by using inhibitors that are known to impair SK activity such as N,N-dimethylsphingosine (DMS) (Edsall et al., 1998; Orr Gandy and Obeid, 2013; Yatomi et al., 1997) and 4-[[4-(4-chlorophenyl)-2-thiazolyl]amino]phenol (SKI-II) (French et al., 2003; Orr Gandy and Obeid, 2013). As shown in Fig. 2A, inhibition of SK with these inhibitors led to a marked decrease in the expression level of MV N protein in H358 cells, which express Nectin-4, the epithelial cell receptor for MV (Muhlebach et al., 2011; Noyce et al., 2011). To exclude the possibility that SK inhibitors exhibit cytotoxic effects, which would eventually interfere with virus propagation, we performed a trypan blue exclusion assay to compare the percentage of viable cells between SKI-II-treated and untreated cells in the presence or absence of MV infection. As shown by the percentage of viability of H358 cells in Fig. S1, SKI-II did not exhibit any significant cytotoxicity in our experimental condition, indicating that the impaired virus replication is not due to the altered cell viability. Since MV is able to use SLAM as well as Nectin-4 as a receptor to infect cells, SLAM-expressing B95-8 cells were infected with MV and incubated with the inhibitor SKI-II. The SK inhibitor displayed a similar inhibitory effect on virus replication in B95-8 cells (Fig. 2B). This result suggests that SK acts as a pro-viral factor in MV replication and importantly, this phenomenon occurs irrespective of the cellular receptor usage by MV, as we observed the SKI-II-mediated inhibition of viral replication in cells expressing either SLAM or Nectin-4. Next, we performed flow cytometric analysis to assess the level of MV proteins expressed on the surface of infected cells on a single cell basis. SKI-II treatment decreased the expression level of MV proteins on the surface of infected cells compared to the untreated cells (Fig. 2C), as indicated by the decrease of mean fluorescence intensity (MFI) (309–193). Also, SK inhibition suppressed the replication of JW strain of wild type MV (Fig. 2D), demonstrating the importance of SK in promoting the replication of both wild type and the attenuated strains of MV.

MV non-structural proteins such as V and C proteins are involved in modulating host defense mechanisms including type I IFN response (Ramachandran and Horvath, 2009; Shaffer et al., 2003). In order to determine if there is a role of MV V or C protein in SK-mediated modulation of MV replication, we used recombinant viruses that lack the V or C gene namely the V^- or C^- viruses (Patterson et al., 2000). The replication of V or C-deficient viruses was diminished by SK inhibition similar to the V and C sufficient MV (Fig. 2E). This result indicates that both MV V^- and MV C^- are sensitive to SK inhibition and that V and C proteins do not play a key role in SK-mediated regulation of MV replication. Furthermore, we used a small interfering RNA (si-RNA) approach to knockdown SK1 levels to further confirm our results. Knockdown of SK1 also reduced the level of pSK1 and resulted in a strong

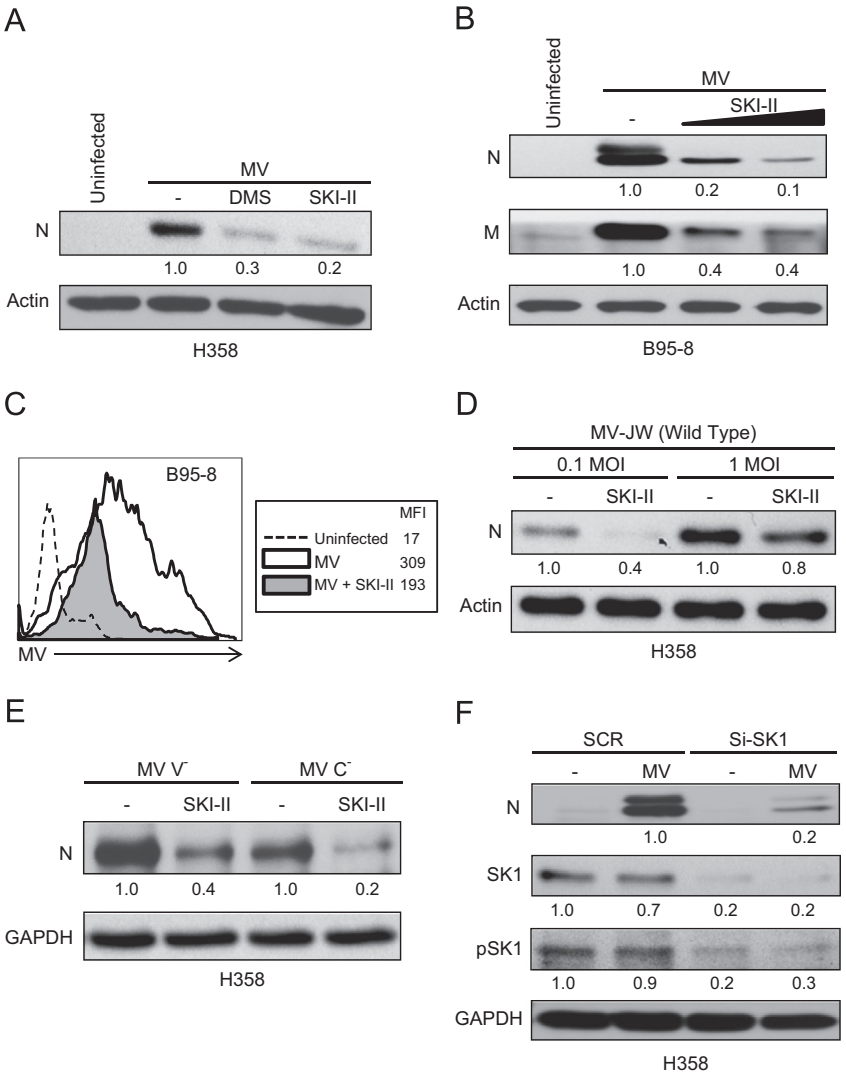

Fig. 2. Inhibition of SK impairs the expression of MV proteins. (A) H358 cells were infected with MV at 0.1 MOI and were co-treated with DMS (20 μM), SKI-II (20 μM), or the solvent, DMSO (−). At 1 dpi, Western blotting was performed to detect MV N protein expression levels. The level of N protein in the solvent treated (−) condition was set to 1.0. (B) B95-8 cells were infected with MV at 0.1 MOI and co-treated with SKI-II at 5 or 10 μM or the solvent (−). At 1 dpi, cell lysates were harvested to perform Western blot analysis to detect N and M protein levels. The level of M and N proteins in the solvent treated (−) condition was set to 1.0. (C) B95-8 cells were infected with MV at 0.5 MOI and at 3 dpi, cells were incubated with a polyclonal antibody to detect measles virus proteins expressed on the surface of the cells by flow cytometry. Dotted histogram represents uninfected cells; open histogram represents MV-infected, the solvent-treated cells; and filled histogram represents MV-infected, SKI-II-treated cells. The levels of mean fluorescence intensity (MFI) of each sample are depicted. (D) H358 cells were infected with wild type MV of JW strain at 0.1 or 1 MOI. Infected cells were co-treated with either SKI-II (20 μM) or its solvent (−). At 1 dpi, Western blotting was performed to detect N protein. The level of N protein in the solvent treated (−) condition was set to 1.0 at both MOIs. (E) H358 cells were infected with V^- or C^- viruses at 0.1 MOI and were either treated with SKI-II (20 μM) or the solvent (−). At 1 dpi, Western blot analysis was performed to detect N protein of MV. The level of viral proteins in the V^- or C^- infected and solvent treated (−) condition was set to 1.0. (F) H358 cells were transfected with siRNA targeting SK1 (si-SK1) or the scrambled si-RNA control (SCR). Transfected cells were infected with MV at 0.1 MOI and harvested for Western blot analysis to detect MV N, SK1, and pSK1 proteins. Relative band intensities of N, SK1 and pSK1 are shown.

decrease in MV N protein levels (Fig. 2F). This data further validates the importance of SK1 in MV replication.

SK inhibition leads to a decrease in the level of MV proteins, which could influence the production of infectious progeny viruses. To investigate this, H358 cells and B95-8 cells were infected by MV, treated with SKI-II or its solvent, and then plaque assays were performed to quantify the MV particles produced from infected cells. The amount of viruses produced from both H358 cells and B95-8 cells was inhibited by SKI-II treatment at 1 or 2 dpi (Fig. 3A and B). Approximately 25 fold fewer viruses were produced from MV-infected, 10 μM SKI-II-treated H358 cells at 2 dpi compared to the untreated cells (Fig. 3A). Similarly, the amount of viruses produced from MV-infected B95-8 cells that were treated with 10 μM SKI-II was about 24 fold less than that from its control cells at 1 dpi (Fig. 3B). These results conclusively indicate that SK inhibition impairs the production of viral proteins and ultimately leads to a decrease in the amplification of MV particles.

MV infection increases the level and activation of SK1 in B95-8 cells

Our results indicate that SK1 has a positive effect on MV replication, as it favors MV propagation in cells. Therefore, there is a possibility that MV infection could regulate the activation/expression of cellular SK1 for its own fitness. To test this possibility, we monitored the expression and phosphorylation of SK1 in B95-8 cells following MV infection. MV infection increased the phosphorylation of SK1 and also the expression level of SK1 protein over time (Fig. 4). The increase of pSK1 is greater (approximately 2.0 fold) than the enhanced expression of SK1 by

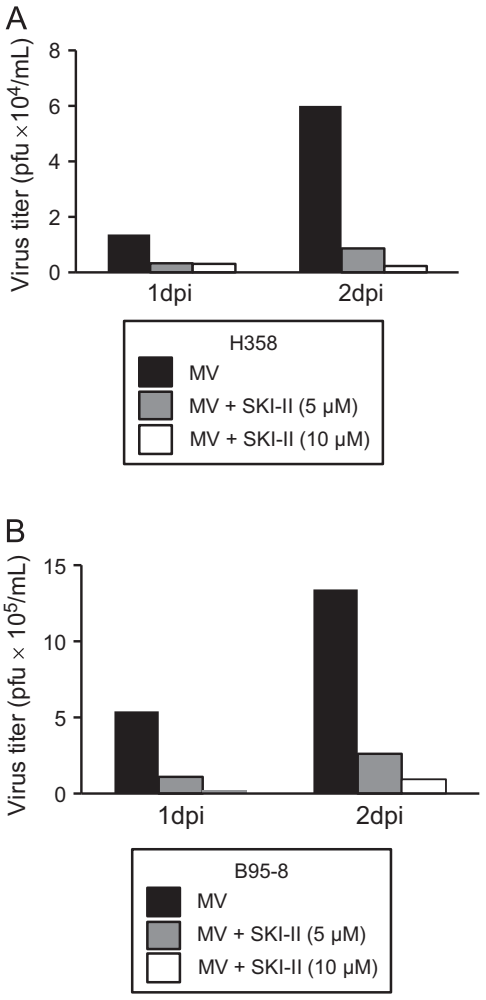

Fig. 3. SK inhibition interferes with infectious MV production. (A) H358 cells were infected with MV-Ed (MV) at 0.1 MOI and co-treated with solvent (−) or SKI-II at 5 μM or 10 μM. At 1 and 2 dpi, samples were harvested. Plaque assay was performed on Vero cells to quantify the amount of infectious virus produced. (B) B95-8 cells were infected at 0.5 MOI of MV and were co-treated with solvent (−) or SKI-II at 5 μM or 10 μM. At 1 and 2 dpi, samples were harvested to perform plaque assays.

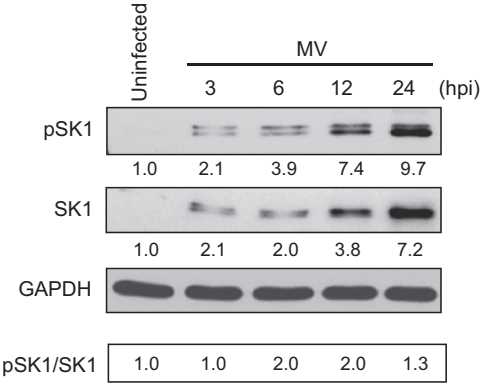

Fig. 4. MV infection increases the level/activation of SK1 in B95-8 cells. B95-8 cells were infected with 1 MOI of MV and cell lysates were harvested at the indicated time points post-infection. Western blotting was performed to detect pSK1 and SK1 proteins. The levels of pSK1 and SK1 proteins in the uninfected condition were set to 1.0. The ratio of the band intensities pSK1/SK1 is shown separately.

infection when their increased intensities were compared (pSK1/SK1). This indicates that the elevated level of pSK1 is not just due to the heightened level of SK1 expression. MV-induced expression/activation of SK1 could enhance MV replication. However, little increase of SK1 expression/phosphorylation was observed in H358 cells following MV infection (data not shown). These results suggest that MV regulates SK1 expression/activation in a cell-type specific manner.

MV-induced activation of NF-κB signaling, which is needed for efficient MV replication, is modulated by SK inhibition

It is possible that SK inhibitors regulate the level of sphingolipids that are components of cellular membranes and affect the initial entry step of MV into the cells. To address this, cells were infected with MV for 1 h and washed to remove any unbound virus particles from the cell surface. After 1 or 3 hours post-infection (hpi), SKI-II was supplied to the infected cells. Addition of SKI-II even after 3 h of MV-cell interaction could still potently inhibit MV replication (Fig. S2), suggesting that the inhibition mainly occurs at the post-entry levels of MV replication.

Recently, SK1 as well as intracellular S1P were reported to interact with TRAF2 and increase TNF-induced activation of the NF-κB pathway (Alvarez et al., 2010). Also, there is a report demonstrating that NF-κB activity is elevated in A549 cells following MV infection (Helin et al., 2001). Therefore, we defined the function of SK1 in MV-induced activation of NF-κB pathway. We detected phosphorylation of p65 (RelA) as one of our read-outs for NF-κB activation, since phosphorylated p65 is a major component of the active NF-κB transcription complex (Gilmore, 2006). Notably, MV increased the activation of p65 at 6, 9, 12 and 30 hpi and interestingly, this increase was impaired by the treatment with SKI-II in B95-8 cells (Fig. 5A). However, the level of total p65 did not change by the SKI-II treatment (Fig. 5A). This implies that SK inhibition leads to a decrease in MV replication, which correlated well with a reduction of activation of NF-κB signaling. Similar results were also reproducible when H358 cells were used (Fig. 5B). We observed that MV induced the phosphorylation of p65 and IKKα/β, which is the kinase upstream of p65 and necessary for the activation of p65. In addition, a decrease in the phosphorylation status of these components was detected when SK was inhibited (Fig. 5B). It is known that the activated p65 subunit translocates to the nucleus and binds to NF-κB promoter elements to activate the transcription of target genes (Gilmore, 2006). Therefore, we have used a reporter plasmid to measure NF-κB promoter activity. SK inhibition decreased NF-κB promoter activity induced by the MV V^- (Fig. 5C), further supporting our conclusion that SK inhibition interferes with NF-κB signaling upon MV infection. These observations led us to speculate that MV-induced activation of NF-κB signal pathway contributes to the efficient MV replication. To investigate this, NF-κB-specific inhibitors were utilized and their effect on MV replication was monitored. As indicated in Fig. 5D, when H358 cells were pre-treated with Wedelolactone that inhibits the kinase activity of IKKα/β (Idris et al., 2009) prior to MV infection, there was a strong decrease in MV N protein levels. Similar inhibition of MV replication by Wedelolactone was observed in B95-8 cells (Fig. S3). To confirm the result, we tested another inhibitor of IKKα/β, Bay-11-7082 (Bay) that prevents the phosphorylation and subsequent degradation of IκB (Pierce et al., 1997). The treatment with Bay reduced the expression of MV N protein when the cells were infected by MV at 0.1 or 1 MOI (Fig. 5E). Thus, we could conclude that MV-induced activation of NF-κB pathway is crucial for efficient virus replication and that SK inhibition could display its anti-viral effect, at least in part, by suppressing the MV-induced NF-κB signal pathway.

Following this observation, the next issue we addressed was the possible role of type I interferon (IFN) in SK-mediated MV replication, since NF-κB activation can lead to type I IFN production. Therefore, we used Vero cells which are known to be

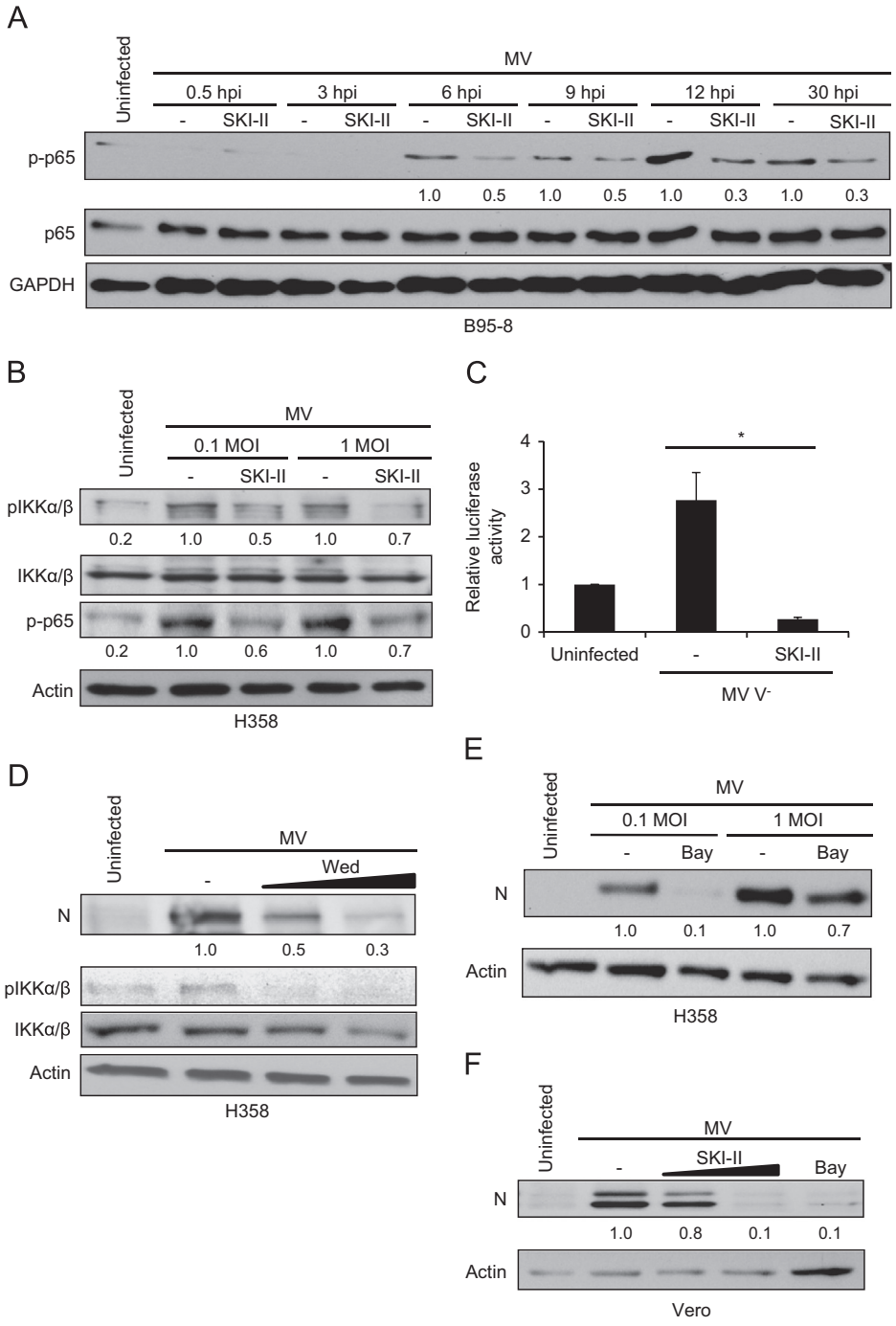

Fig. 5. MV-induced activation of NF-κB signaling, which is needed for efficient measles virus replication, is modulated by SK inhibition. (A) B95-8 cells were infected with MV at 0.5 MOI and were co-treated with SKI-II (10 μM) or its solvent (−). Cell lysates were harvested at various time points as indicated. Western blotting was performed to detect the p-p65 and p65 proteins. The level of p-p65 proteins in the solvent treated condition (−) was set to 1.0 at the time points shown. (B) H358 cells were infected with MV at 0.1 MOI or 1 MOI as indicated and were co-treated with solvent (−) or SKI-II (20 μM). At 1 dpi, cell lysates were harvested for Western blotting to detect p-p65, pIKKα/β, and IKKα/β levels. The levels of p-p65 and pIKKα/β in the solvent treated (−) condition were set to 1.0 for each MOI. (C) H358 cells were transfected with pGL3-NF-κB plasmid along with pRL-CMV control plasmid. One day later, cells were infected with 0.5 MOI of MV V⁻ and co-treated with SKI-II at 20 μM or the solvent (−). At 1 dpi, cells were analyzed for luciferase activity. Relative luciferase values are shown. Values represent mean ± SEM ($n=3$). *$p < 0.05$. (D) H358 cells were pre-treated with Wedelolactone at 50 and 75 μM or solvent (−) for 30 min followed by infection with MV at 0.1 MOI. At 1 dpi, Western blotting was performed to detect pIKKα/β, IKKα/β, and viral N protein levels. Relative intensities of each band of N protein are shown. (E) H358 cells were pre-treated with 20 μM Bay-11-7082 (Bay) or the solvent (−) for 30 min and then infected with MV at 0.1 MOI or 1 MOI. At 1 dpi, Western blotting was performed to detect N protein levels. The level of N proteins in the solvent treated condition (−) was set to 1.0 at each MOI. (F) Vero cells were infected with MV at 0.1 MOI and were either treated with SKI-II (25 μM or 50 μM) or Bay-11-7082 (20 μM) or the solvent (−). At 1 dpi, cell lysates were harvested to perform Western blot analysis to detect N protein. Relative intensities of each band of N protein are shown.

deficient in type I IFN production (Chew et al., 2009). Interestingly, SKI-II or Bay-mediated inhibition of MV replication was recapitulated in Vero cells (Fig. 5F), suggesting that the anti-viral effect observed upon SK inhibition does not mechanistically involve the production of type I IFN in itself. These results demonstrate that SK1-NF-κB pathway is needed for effective MV replication and this phenomenon is independent of the production of type I IFN.

Discussion

Our findings establish a pro-viral role of SK1 in the replication of MV. We show that overexpression of SK1 promotes efficient MV replication, whereas the enforced inhibition of SK1 activation/expression represses MV replication.

Several studies have suggested that the uptake of viruses into the cell can be influenced by the sphingolipid composition of the cell membrane (Chazal and Gerlier, 2003). For instance, MV interaction with DC-SIGN induces sphingomyelinase activation and ceramide generation, which enhances MV uptake into dendritic cells (Avota et al., 2011). In contrast, the entry of human immunodeficiency virus (HIV) (Finnegan et al., 2004) and hepatitis C virus (HCV) (Voisset et al., 2008) into cells was shown to be inhibited by ceramide. Since SK could regulate the balance of sphingolipid levels including ceramide, sphingosine, and S1P (Snook et al., 2006), it is conceivable that inhibition of SK enzymatic activity influences the uptake/entry step of MV into the cells. However, addition of the SK inhibitor even at 3 h after viral infection of the cells could potently inhibit MV replication (Fig. S2). This suggests that the inhibition of virus replication may not occur at the level of MV-interaction with the cellular receptor but presumably at a stage post MV entry. We have measured the level of viral RNAs to determine if the inhibition is observed at the level of viral RNA synthesis. However, there was no significant change in MV N RNA levels (both the positive and negative sense N RNAs) between SK inhibitor-treated cells and the solvent-treated control (data not shown). This suggests that SK inhibition might regulate MV replication at a post-transcriptional level. The exact point in MV life cycle where the inhibition is initiated remains to be further explored.

Recent findings for SK-mediated regulation of TNF-induced NF-κB signaling led us to hypothesize that SK inhibition regulates host NF-κB signal pathway upon MV infection. Indeed, our results suggest that the antiviral effect exhibited by SKI-II directly correlated with SK-mediated modulation of the NF-κB pathway. Importantly, we have shown that NF-κB pathway is critical for efficient MV replication (Fig. 5D and E). To the best of our knowledge, this is the first report to reveal the crucial role played by the NF-κB pathway in promoting MV replication. These findings provide a new mechanism of how MV uses the host cellular machinery to facilitate its own replication.

Several viruses are thought to incorporate/hijack the NF-κB pathway into their life cycle for their own benefits in replication/pathogenesis (Hiscott et al., 2001). One well-known example is influenza virus infection wherein NF-κB signaling plays a critical role in promoting influenza viral RNA synthesis (Kumar et al., 2008). However, NF-κB signaling could regulate anti-viral cellular processes including host protective immune responses (Hayden and Ghosh, 2012). For instance, MV RNA is sensed by cellular pattern recognition receptors (PRRs) such as RIG-I and MDA-5 (Ikegame et al., 2010), which could lead to NF-κB-mediated innate anti-viral immune responses by inducing the synthesis of pro-inflammatory cytokines and type I IFNs to control MV spread. In response to this host protective signaling, many viruses encode for proteins to block/antagonize this pathway so that they can establish a more productive infection in the cells. Some examples include influenza NS1 protein (Wang et al., 2000) and HCV core protein (Joo et al., 2005). Similarly, MV V protein was reported to inhibit NF-κB signaling by binding to the Rel homology domain (RHD) of the p65 subunit to disrupt its nuclear translocation (Schuhmann et al., 2011). Also, the P protein of MV suppresses NF-κB signaling by up-regulating A20, which is a negative regulator of NF-κB, to remain “immunologically silent” in monocytes but this phenomenon was not found in epithelial cells (Yokota et al., 2008). This report indicates that a cell type-dependent regulation of NF-κB pathway by MV may exist.

Thus, MV activates NF-κB to facilitate its replication, while MV seems to suppress activation of NF-κB signaling to some extent. It is possible that MV-induced activation of NF-κB signaling is required for the synthesis of viral proteins and then newly produced MV proteins suppress the anti-viral function of NF-κB. Therefore, an optimal level of NF-κB activation that facilitates MV replication seems to be maintained in the host cells. These observations suggest a dual role for NF-κB pathway during MV replication. MV might differentially regulate the cellular NF-κB signaling depending on various factors such as the time point of NF-κB activation during MV replication cycle, cell type specificity, and the extent of NF-κB signaling it activates. We have recently published that SK1 has pro-viral role in influenza virus replication and the mechanisms also involve SK-mediated regulation of NF-κB pathway, which is essential for influenza viral RNA synthesis (Seo et al., 2013). Also during HIV infection, NF-κB is known to directly bind to the HIV long terminal repeat to promote viral transcription and HIV gene expression (Kwon et al., 1998). The mechanistic details of how the NF-κB pathway aids MV replication remains to be further explored.

MV non-structural proteins V and C are known to be involved in subverting the host immune response, particularly by antagonizing the antiviral type I IFN system (Ramachandran and Horvath, 2009; Shaffer et al., 2003). Additionally, these proteins were shown to suppress the NF-κB pathway (McAllister et al., 2010; Schuhmann et al., 2011). However, V and C-deficient viruses still remained sensitive to SK inhibition. This finding leads us to speculate that V and C proteins might be dispensable for MV in utilizing SK1 as a pro-viral factor to aid its own replication. Furthermore, it is interesting to note that although NF-κB signaling can lead to type I IFN production, our results show that SK inhibition's anti-viral mode of action is independent of the production of type I IFN (Fig. 5F).

MV infection increases the activation of SK1 as well as the expression level of SK1 in B95-8 cells (Fig. 4). It is possible that the MV genome and/or viral protein(s) could trigger the activation of SK1. Indeed, LPS-mediated stimulation of its receptor TLR4 has been reported to activate SK1 (Pchejetski et al., 2011). Therefore, PRR-mediated sensing of MV components could lead to activation of SK1, which remains to be further explored. However, MV-induced phosphorylation of SK1 was not observed in H358 cells, suggesting that cell type-specific regulation may exist. Conceivably, B95-8 cells, but not H358 cells, efficiently produce cytokines such as TNF-α upon MV infection, which may contribute to the prominent activation of SK1. We and other investigators have reported that viruses regulate SK1 expression or activation. Respiratory syncytial virus (Monick et al., 2004) and influenza virus increase SK1 activation/expression (Seo et al., 2013), whereas bovine viral diarrhea virus (Yamane et al., 2009) and dengue virus (Wati et al., 2011) decrease SK1 activation. Thus, SK1 activation/inactivation is dependent on the type of viruses invading the host cells and seems to be an important regulatory event occurring in virus-infected cells.

The activation of SK1 that we observe following MV infection can result in the intracellular accumulation of S1P. The S1P thus generated is known to function in two possible manners (Spiegel and Milstien, 2011). It can be secreted out of the cells and then bind to canonical S1P receptors to trigger SIP receptor-mediated signaling events. The other way by which S1P functions is to act intracellularly as a second messenger. Indeed, recent studies have delineated the intracellular role of S1P, which is binding to TRAF2 and mediating TNF-induced NF-κB activation (Alvarez et al., 2010). In our experimental system, we find that, exogenously supplied S1P does not have any effect on MV replication (Fig. 1B). Therefore, SK1 enzyme mediated generation of intracellular S1P and the subsequent intracellular signaling events appear to regulate MV replication.

In this study, we have demonstrated that SK1 expression/activation is required for efficient MV replication and propagation. Further, our study has revealed a crucial role of the NF-κB pathway in MV replication and has established a link between SK1 and NF-κB pathway upon MV replication. Development of small animal models that manifest the clinical and biological aspects of a natural measles infection would allow us to determine the role of SK1 and NF-κB pathway in vivo. Collectively, our observations provide new insights into the MV-host interactions and further provide a basis for the development of novel therapeutic strategies to control MV infection.

Materials and methods

Viruses and cells

Edmonston MV (MV-Ed), the vaccine strain of MV (Hahm et al., 2007) and V or C-deficient MV (Patterson et al., 2000) were amplified on Vero cells. Wild type MV of the JW strain was amplified by passage on B95-8 cells as described previously (Hahm et al., 2003; Manchester et al., 2000). HEK293 cells and SK1 cells (Min et al., 2007) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech) as explained elsewhere. NCI-H358 cells (Human bronchioalveolar carcinoma cells) were purchased from ATCC and were maintained in RPMI 1640 medium supplemented with D-glucose to a final concentration of 4.5 g/L, 1% HEPES, 1% sodium pyruvate, and 1% L-glutamine. B95-8 cells (Marmoset B-cell line) were maintained in RPMI 1640 medium (Mediatech). Vero cells were maintained in DMEM medium. Cells were cultured in a CO_2 incubator at 37 °C and all media were supplemented with 10% fetal bovine serum (HyClone) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Mediatech).

Western blot analysis

Specific antibodies against measles virus proteins namely nucleoprotein (N) and matrix protein (M), SK1, pSK1, pIKKαβ, IKKαβ, p65, p-p65, actin, and GAPDH were purchased from Cell Signaling Technology, Abcam, Santa Cruz Biotechnology, and ECM Biosciences. Total proteins were harvested using 2 × sample buffer containing β-mercapto-ethanol and after heat inactivation at 95 °C, equal amounts of protein samples were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels to resolve proteins and then transferred to nitrocellulose membrane (PROTRAN-NC, Whatman). The membrane bound antibodies were detected using Enhanced Chemiluminescence substrate solution (Thermo scientific). The relative intensities for each band were determined based on the control protein expression (Actin or GAPDH) by densitometry using Image J software and the values obtained are depicted below each blot. All the presented data were repeated at least twice with independent experimental settings.

Plaque assay

For titration of viruses, the cell lysates with the supernatants were subjected to 2 cycles of freezing and thawing to collect cell-associated MV along with the viruses released into the supernatant. Monolayers were prepared by seeding 4×10^5 Vero cells in 2 mL of growth medium on 6 well plates (Corning). After overnight incubation, monolayers were infected with the virus sample that was serially diluted in complete medium. Viruses were allowed to infect monolayers for 1 h at 37 °C, 5% CO_2, shaking plates every 15 min. After viruses were adsorbed, a 2 mL agarose-containing overlay was added to each well. After the overlay solidified, the plates were incubated for 5 days at 37 °C, 5% CO_2. On day 5, cells were fixed with 25% formalin and stained with crystal violet. The numbers of plaques in each well were counted and titers were calculated.

Inhibitors

To inhibit sphingosine kinase activity, SK-specific inhibitors SKI-II (Sigma-Aldrich) and N,N,-dimethylsphingosine (Cayman Chemical) were used. To inhibit NF-κB signaling, Bay-11–7082 (Sigma-Aldrich) or Wedelolactone (Sigma-Aldrich) were used. DMSO was used as solvent to prepare all of the inhibitors mentioned.

RNA interference

Small interfering RNAs (siRNA) targeting human SK1 (siSK1) was purchased from Thermo Scientific (ON TARGET plus SMART pool siRNA). Non-targeting scrambled RNA was purchased from Dharmacon and used as control. Cells were reverse transfected with 50 nM si-SK1 using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. At day 3 post-transfection, cells were split and transfected once again with siRNA. One day later, transfected cells were infected with MV and harvested at 2 dpi for analysis.

Sphingolipids

Sphingosine 1-phosphate (S1P) was purchased from Cayman Chemicals. 3% tissue culture grade-bovine serum albumin (BSA) (Sigma Aldrich) was used as solvent to dissolve S1P.

Flow cytometric analysis

For detection of measles virus proteins by flow cytometry, MV-infected B95-8 cells were incubated with a human polyclonal serum to MV (Hahm et al., 2003, 2007) which was used as the primary antibody. The cells were thoroughly washed and then stained with a PE-conjugated donkey anti-human IgG antibody (Jackson Immuno Research Laboratories). Data were collected by a CyAn ADP flow cytometer (Beckman Coulter) and were analyzed with FlowJo (Treestar) software. Similar result was obtained with two independent experiments.

NF-κB luciferase reporter assay

H358 cells were co-transfected with 100 ng of NF-κB promoter-luciferase reporter plasmid (pGL3-NF-κB) (Nakhaei et al., 2009) and 10 ng of Renilla luciferase plasmid (pRL-CMV) which was used as a transfection control. After 24 h, transfected cells were infected with 0.5 MOI of MV V^- and co-treated with SKI-II or its solvent. At one day post-infection, cells were lysed to measure Renilla and firefly luciferase activities using the dual luciferase reporter assay system (Promega), according to manufacturer's instructions. The averages were compared using a bidirectional, unpaired Student's *t*-test.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.11.039.

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