THE AMERICAN UNIVERSITY OF BEIRUT

A NOVEL ROLE FOR NEUTRAL SPHINGOMYELINASE DURING INFLUENZA A VIRUS REPLICATION

by

WAFAA BILAL AL SOUSSI

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BY

WAFAA BILAL AL SOUSSI

Approved by:

Dr. Hassan Zaraket, Assistant Professor — Advisor

Department of Experimental Pathology, Immunology, and Microbiology

Dr. Hiba El Hajj, Assistant Professor — Member of Committee

Departments of Internal Medicine and Experimental Pathology, Immunology, and Microbiology

Dr. Ghassan Dbaibo, Professor — Member of Committee

Departments of Pediatrics and Adolescent Medicine and Biochemistry

Dr. Margret Shirinian, Assistant Professor — Member of Committee

Department of Experimental Pathology, Immunology, and Microbiology

Date of thesis/dissertation defense: July 27, 2017

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Influenza A virus (IAV) is one of the most common causative agents of acute respiratory tract infections worldwide. It causes significant morbidity and mortality during its seasonal outbreaks and can occasionally cause pandemics. Current influenza antiviral drugs target viral proteins, which can quickly evolve resistance to existing drugs. Therefore, targeting host cell factors during infection is a promising antiviral approach as their genes are less prone for mutation, and thus the selection of resistance. Numerous studies have demonstrated the role of sphingolipids in various aspects of the life cycles of several viruses including attachment and fusion, intracellular transport, replication, and budding. Previous studies conducted in our laboratory have shown that IAV infection leads to increase in cellular ceramide. Knowing that neutral sphingomyelinase (nSMase) is a key player in stress-induced production of ceramide by hydrolyzing the membrane lipid sphingomyelin (SM), we hypothesized that it could play a role during IAV infection. Therefore, the aim of this study was to investigate the role of nSMase during IAV infection in A549 cells, human lung adenocarcinoma epithelial cells.

We first investigated if IAV induces ceramide accumulation at the plasma membrane and whether this accumulation is mediated by nSMase. This was addressed by assessing ceramide accumulation in cells infected with IAV in the presence or absence GW4869, a nSMase inhibitor, by using confocal microscopy. In addition, we measured virus replication upon inhibition of nSMase. Viral titers were determined using plaque assay. Further, we assayed the activity of nSMase in IAV-infected lung epithelial cells at different time points using Amplex® Red Sphingomyelinase assay kit. Our results indicate that IAV induces ceramide accumulation at the plasma membrane. Inhibition of nSMase prevented the accumulation of ceramide in infected cells, suggesting that ceramide increase is mediated by nSMase. Moreover, nSMase inhibition reduced IAV replication in A549 cells. Therefore, nSMase activity is required for efficient IAV replication. Notably, nSMase activity was not further enhanced due to IAV infection compared to uninfected cells. This indicates that basal activity of nSMase is sufficient and required for efficient IAV replication.

In this study, we show evidence of a novel role for nSMase during IAV replication. The inhibition of this pathway may provide new avenues for IAV antiviral drug development.

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INTRODUCTION

A. Influenza A virus (IAV)

1. Biology of influenza viruses

Influenza viruses comprise a major group of respiratory viral pathogens that continue to pose a significant threat to humans worldwide (Gaur, Munjal, & Lal, 2011). They belong to the *Orthomyxoviridae* family and are divided into four main genera (A, B, C and D). These genera are classified based on differences in two internal glycoproteins termed nucleoprotein (NP) and matrix (M1) and exhibit different degrees of antigenic variations, host range, and pathogenicity (Vemula et al., 2016).

Influenza A viruses (IAVs) possess high antigenic diversity and undergo faster evolution than the other three genera (Mak, Jayawardena, & Poon, 2012). IAVs are further classified based on genetic diversity of their viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (Vemula et al., 2016). Sixteen HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified (Taubenberger & Morens, 2010). Of these, three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes have caused human epidemics and/or pandemics since the last century (Bouvier & Palese, 2008; Mak et al., 2012).

2. Influenza burden

In spite of the availability of vaccines and antiviral medications, influenza, a respiratory disease caused by influenza viruses, still poses a significant toll on global morbidity and mortality (Fischer, Gong, Bhagwanjee, & Sevransky, 2014). Influenza A and B have been implicated in seasonal epidemics. The World Health Organization (WHO) estimates that each year influenza affects about 5 to 15% of the world's population causing about 250,000 to 500,000 deaths (Vemula et al., 2016). Occasionally and upon undergoing a major antigenic change, influenza viruses can cause pandemics (Mak, Jayawardena, and Poon 2012). The direct and indirect healthcare costs and the loss of productivity linked with influenza also result in huge economic losses on the global level as well (Mak et al., 2012).

Epidemics occur when point mutations accumulate during replication. Amino acid changes in the antigenic regions of the viral surface glycoprotein HA result in the phenomena known as "antigenic drift". These changes allow the viral strains to shun pre-existing host immunity causing seasonal outbreaks (Taubenberger & Morens, 2010). Influenza pandemics, however, happen at unpredictable intervals due to "antigenic shifts". These shifts in the antigenic properties of IAV occur when a virus acquires an antigenically novel HA of a new subtype through reassortment. This leads to high infection attack rates of volatile brutality (Mak et al., 2012; Taubenberger & Morens, 2010). Over the past hundred years, the world has witnessed four catastrophic pandemics starting with the 1918 Spanish flu, the 1957 Asian flu, the 1968 Hong Kong flu, and the novel 2009 H1N1 pandemic (H1N1pdm09). The most grievous pandemic among the above four is the mother of pandemics, the 1918 Spanish flu, which claimed more than

40 million lives and achieved a fatality rate of about 2.5% (Taubenberger & Morens, 2006).

3. IAV structure and genetic composition

IAVs are pleomorphic, forming spherical particles that are 100 nm in diameter (Figure 1) as well as elongated, filamentous particles reaching over 300 nm in length. They are studded with glycoprotein spikes, the HA and the NA, which protrude from a host cell-derived lipid envelope, along with a smaller number of matrix M2 ion channels. Underlying the envelope and its three projections is a matrix of M1 protein. Internal to M1, the nuclear export protein (NEP) or nonstructural protein 2 (NS2) and the ribonucleoprotein (RNP) complex are found. Ribonucleoproteins (RNPs) comprise the viral RNA molecules, RNA polymerase complex proteins, PB1, PB2 and PA, and multiple copies of the nucleoprotein (NP) (Bouvier & Palese, 2008).

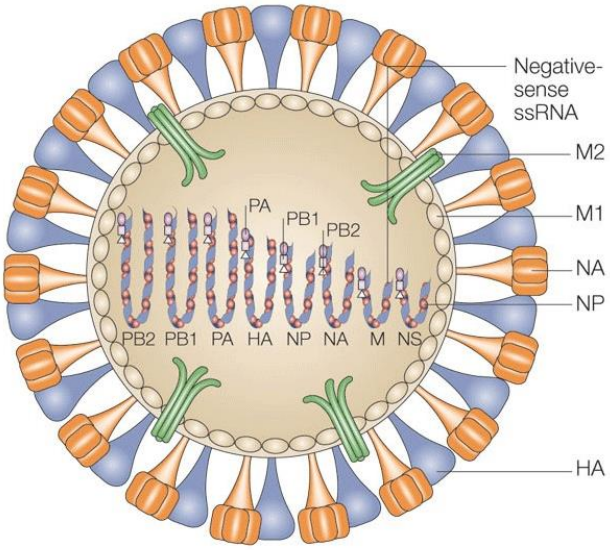


Figure 1. Structure and genetic makeup of influenza A virus (Horimoto & Kawaoka, 2005) IAV is an enveloped, negative-sense, single stranded RNA virus. The envelope encloses eight gene segments coated by multiple copies of a nucleoprotein (NP) and bound to a trimeric RNA-dependent RNA polymerase complex. The NP and the components of the polymerase complex are expressed each from its respective segment. The hemagglutinin (HA) segment encodes the receptor-binding protein HA, and the neuraminidase (NA) segment encodes the sialic acid-cleaving protein NA. The matrix segment encodes the matrix M1 protein and the proton-selective ion channel M2. In addition, non-structural proteins NS1 and NS2 are expressed from the non-structural segment.

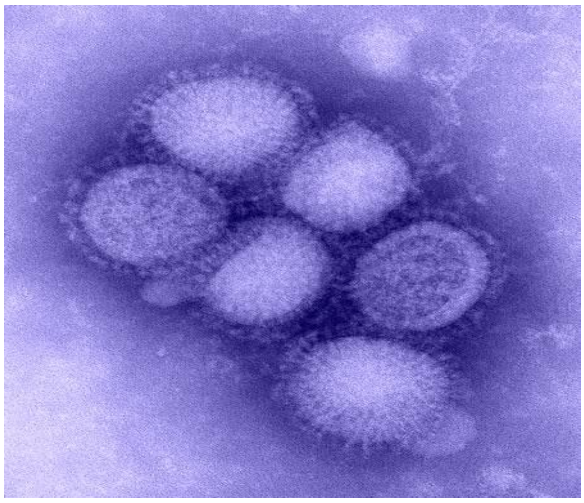


Figure 2. Electron micrograph of influenza A virus ("CDC H1N1 Flu | Images of the H1N1 Influenza Virus," n.d.) A characteristic feature of IAV is two types of glycoprotein spikes projecting from the lipid membrane of the viral envelope: the hemagglutinin (HA) and the neuraminidase (NA).

The viral genome lying within the virus interior comprises eight negative-sense, single stranded viral RNA (vRNA) segments. These segments are numbered in decreasing order of length and encode for at least 11 functionally essential proteins (Table 1). (Bouvier & Palese, 2008; König et al., 2010; Taubenberger & Kash, 2010).

Table 1. Influenza A virus proteins and their functions (adapted from Rossman & Lamb, 2011)

Genomic Segment	**Encoded protein(s)**	**Function**
1	PB2	RNA polymerase subunit
2	PB1 PB1-F2	RNA polymerase subunit Induction of host-cell apoptosis
3	PA	RNA polymerase subunit
4	HA	Surface glycoprotein, antigenic determinant, receptor binding and membrane fusion activity
5	NP	RNA-binding protein, nuclear import regulation
6	NA	Surface glycoprotein, enzymatic cleavage of sialic acid, release of progeny virions
7	M1 M2	Matrix protein, bridging interactions between the viral envelope and the RNP core Proton-selective ion channel, virus uncoating, virus assembly and budding
8	NS1 NS2 (NEP)	Multi-functional protein, major role in evasion of the host immune system Export of newly synthesized viral RNPs

4. IAV replication cycle

Influenza virus replication cycle is summarized in figure 3. The replication cycle includes 5 major steps: attachment, endosomal uptake and fusion, replication, and assembly and budding.

a. Virus attachment

The IAV replication cycle is initiated by the recognition of N-acetylneuraminic (sialic) acid on the host cell surface (Ramos and Fernandez-Sesma 2012). The sialic acid moieties, commonly encountered at the termini of glycoconjugates, are recognized and bound by the viral HA spikes. The HA proteins of human IAVs have a preferential specificity for α-2,6-linkages, whereas those of avian IAVs preferentially recognize α-2,3-linkages. (Couceiro, Paulson, & Baum, 1993; van Riel et al., 2007).

b. Virus entry

Following attachment of the IAV by multiple copies of the HA protein, the virus is uptaken by receptor-mediated endocytosis. First, acidification of the endosomal compartment triggers conformational changes in the HA molecules exposing a fusion peptide. This peptide inserts itself into the endosomal membrane and mediates the formation of a fusion pore upon fusing the host and virus membranes. Second, the acidic environment of the endosome activates the M2 ion channels allowing entry of hydrogen ions into the viral particle. Consequently, acidification of the viral core disrupts protein-

protein interactions and promotes the release of RNPs from the viral matrix M1 through the fusion pore into the host cell cytoplasm (Bouvier & Palese, 2008; Samji, 2009).

c. Synthesis of viral RNA

Transcription and replication of virus genome take place in the nucleus. After uncoating, the vRNPs are trafficked to the host cell nucleus by means of nuclear localization signals (NLSs). These NLSs are found within the proteins that make up the vRNPs, with NP established as the major factor in vRNP import (Cros & Palese, 2003; Samji, 2009). The incoming vRNPs interact with karyopherins, cellular proteins involved in nuclear import, through NP, and are finally trafficked through the nuclear membrane at specific locations called the nuclear pore complexes (NPCs) (Cros & Palese, 2003).

All influenza virus RNA synthesis occurs in the nucleus. The viral RNA-dependent RNA polymerase, which is imported into the nucleus as part of the RNPs, utilizes the negative-sense strands of RNA as a template to produce two positive-sense species: messenger RNA (mRNA) that is subsequently used for protein synthesis, and complementary RNA (cRNA) which is eventually transcribed into more copies of genomic vRNA (Bouvier & Palese 2008). In either case, the PB1 subunit catalyzes RNA synthesis within its internal active site cavity (Pflug, Lukarska, Resa-Infante, Reich, & Cusack, n.d.).

Transcription is initiated by a process called “cap-snatching”. The viral RNA polymerase binds to the 5’ methylated caps located at the ends of precursors of cellular mRNAs and “snatches” 10 to 15 nucleotides 3’ to the cap structure. This capped RNA fragment serves as a primer for viral mRNA synthesis. Unlike host cell mRNA, which is

polyadenylated by a specific polymerase, viral mRNA is polyadenylated through the transcription of a stretch of 5 to 7 uracil residues in the negative-sense vRNA into a string of adenosines that form the poly-A tail (Samji, 2009). Once polyadenylated and capped, viral mRNA can be exported out of the nucleus and translated like host mRNA. Nuclear export is mediated by M1 and NS2 viral proteins (Bouvier & Palese, 2008).

d. Synthesis of viral proteins

Viral proteins are synthesized on ribosomes into the endoplasmic reticulum, where they undergo proper folding. Subsequently, proteins are trafficked to the Golgi apparatus for post-translational modification (Bouvier & Palese, 2008). When the newly made proteins and genetic material are produced, all three envelope proteins assemble on the plasma membrane, whereas non-envelope proteins locate themselves just beneath the modified membrane for packaging (Van-Tam & Sellwood, 2009).

e. Packaging of RNA and virus assembly

An infective viral particle contains all eight RNA segments. Recent evidence suggests a selective mechanism for vRNA recruitment into virions, by which specific structures ensure the incorporation of all eight segments into the majority of virus particles (Fujii, Goto, Watanabe, Yoshida, & Kawaoka, 2003).

f. Virus budding and release

IAV budding takes place at the plasma membrane, possibly initiated by the building up of M1 matrix protein underneath the lipid bilayer. When budding is

complete, virus particles are actively released by the sialidase activity of the NA protein. The latter splits off sialic acid on cell-surface glycoproteins, which holds the HA of the newly synthesized virions, and thus aids in their final release (Bouvier & Palese, 2008).

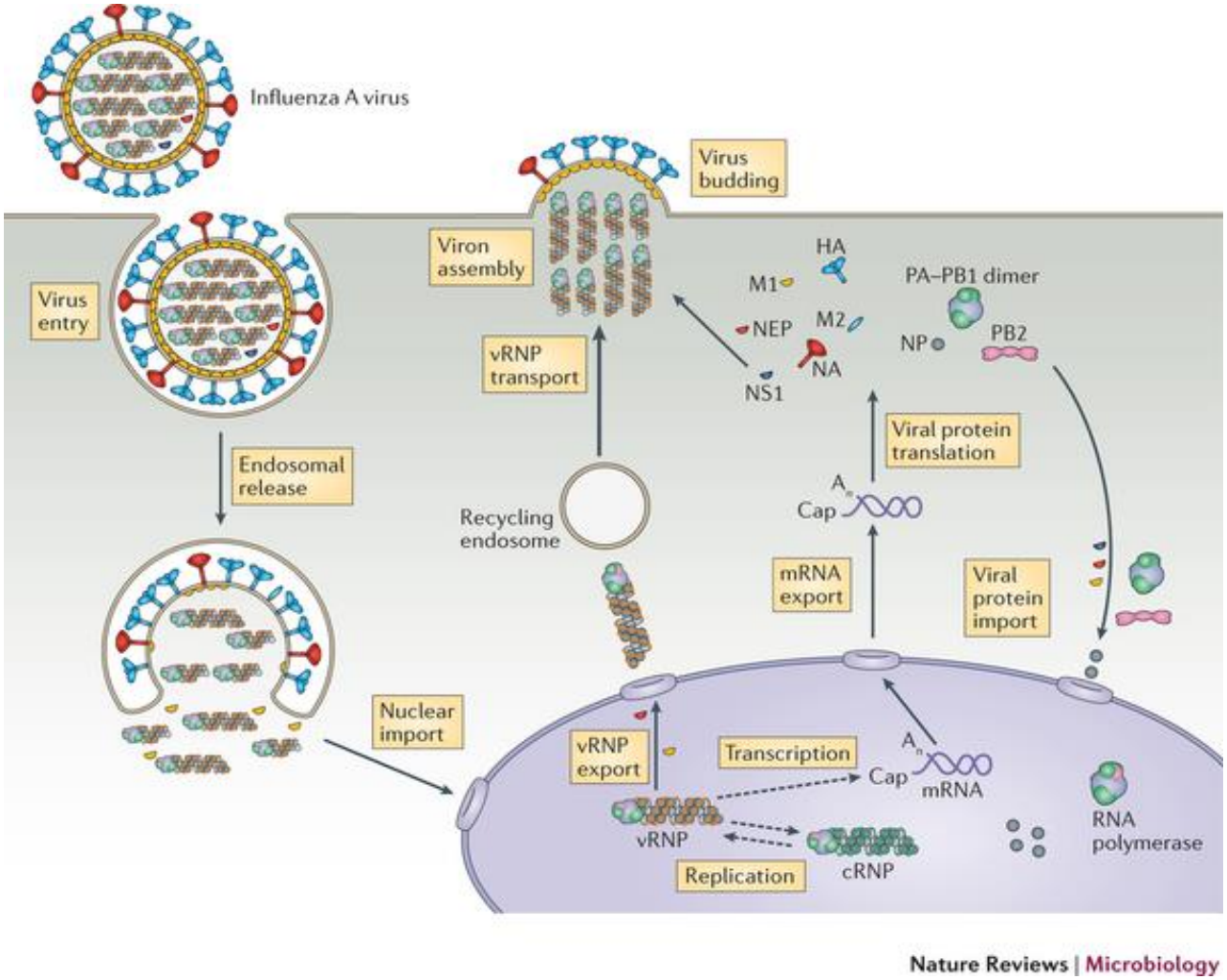


Figure 3. Influenza A virus life cycle (Velthuis & Fodor, 2016) The IAV replication cycle is initiated when the viral hemagglutinin (HA) attaches to sialic acid residues on the host cell surface, and the virus is endocytosed. Acidification of the endosomal compartment triggers both, conformational changes in the hemagglutinin (HA) molecules mediating the fusion of virus and endosomal membranes, and activation of the M2 ion channels allowing entry of hydrogen ions into the virus particle, facilitating the export of ribonucleoproteins (RNPs) into the cytoplasm. Afterwards, RNPs are trafficked to the nucleus, the site of all viral RNA synthesis. Once processed, viral mRNA is exported to the cytoplasm and translated like host mRNA. Finally, viral proteins and genome assemble into progeny viruses that bud at the plasma membrane.

5. Reservoirs, reassortment and host tropism

Except for the recently discovered novel bat influenza viruses (Tong et al., 2012, 2013), all type A viruses are believed to have evolved from an aquatic avian reservoir but can infect a wide range of warm-blooded animals as well (Taubenberger & Morens, 2010; Webster, Bean, Gorman, Chambers, & Kawaoka, 1992).

Reassortment has been shown to play a key role in IAV evolution (Dugan et al., 2008). Owing to its segmented genome, co-infection of one host cell with two different IAVs can lead to genome segment exchange and result in progeny viruses enclosing segments of both parental ones. Pandemic viruses can emerge as an outcome of reassortment. For example, the H1N1pdm09 virus emerged as a result of reassortment events involving avian, swine and human influenza viruses (Mak et al., 2012).

Viral attachment to the host cell is a major restriction factor that prevents avian influenza from infecting humans. The majorities of avian viruses replicates either poorly or are even non-infectious to humans. In general, avian influenza viruses preferentially bind to α-2,3-linked sialic acids, which are common in epithelial cells in the intestine of avian species (Ito & Kawaoka, 2000; Rogers & Paulson, 1983; Rogers, Pritchett, Lane, & Paulson, 1983), whereas human influenza viruses efficiently bind to α-2,6-linked sialic acids, which are predominant in epithelial cells in the human respiratory tract (Couceiro et al., 1993; Shinya et al., 2006; van Riel et al., 2007). Interestingly, both avian and human influenza virus receptors are present in pigs, which may explain why pigs serve as

"mixing vessels" for the co-infection, replication, and reassortment among avian, human, and swine viruses (Ma, Kahn, & Richt, 2008).

6. Pathogenesis and immunity

IAV can be transmitted either by direct contact or aerosol (Milton, Fabian, Cowling, Grantham, & McDevitt, 2013). Once deposited in the respiratory tract, the virus gains access to the respiratory epithelium by breaking down mucins in respiratory tract secretions (Matrosovich, Matrosovich, Gray, Roberts, & Klenk, 2004). Afterward, replication is initiated, and the viral progeny is shed. Virus replication continues, leaving in its wake destroyed cells and desquamated superficial mucosa, until being controlled and cleared by the host immune system (Van-Tam & Sellwood, 2009).

The first line of defense against influenza is formed by the innate immune system which encompasses physical barriers and cellular responses. In case the virus overcomes physical barriers in the airways, epithelial cells can sense infection and initiate an antiviral response that eventually results in the release of pro-inflammatory cytokines and recruitment of white blood cells (van de Sandt, Kreijtz, & Rimmelzwaan, 2012). These will take up, destroy, and process the virus antigens and initiate the adaptive immune system (van de Sandt et al., 2012).

In an uncomplicated case, the resulting edema and mononuclear cell infiltration of the invaded tissue contribute to symptoms including nonproductive cough, sore throat, and nasal discharge. These are accompanied by systemic symptoms like fever, muscle aches, malaise general fatigue (Taubenberger & Morens, 2008).

7. Complications of infection

The clinical course of influenza can result in severe illnesses and complications in high risk populations such as infants, elderly, and those with underlying chronic conditions. Complications may include primary viral or secondary bacterial pneumonia, hemorrhagic bronchitis, and cardiovascular events which may be severe enough to cause death (Taubenberger & Morens, 2008).

8. Vaccination and treatment

Annual vaccination is an effective approach to prevent seasonal influenza (Havers, Fry, Peacock, & Finelli, 2014). However, vaccines are only effective when they match the predicted prevailing strain and hence need to be updated regularly (Vries, Altenburg, & Rimmelzwaan, 2015; Wiersma, Rimmelzwaan, & de Vries, 2015). This is not very practical and affords poor or no protection in case the vaccine strain was miss-matched due to the continuous emergence of drift variants among circulating influenza strains. Furthermore, seasonal influenza vaccines fail to confer protection against novel pandemic influenza viruses (Vries et al., 2015). Currently used influenza vaccines primarily aim at the production of antibodies against the HA surface glycoprotein and to a lesser extent, the NA glycoprotein (Fiore, Bridges, & Cox, 2009).

Antiviral therapy is available for both prophylaxis and treatment of influenza (Kamali & Holodniy, 2013; Vries et al., 2015). Currently, two classes of antiviral medications are available for treatment: neuraminidase inhibitors (NAIs) and adamantanes or ion-channel blockers (Kamali & Holodniy, 2013). However, the high rate

of resistance to the latter class rendered them ineffective and thus not recommended for clinical use (Dong et al., 2015). The majority of currently circulating influenza strains remain susceptible to NAIs despite the detection of sporadic cases or outbreaks of NAI-resistant viruses. Other promising targets are currently being investigated including the influenza RNA polymerase and the nucleoprotein (NP) (Kamali & Holodniy, 2013). Because viral targets are prone to frequent mutations and potential selection of resistant mutations, host factors involved in virus replication are being investigated as potential antiviral targets.

B. Sphingolipids

1. Composition and architecture of the plasma membrane

The plasma membrane of the cell defines its periphery, having the central function of separating the inner cellular contents from the surrounding. However, it also has crucial roles in communication processes such as subcellular trafficking, signal transduction, and metabolic exchange. Like all the membranes, the plasma membrane is constructed of a basic structural element, the lipid bilayer, with proteins protruding on both sides (Milhas, Clarke, & Hannun, 2010).

Plasma membrane lipids are asymmetrically distributed within the bilayer such that the compositions of the extracytosolic and cytosolic leaflets noticeably differ. The outer leaflet is shown to be enriched with choline-containing lipids (phosphatidylcholine and sphingomyelin) and glycosphingolipids, whereas the inner one is abundant in phosphoinositides, phosphatidylserine, phosphatidylethanolamine and phosphatidic acid

(Ikeda, Kihara, & Igarashi, 2006). Accordingly, metabolism of lipids within each leaflet may have different physiological consequences (Milhas et al., 2010).

Although the plasma membrane is primarily considered as a fluid-mosaic membrane (Singer & Nicolson, 1972), it was demonstrated that clusters of lipids in an ordered state do exist in the generally disordered milieu of the membrane. These clusters of lipids or membrane microdomains are termed "lipid rafts" (Pike, 2009).

2. Lipid rafts

Lipid rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes (Pike, 2006). These rafts form from the tight hydrophobic interactions between their sphingolipid and cholesterol molecules (Figure 4). Sphingolipids associate laterally with one another primarily through hydrophobic interactions between their side chains, whereas cholesterol plugs any voids between the associated sphingolipids (Cremesti, Goni, & Kolesnick, 2002).

Lipid analysis has revealed that cholesterol levels in rafts are generally double those found in other regions of the plasma membrane (Pike, 2009). Likewise, up to 70% of the total cellular sphingomyelin (SM), the most prevalent membrane sphingolipid, resides in rafts (Cremesti et al., 2002).

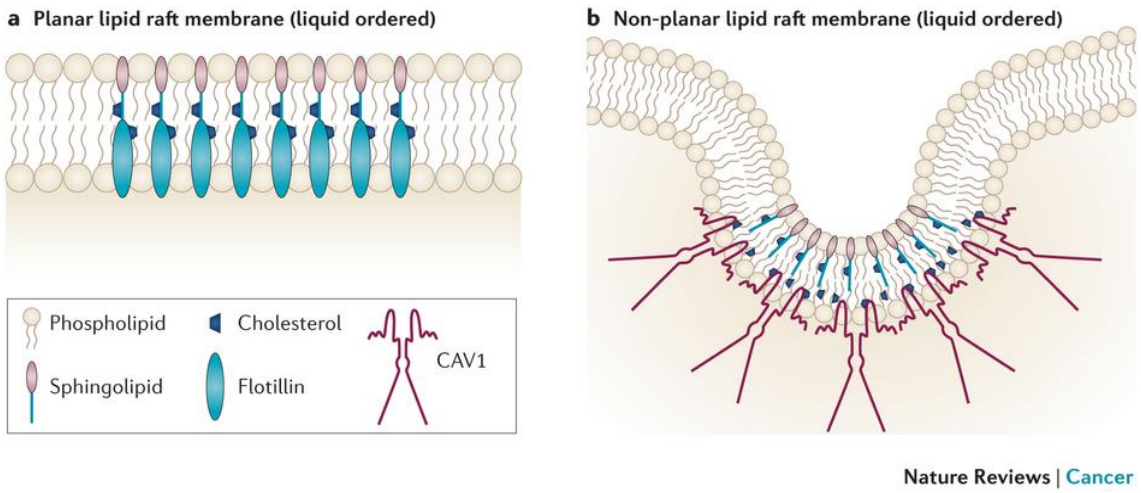


Figure 4. Planar and non-planar lipid raft microdomains (Martinez-Outschoorn, Sotgia, & Lisanti, 2015) Two types of lipid rafts have been proposed: planar lipid rafts and non-planar lipid rafts. Planar lipid rafts are continuous with the plasma membrane and contain flotillin proteins. On the other hand, non-planar rafts are inward folding of the plasma membrane that contain caveolin proteins.

3. Sphingomyelin

SM is a major component of biological membranes in eukaryotic cells (Slotte & Ramstedt, 2007). SM is comprised of a highly hydrophobic ceramide backbone and a hydrophilic phosphorylcholine head group (Zhang, Li, Becker, & Gulbins, 2009).It is synthesized on the luminal side of the plasma membrane or the Golgi apparatus. Accordingly, SM resides predominantly to anticytoplasmic leaflets of the cell membrane and intracellular vesicles, which helps in the generation of bilateral asymmetry and membrane microdomains (Zhang et al., 2009).

SM is synthesized by the action of sphingomyelin synthases (SMS), which catalyze the transfer of a phosphorylcholine headgroup from phosphatidylcholine to ceramide. At least two members of this family exist in most mammalian species:

sphingomyelin synthase 1 (SMS1), which is present in the trans-Golgi, and sphingomyelin synthase 2 (SMS2), which is localized to both the trans-Golgi and the plasma membrane (Gault, Obeid, & Hannun, 2010).

Not only is it a constituent of lipid rafts, but SM can be broken down through the hydrolysis of the phosphorylcholine head group by the sphingomyelinase (SMase) family to produce ceramide, a bioactive lipid in its own right (Gault et al., 2010; Milhas et al., 2010). The release of ceramide results in the formation of small ceramide-enriched membrane domains and thus alters the biophysical characteristics of membranes (Zhang et al., 2009).

4. Ceramide

Ceramide is a sphingolipid that has been recognized as an important signaling molecule, shown to mediate vital cell processes as diverse as cycle arrest, apoptosis, senescence, and stress responses (Kitatani, Idkowiak-Baldys, & Hannun, 2008).

a. Ceramide structure

Structurally, ceramide comprises a fatty acid of variable chain length bound via an amide linkage to a sphingoid base (Figure 5). In mammalian cells, the fatty acid chain length generally varies from 16 to 26 carbons. These fatty acids can be either saturated or monounsaturated, and may sometimes possess a hydroxyl group linked either to C2 (α-hyrdroxy fatty acids) or to the terminal carbon atom (ω-hydroxy fatty acids) (Cremesti et al., 2002).

Ceramide is the structural parent of all sphingolipids. Derivatives of ceramide differ in their head groups. For instance, SM contains phosphocholine as its polar head group, whereas cerebrosides have a single sugar connected to the ceramide moiety. (Boyle, 2005).

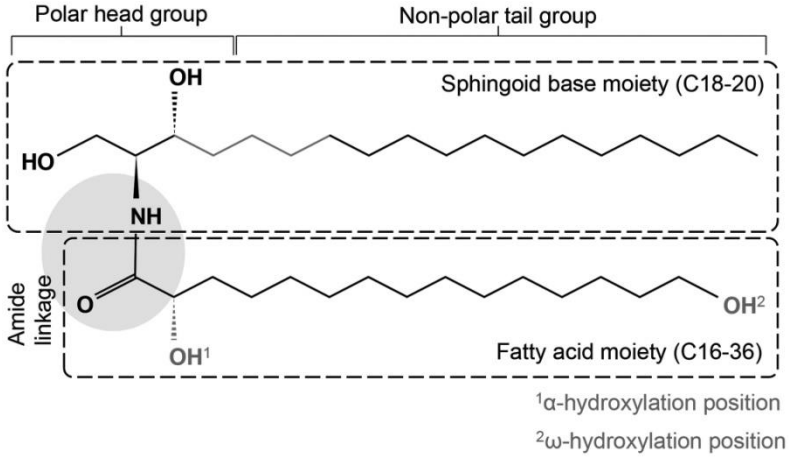


Figure 5. Basic chemical structure of ceramides (Cha et al., 2016) Ceramide is composed of a long-chain sphingoid base bound via an amide linkage to a fatty acid which is either saturated or unsaturated. The fatty acid may sometimes possess a hydroxyl group linked either to C2 or to the terminal carbon atom.

b. Biological implications of ceramide

Ceramide is known to play a key role in the signal transduction of a variety of extracellular stimuli that lead to diverse responses including cell differentiation, cell cycle arrest, and apoptotic cell death (Hannun, 1996). This is most probably a result of ceramide engaging different molecular targets. These include kinases, phosphatases, oncogenes, and transcription factors (Ballou, Lauledeerkind, Rosloniec, & Raghow, 1996; Hannun, 1996; Mathias, Peña, & Kolesnick, 1998; Riboni, Viani, Bassi, Prinetti, & Tettamanti, 1997). The effect of ceramide on cell cycle arrest is mediated by the dephosphorylation of the retinoblastoma (Rb) protein, which consequently activates the

growth suppressor pathway (Dbaibo et al., 1995; Jayadev et al., 1995). Moreover, ceramide can act as a second messenger in activating the apoptotic cascade (Dbaibo, Obeid, & Hannun, 1993; Obeid, Linardic, Karolak, & Hannun, 1993). Among the best characterized ceramide targets are ceramide- activated protein phosphatases (CAPPs), which include protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) (Chalfant et al., 1999). PP1 is a serine/threonine protein phosphatase responsible for the dephosphorylation of serine/arginine- rich proteins including splicing proteins (Chalfant et al., 2002). Precisely, ceramide induces PP1-mediated dephosphorylation of splicing proteins leading to the generation of the pro-apoptotic splice variants, Bcl-x(s) and caspase 9 (Chalfant et al., 2002). On the other hand, PP2A contributes to the mitochondrial signaling pathway by dephosphorylating anti-apoptotic proteins such as BCL-2 and Bad (Dobrowsky, Kamibayashi, Mumby, & Hannun, 1993; Ruvolo, Deng, Ito, Carr, & May, 1999). As a result, ceramide- induced PP2A activity leads to cytochrome c release and subsequent cell death (Ruvolo et al., 1999).

Ceramide has been also shown to be a major modulator of membrane domains. Ceramide molecules spontaneously self-associate and give rise to ceramide-enriched membrane microdomains. The latter are able to fuse with one or a few ceramide-enriched membrane macrodomains, so called platforms (Holopainen, Subramanian, & Kinnunen, 1998). Generally, ceramide-enriched membrane platforms mediate receptor reorganization and recruitment of intracellular signaling molecules in response to various stimuli. In this regard, ceramide domains can thus function in multiple signaling pathways rather than being part of a specific signaling cascade and enable receptors to effectively transmit the activation signal (Bollinger, Teichgräber, & Gulbins, 2005).

c. Ceramide production

Three major pathways account for ceramide production within a cell (Figure 6). These are the *de novo* synthesis, salvage, and the SM hydrolysis pathways (Bikman & Summers, 2011; Kitatani et al., 2008).

The *de novo* synthesis occurs at the cytosolic leaflet of the ER where a series of enzymatic reactions generate ceramide from non-sphingolipid precursors. Through the sequential action of serine palmitoyltransferase, 3-ketodihydrosphingosine reductase, and (dihydro) ceramide synthase, cytosolic serine and palmitoyl CoA molecules are converted into dihydroceramide. Following its generation, dihydroceramide is acted on by dihyroceramide desaturase, which introduces a trans double bond and thus converts this transient intermediate into a biologically active ceramide (Gault et al., 2010).

In the salvage pathway, complex sphingolipids are required as an initial supply of ceramide. Through a series of events, higher-order sphingolipids are broken down eventually into sphingosine. The latter is then reused through reacylation to generate ceramide (Kitatani et al., 2008).

Ceramide can be also generated from hydrolysis of the phosphorylcholine head group from SM through the action of a small family of SMase enzymes. SM is the most prevalent sphingolipid and is thus an important source of ceramide (Bikman & Summers, 2011).

The biological effects of ceramide can be controlled by its subcellular localization. For instance, ceramide generated at the plasma membrane triggers pathways associated with growth arrest, oxidative stress-mediated cell death, and lipid raft function, whereas that generated in the mitochondria is involved in the regulation of processes leading to intrinsic cell death (Saddoughi, Song, & Ogretmen, 2008).

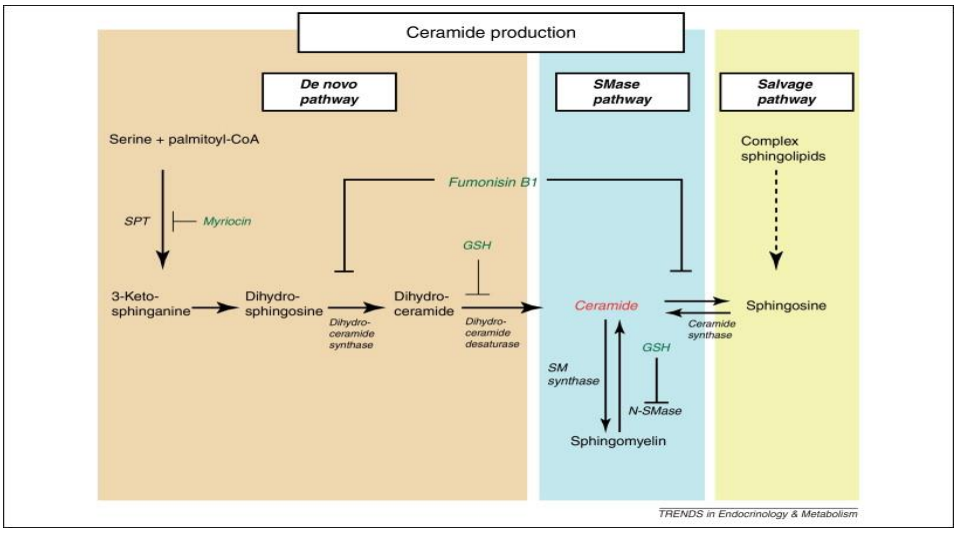


Figure 6. Cellular pathways involved in the production of ceramide (Pagadala, Kasumov, McCullough, Zein, & Kirwan, 2012) Ceramide can be produced by three pathways. *de novo* ceramide synthesis relies on the presence of serine and palmitoyl-CoA, while sphingomyelin (SM) hydrolysis and the salvage pathway rely on the presence of an initial supply of ceramide precursors. *de novo* ceramide synthesis begins with the condensation of serine and palmitoyl-CoA by serine palmitoyl transferase (SPT) yielding 3-ketosphinganine, which is then reduced and acetylated into dihydroceramide. The latter is is subsequently desaturated by dihydroceramide desaturase to form ceramide. In the sphingomyelinase (SMase) pathway, ceramide is generated from the hydrolysis of sphingomyelin (SM).

5. Sphingomyelinases

Sphingomyelinases (SMases) are enzymes that catalyze the breakdown of SM into ceramide and phosphorylcholine, which among other functions lead to altered membrane properties. (Goñi & Alonso, 2002). To date, a number of SMases have been

identified (Samet (Glick) & Barenholz, 1999). These are classified into three types based on their pH optimum (Table 2) (Samet (Glick) & Barenholz, 1999).

a. Acid sphingomyelinases

Acid sphingomyelinase (aSMase) was the first SMase to be de described in mammalian cells (Barnholz, Roitman, & Gatt, 1966). It is a soluble enzyme that catalyzes the hydrolysis of SM to ceramide at an optimum pH of 5, suiting its localization in lysosomes (Jenkins et al., 2011). The absence of aSMase is responsible for Niemann-Pick disease, a lysosomal storage disorder characterized at the cellular level by the buildup of SM within lysosomes (Schuchman, 2007). Interest in aSMase expanded beyond its role in lysosomal SM turnover with the discovery that cells from aSMase-deficient mice resisted apoptosis in response to various stimuli (Santana et al., 1996). Subsequently, aSMase was suggested to be involved in the signal transduction pathway and a variety of stress responses (Jenkins, Canals, & Hannun, 2009; Smith & Schuchman, 2008). aSMase is the most extensively studied and best characterized of all types, including sequence and cloning (Schuchman, Levran, Pereira, & Desnick, 1992).

sSMase enzyme arises from the aSMase gene (*SMPD1*) via alternative trafficking of a single protein precursor. This precursor can be targeted either to the lysosomal compartment (aSMase) or to the Golgi secretory pathway (sSMase) (Jenkins et al., 2011). sSmase, unlike aSMase, is activated by physiologic levels of Zn^{2+}, although the sSMase from endothelial cells is partly Zn^{2+}-independent. It functions best at acidic pH but can also hydrolyze certain substrates, such as atherogenic lipoproteins, at neutral pH. sSmase has been proposed to have a role in cardiovascular pathophysiology (Tabas, 1999).

b. Alkaline sphingomyelinases

Alkaline sphingomyelinase (alkSMase) is present in the intestinal tract and human bile. It is responsible for digestion of dietary SM and for hydrolysis of SM in the mucosal membrane. In contrast to other types of SMase, this enzyme is known for its tissue specific expression, bile salt dependency, divalent ion independency, and trypsin resistance (Duan, 2006).

c. Neutral sphingomyelinases

Neutral sphingomyelinase (nSMase) catalyzes the hydrolysis of SM into ceramide at a neutral pH optima (7.4). Notably, nSMase exists as two enzymatic forms: a magnesium (Mg^{2+})-dependent and a Mg^{2+}-independent (Levade & Jaffrézou, 1999).

Mg^{2+}-dependent nSMase is a membrane-associated protein in mammals. The enzyme requires millimolar concentrations of divalent cations (Mg^{2+} or Mn^{2+}) for optimal activity. It is generally expressed in all mammalian tissues, but the highest expression is detected in the brain (Goñi & Alonso, 2002). The molecular mass is about 92 kDa, although smaller isoenzymes, possibly proteolytic products, are found. Mg^{2+}-dependent nSMase is activated by several stimuli, including oxidative stress, pro-inflammatory stimuli, amyloid beta (Aβ), radiation, chemotherapeutic agents, and pathogens (Barth, Gustafson, & Kuhn, 2012; Clarke, Cloessner, Roddy, & Hannun, 2011; Clarke & Hannun, 2006; Karakashian, Giltiay, Smith, & Nikolova-Karakashian, 2004).

Mg^{2+}-dependent nSMase has been implicated in several biological processes including apoptosis, cell growth, and inflammation (Clarke & Hannun, 2006).

Additionally, a role for nSmase has been revealed in cardiac failure, pulmonary pathophysiology, and neurologic disorders. Furthermore, studies from knockout mice have provided strong evidence for a role of nSMase in maturation or development of bones as these show embryonic dwarfism (Stoffel, Jenke, Blöck, Zumbansen, & Koebke, 2005). Several lines of evidence have also indicated that nSMase plays a key role in the infection of mammalian cells with microbial pathogens (Jan, Chatterjee, & Griffin, 2000; Jana & Pahan, 2004; Mueller, Avota, Collenburg, Grassmé, & Schneider-Schaulies, 2014).

Mg^{2+}-independent nSMase is poorly known. It is exemplified by the cytosolic isoform identified in HL-60 cells and the 53 kDa isoform identified in rabbit skeletal muscle (Ghosh, Sabbadini, & Chatterjee, 1998; Okazaki, Bielawska, Domae, Bell, & Hannun, 1994).

Table 2. Summary of the properties of the SMase family

	Acidic		**Alkaline**	**Neutral**	
	aSMase	sSMase	alkSMase	Mg^{2+}-dependent nSMase	Mg^{2+}-independent nSMase
Localization	lysosome	Golgi secretory pathway	Intestinal tract and human bile	Membrane-associated	Poorly known
Ion dependency	none	Zn^{2+}	none	Mg^{2+}(or Mn^{2+})	none
Function	Several biological processes including signal transduction and stress responses	Cardiovascular pathophysiology	Digestion of dietary and mucosal membrane SM/anti-proliferative and anti-inflammatory roles	Several biological processes including apoptosis, cell growth, and inflammation	Poorly known
Lack of enzyme	Niemann-Pick disease	Defective cholesterol trafficking and efflux	Colon carcinogenesis	Severe chondrodysplasia and dwarfism	Poorly known

C. Viral infections and sphingolipids

Viruses are obligatory intracellular pathogens. Accordingly, viruses cross plasma membranes at least twice, for cell entry and exit. Furthermore, a number of viruses exploit membranes during genome replication and assembly (Lorizate & Kräusslich, 2011). Sphingolipids are major plasma membrane constituents and, as such, their local segregation and metabolites are tightly linked to all membrane-involving processes. They directly affect biophysical properties of membranes altering membrane curvature, fusogenicity, vesiculation, as well, as signal transduction (Figure 7) (Schneider-Schaulies & Schneider-Schaulies, 2015).

Lipid rafts, membrane microdomains enriched in cholesterol and sphingolipids, are known to be involved in various cellular events such as synthetic traffic, endocytic traffic, and signal transduction. Thus, it is logical that rafts are involved in the virus entry, assembly, or/and budding processes during virus life cycles (Chazal & Gerlier, 2003).

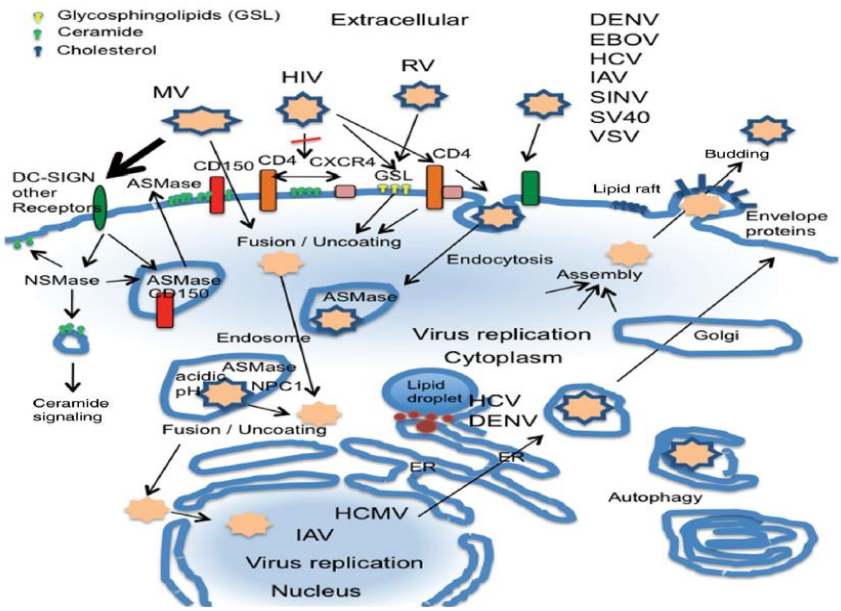


Figure 7. Membrane and components involved in virus replication (Schneider-Schaulies & Schneider-Schaulies, 2015). A number of viruses such as Measles virus (MV), Human immunodeficiency virus (HIV), Rhinovirus (RV), Dengue virus (DENV), Sindbis virus (SV) and Ebolavirus (EBOV) activate acid and/or neutral sphingomyelinase, which alter membrane lipid composition and subsequently influence virus uptake. Glycolipids can also function as receptors for the uptake of several viruses such as HIV and RV. In addition, lipid droplets and endoplasmic reticulum (ER) membrane are crucial for replication and assembly of Hepatitis C virus (HCV) and DENV. Herpes virus uses nuclear and ER membranes for efficient intracellular budding prior its transfer to the host-cell surface.

1. IAV and lipid rafts

The involvement of lipid rafts in virus entry, assembly, and budding are demonstrated on the effects of raft-disrupting reagents and the detection of viral proteins and viral cellular receptors in the replication process (Takahashi & Suzuki, 2011). Several viruses including Human coronavirus, Ebola virus, and rotavirus utilize raft-dependent pathways for their cell entry (Arias et al., 2002; Guerrero, Zárate, Corkidi, López, & Arias, 2000; Nichols et al., 2001; Nomura et al., 2004). Similarly, for the assembly and budding processes of many viruses, lipid rafts act as sites for assembly of

viral proteins and thus facilitate production of infective viral progeny (Takahashi & Suzuki, 2011). For instance, the measles virus M protein associates with lipid rafts and with hemagglurinin-fusion (H-F) protein complexes associated with lipid rafts to ensure efficient assembly prior to budding (Manié, de Breyne, Debreyne, Vincent, & Gerlier, 2000; Vincent, Gerlier, & Manié, 2000). Ebola virus and Marburg virus also utilize lipid rafts as a platform for assembly and budding (Bavari et al., 2002). Furthermore, a role of lipid rafts in signal transduction has been investigated for a number of viruses including Human T cell leukemia virus type 1 (HTLV-1) and Dengue virus (DEN) (Huang, Ren, Guan, Jiang, & Cheng, 2009; Silva et al., 2011).

IAV utilizes lipid rafts in several aspects of its infectious cycle. Binding of IAV to cells leads to clustering of lipid rafts, suggesting that binding results in re-arrangement of signaling platforms and virus internalization (Eierhoff, Hrincius, Rescher, Ludwig, & Ehrhardt, 2010). Moreover, influenza budding was enhanced by disrupting the lipid rafts with cholesterol depletion (Barman 2007). Additionally, influenza virus HA and NA proteins associate with lipid rafts giving rise to regions of assembly and budding (Leser & Lamb, 2005). It has been suggested that HA clustering in rafts is necessary so that HA is incorporated in progeny virions at adequate concentrations and thus enables efficient virus-cell fusion in the subsequent infection cycle (Takeda, Leser, Russell, & Lamb, 2003). Taken together, these data suggest that lipid rafts play critical roles during both early and late stages of the IAV life cycle.

2. Viral infections and nSMase

Accumulating evidence suggests that lipid rafts are the specific sites for ceramide production in response to various stimuli. Whereas several studies highlight the

involvement of lysosomal aSMase in raft-associated ceramide generation, other studies reveal a role for Mg^{2+}-dependent nSMase in this respect (van Blitterswijk, van der Luit, Veldman, Verheij, & Borst, 2003; Zundel, Swiersz, & Giaccia, 2000).

Several bacteria and viruses infect cells by exploiting the Mg^{2+}-dependent nSMase-ceramide system (Table 3) (Faulstich et al., 2015; Martín-Acebes et al., 2014). nSMase has been shown to be involved in the internalization of pathogens, the induction of apoptosis in host cells, the intracellular activation of signaling pathways, and the release of cytokines in response to infection (Grassmé & Becker, 2013). The accumulation of ceramide in the plasma membrane creates platforms for clustering of receptor and signaling molecules (Stancevic & Kolesnick, 2010; Zhang et al., 2009).

Table 3. Summary of the roles of nSMase during some microbial infections

Pathogen group	Pathogen	Role of nSMase	Reference
Viruses	West Nile virus	Budding	(Martín-Acebes et al., 2014)
	Measles virus	Immune suppression	(Mueller et al., 2014)
	Sindbis virus	Apoptosis	(Jan et al., 2000)
	Human immunodeficiency virus type 1	Apoptosis	(Jana & Pahan, 2004)

Bacteria	*Neisseria gonorrhoeae*	Invasion	(Faulstich et al., 2015)

a. West Nile virus and nSMase

A study by Martín-Acebes et al. attempted to investigate whether infection with West Nile virus (WNV), a flavivirus responsible for outbreaks of febrile illness and meningoencephalitis, would affect glycerophospholipid and sphingolipid components of cellular membranes (Martín-Acebes et al., 2014). Cells infected with WNV showed an increase in the content of ceramide and SM. Further investigation demonstrated a requirement for nSMase function and its activity-induced ceramide production during the biogenesis of WNV. Ceramide segregated into domains triggering membrane bending and budding processes. The significance of these findings was underscored by experiments demonstrating that pharmacological inhibition of nSMase activity or depletion of nSMase by RNA interference reduced the production of WNV (Martín-Acebes et al., 2014).

b. Measles virus and nSMase

A study conducted by Mueller et al. aimed to elucidate the role of nSMase in Measles virus (MV)-induced inhibition of T-cell activation (Mueller et al., 2014). Genetic ablation of the enzyme greatly facilitated T-cell hyper-responsiveness indicating that nSMase acts to inhibit T-cell responses. The study further showed that cells exposed to MV exhibited prolonged and exaggerated nSMase activation. This was associated with

actin cytoskeletal paralysis and loss of expansion. Therefore, the study demonstrates how nSMase activity may account for immune suppression (Mueller et al., 2014).

c. Sindbis virus and nSMase

Sindbis virus (SV) is a positive-sense RNA virus causing encephalitis by infecting and inducing the death of neurons (Jan et al., 2000). Cells infected with SV displayed increased levels of ceramide which peaked between 2 and 6 hpi. The increase in ceramide was shown to be the result of both aSMase and nSMase activation, which contributed to SV-induced apoptosis (Jan et al., 2000).

d. Human immunodeficiency virus type 1 and nSMase

nSMase has been also implicated in the replication of human immunodeficiency virus type 1 (HIV-1) (Jana & Pahan, 2004). HIV-1 coat protein gp120 mediates apoptosis and cell death in primary neurons through redox-regulated activation of nSMase. Antisense knockdown of nSMase markedly inhibited gp120-induced apoptosis and cell death. Likewise, the HIV-1 regulatory protein Tat also induces neuronal cell death via nSMase (Jana & Pahan, 2004).

Despite the abundance of literature investigating the role of lipid rafts, the major site for ceramide production, in IAV infection, none has been done to determine the role of ceramide and the enzymes leading to its generation. Unpublished data from our laboratory have indicated an increase in total ceramide in human lung adenocarcinoma epithelial (A549) cells infected with IAV. Because multiple metabolic pathways converge upon ceramide, we decided to investigate the effect of the de novo synthesis pathway and the hydrolysis (SMase) pathway on ceramide production and virus

replication in IAV infected cells. Preliminary data from our lab revealed an imported role for the *de novo* synthesis pathway in IAV-induced ceramide production. Moreover, cells treated with *de novo* synthesis pathway inhibitors, displayed enhanced virus production. We also demonstrated that aSMase negatively modulates IAV infection. Specifically, cells deficient for aSMase expression supported enhanced IAV replication.

However, the role of nSMase-ceramide pathway during IAV infection has not yet been assessed. Because targeting sphingolipid metabolism is currently considered a suitable therapeutic strategy for treatment of viral infections like HCV infection (Amemiya et al., 2008; Katsume et al., 2013; Sakamoto et al., 2005), knowledge of the role of nSMase during influenza replication may point to the nSMase as a potential cellular target suitable for antiviral design against IAV. The current study thus investigates the role of nSMase during IAV infection and paves the way for further studies regarding the underlying mechanisms.

CHAPTER II

MATERIALS AND METHODS

A. Experimental design

1. Cell culture

The human lung adenocarcinoma epithelial cells (A549) and Madin-Darby canine kidney cells (MDCK) were obtained from St Jude Children's Research Hospital and BEI Resources, respectively. These cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin antibiotics (Sigma) in a humidified atmosphere containing 5% CO_2 at 37°C.

2. IAV propagation

The influenza a virus A/Puerto Rico/8/34 strain was obtained from Dr. Richard Webby, St. Jude's Children's Hospital.

A/Puerto Rico/8/34 (H1N1) (PR8) viruses were grown in embryonated chicken eggs. In brief, virus stock was inoculated into the allantoic cavity using a needle. After 2 days of incubation at 37°C, the eggshell was carefully opened, and the fluid containing the virus was harvested, aliquoted and transferred to -80°C for storage.

The viruses were then grown on MDCK cells in virus infection medium (VIM). One day before infection, the cells were plated at $3x10^6$ per $150mm^2$ culture dish. Infection was done in minimal medium to allow for maximal viral adsorption. The viral inoculum was incubated with the monolayer for 1 hour at 37°C with gentle shaking every 15 minutes before the addition of 25ml of fresh VIM containing 1 μg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone [TPCK]-trypsin. The medium was harvested at 48-72 hours post infection and centrifuged at 1500 rpm for 3 minutes. Finally, the virus-rich supernatant was stored in aliquots at -80°C until further use. Viral titers were determined by plaque assay on monolayers of MDCK cells.

3. IAV infection

For infections, A549 cells were washed out twice with PBS++ (phosphate buffered saline with calcium and magnesium ions). The viral inoculum was incubated with cell monolayers for 1 hour at 37°C with gentle shaking every 15 minutes. After incubation, the inoculum was removed and unbound viral particles were discarded by washing twice with acidic PBS++. VIM containing 0.2 μg/ml [TPCK]-trypsin was added and, following incubation for different periods of time, the supernatant containing IAV was collected and stored in aliquots at -80°C until titration.

4. IAV titration

The infectivity titers were determined at 24 hours post-infection (hpi) by plaque assay on MDCK cells. Cells were plated at $0.8x10^6$ in 2 ml DMEM/10% FBS in 6-well tissue culture plates one day prior to the assay. On the day of the assay, homogeneously

confluent monolayers were observed. Ten-fold serial dilutions (10^{-1}-10^{-6}) of the virus stocks or samples were prepared in VIM and put on ice. For the assay, media was aspirated and cells were washed out twice with PBS++. The wash medium was then removed, and 200 µl of each dilution was added onto its corresponding well. Cells were incubated for an hour at 37°C with gentle shaking every 15 minutes to allow for homogeneous adsorption of the virus. The cells were then overlaid with 2 ml of 0.5% agarose (Invitrogen) in 1x MEM containing 1 µg/ml TPCK-trypsin and incubated for 72 hours at 37°C. After incubation, cells were fixed and stained with crystal violet solution (0.5% crystal violet, 10% ethanol, 20% formaldehyde) and then washed out with tap water. Plaques were counted, and the infectivity titers were determined as follows:

Virus Titer (PFU/ml) = Number of plaques/ (Dilution factor x Volume of virus added (ml))

5. Drug preparation and cytotoxicity assay (MTT Assay)

GW4869 (Sigma) stocks (1.5 mM) were prepared in dimethyl sulfoxide (DMSO), aliquoted, and stored at -80°C. Right before use, stock suspensions were solubilized by the addition of 5% methane sulfonic acid (MSA) (Sigma). Therefore, a final concentration of 1.43 mM was obtained at the time of the experiments.

For determining the cytotoxicity of GW4869, A549 cells were plated at 70% confluence ($2x10^4$ cells per well) in 96-well plates and incubated at 37°C one day prior to drug treatment. The cells were mock-treated or treated with the drug in dilutions with a final concentration ranging between 1.56 µM and 25 µM. The cells were then incubated

for 24 hours. Twenty µl of 1 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well and incubated for 2 hours. Finally, the reaction was stopped by adding100 µl of acidic isopropanol were added to each well, and absorbance was measured using a Multiskan ELISA reader at 595 nm.

6. Drug treatment

A549 cells were plated at $0.2x10^6$ in 600 µl DMEM/10% FBS in 24-well tissue culture plates and incubated at 37°C one day prior to treatment and infection. The cells were either mock-treated or treated with the GW4869 in dilutions with a final concentration ranging between 0.6256 µM and 20 µM and incubated for 1 or 24 hours. After incubation, media was aspirated and cells were washed out twice with PBS++. The wash medium was then removed, and cells were inoculated with 200 µl of VIM containing both IAV (1 or 0.01 multiplicity of infection (MOI)) and the corresponding drug dilution for a further 60-minute incubation, with gentle shaking every 15 minutes. After infection, the viral inoculum was replaced by 600 µl VIM containing 0.2 µg/ml TPCK and the corresponding GW4869 concentration or VIM alone. Twenty-four hours post-infection, virus progeny was collected and stored at -80°C for further titration using plaque assay.

7. Immunofluorescence microscopy

A549 cells were plated on glass coverslips at $0.25x10^6$ in 600 µl DMEM/10% FBS in 12-well tissue culture plates and incubated at 37°C one day prior to treatment and

infection. The cells were either mock-treated (vehicle alone) or treated with GW4869 (20 μM) and incubated for 1 h. After incubation, media was aspirated and cells were washed out twice with PBS++. The wash medium was then removed, and cells were inoculated with 200 μl of VIM containing both IAV (1 MOI) and the drug for a further 60-minute incubation, with gentle shaking every 15 minutes. After infection, the viral inoculum was replaced by 500 μl VIM containing 0.2 μg/ml TPCK and GW4869 or VIM alone. Twenty-four hpi, virus progeny was collected and stored at -80°C for further titration.

At 8 and 24 hpi, media was aspirated, and cells were washed out twice with PBS++. The wash medium was then removed, and the cells were fixed with 4% paraformaldehyde (PFA) in PBS-- for 15 min at room temperature (RT). To restrict the staining to the plasma membrane, cells were not permeabilized. Then, the cells were blocked in 1% BSA in PBS for 1 h and incubated with an anti-ceramide monoclonal antibody (1:100 in 1% BSA) at 4 °C overnight. After being washed six times with 0.1 % tween in PBS--, the cells were stained with an Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody for 1 h at RT. Nuclei were stained with 4′, 6′-diamidino-2-phenylindole (DAPI). Finally, cells were directly observed using a confocal laser scanning microscope. The fluorescence intensity of the images was processed and quantified using ZEN software.

8. nSmase activity assay

A549 cells were plated at $1x10^6$ in 5 ml DMEM/10% FBS in t-25 flasks and incubated at 37°C one day prior to infection. The cells were either mock-infected or infected with 500 μl of VIM containing IAV at an MOI of 1. The viral inoculum was

incubated with cell monolayers for 1 hour at 37°C with gentle shaking every 15 minutes. At different time points post-infection (0, 15, 30, 45, 60, 120, and 360 min), cells were scraped off the flasks and centrifuged at 1500 rpm for 5 min. After centrifugation, pelleted cells were resuspended in 1 ml PBS-- (phosphate buffered saline without calcium and magnesium ions) and centrifuged again at 800 g for 10 min. The cell pellet was resuspended and lysed, proteins were quantified, and nSMase activity was assayed using the Amplex® Red Sphingomyelinase Assay Kit. Each of the steps above will be described below in details.

a. Protein extraction

Cell pellets were resuspended in 100 µl 1x lysis buffer (0.1 mM Tris-HCl, pH 7.4, plus protease inhibitors) by pipetting up and down several times and then incubated on ice for 1 hr with pipetting or vortexing every 10 min. The homogenate was centrifuged for 10 min at 14,000 g at 4 °C, and the supernatant was collected for protein quantification.

b. Protein quantification

The extracted proteins were quantified using DC™ Protein Assay Kit (Bio-Rad). Briefly, proteins were diluted in distilled water (dH_2O) to a final volume of 25 µl. Working solutions of reagent A (alkaline copper tartrate solution) and reagent S (aqueous SDS solution) were prepared and added to each sample (125 µl per sample) followed by the addition of 1000 µl reagent B (Folin reagent). Sample tubes were vortexed immediately and transferred to a 96-well plate in triplicates. The absorbance of the

developed blue color was read at 750 nm using a microplate reader. Protein concentration was determined by plotting the absorbance vs. concentration of known standards of bovine serum albumin (BSA, Amresco).

c. nSMase activity assay

nSMase activity was analyzed using the Amplex® Red Sphingomyelinase Assay Kit according to the manufacturer's instructions (Molecular Probes, Invitrogen). In brief, cell lysates were diluted with 1x reaction buffer (0.1 M Tris–HCl and 10 mM $MgCl_2$, pH 7.4), and then inoculated into a 96-well plate in duplicates (100 µl/well). The total amount of protein in each well was 50 µg. A working solution of 100 µM Amplex® Red reagent containing 2 U/mL HRP, 0.2 U/mL choline oxidase, 8 U/mL of alkaline phosphatase and 0.5 mM SM was added to each microplate well (100 µl / well) and incubated for 30 min at 37 °C protected from light. Fluorescence was finally measured in a fluorescence microplate reader at 530/590 nm excitation/emission levels.

In this enzyme-coupled assay, SMase activity is analyzed indirectly using Amplex® Red reagent. First, SMase hydrolyses the SM to generate ceramide and phosphorylcholine (Figure 8). Alkaline phosphatase then catalyzes the hydrolysis of phosphorylcholine, and choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with Amplex® Red reagent to yield the highly fluorescent product, resorufin.

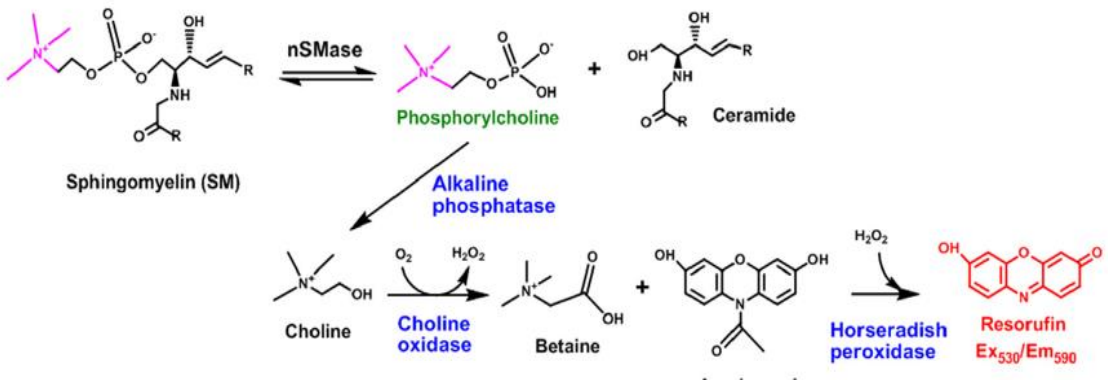


Figure 8. nSMase fluorescence assay consisting of three coupled reactions and Amplex® Red reagent to yield the highly fluorescent product, resorufin (Figuera-Losada et al., 2015).

9. Statistical analysis

All statistical analyses were performed using Microsoft Excel. Statistical significance was determined using Student's t test. Data are representative of two independent experiments. Data are represented as the mean ± SD, and statistical significance ($P < 0.05$) was indicated by an (*) asterisk. In addition, $P < 0.01$ and $P < 0.001$ were marked with two (**) and three (***) asterisks in the figures, respectively.

CHAPTER III

RESULTS

A. GW4869 is not cytotoxic in A549 cells

GW4869 is a potent, cell-permeable, and specific nSMase inhibitor. It displays noncompetitive inhibition as its structure does not resemble that of SM. It was shown to have an *in vitro* IC_{50} of 1 μM for nSMase and no inhibition on aSMase (Canals, Perry, Jenkins, & Hannun, 2011).

In order to determine the working concentration of GW4869, we first evaluated its cytotoxicity on A549 cells by using MTT assay. Briefly, A549 cells were treated with the drug in 2-fold serial dilutions starting at 25 μM. The cells were then incubated with the drug 24 h prior to addition of MTT and spectrophotometric reading. A decrease of more than 20% in cell viability was used to indicate drug cytotoxicity. GW4869 was well tolerated by A549 cells, exhibiting no significant cytotoxicity at the used concentrations from 1.56 to 25 μM (Figure 9).

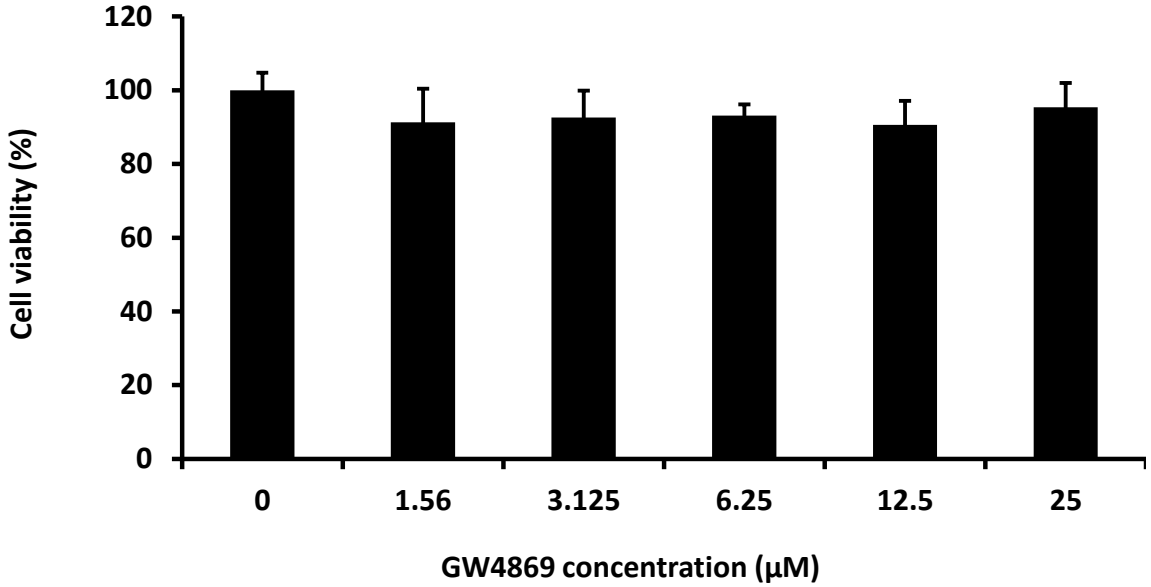


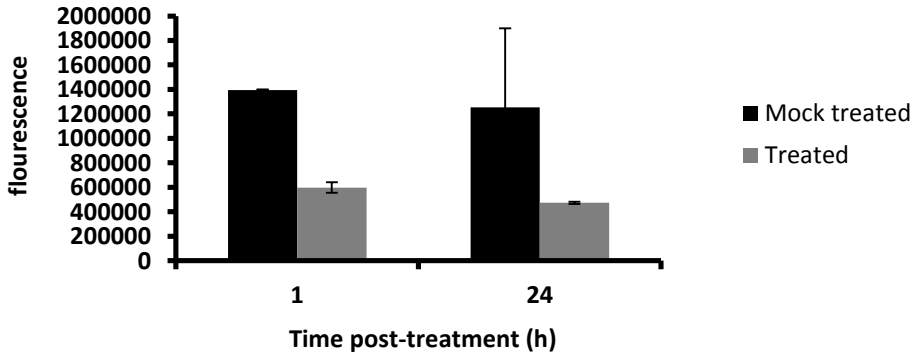
Figure 9. Effect of GW4869 on the viability of A549 cells. Cells were treated with the indicated concentrations of GW4869 and incubated at 37 °C for 24 h. Cell viability was then evaluated by MTT assay. The results are presented as a percentage of control (untreated) cells. A decrease of more than 20% in cell viability was used to indicate drug toxicity. Data are represented as the mean ± SD.

B. nSMase activity may be crucial for accumulation of ceramide at the plasma membrane during IAV infection.

As previously mentioned, preliminary data from our laboratory showed that IAV infection induces ceramide accumulation, and the de novo synthesis pathway of ceramide production was shown to account for this accumulation. Therefore, we investigated if IAV-induced ceramide accumulation is also mediated by nSMase. To address this, we assessed ceramide levels in A549 cells infected with IAV in the presence or absence of nSMase inhibition with GW4869 (20 µM) at 8 and 24 hpi by immunofluorescence staining and confocal microscopy using an antibody directed against ceramide.

First, in order to confirm inhibition of nSMase activity at the selected concentration of GW4869, nSMase activity was detected in A549 cells after incubation in presence of GW4869 for 1 and 24 h. Treatment of cells with 20 µM GW4869 significantly decreased nSMase activity by ~ 60% at 1 h post-treatment (Figure 10). At 24 h post-treatment, nSMase activity was also inhibited by ~ 60% compared to untreated (control) cells, although the drop in activity was not statistically significant (Figure 10).

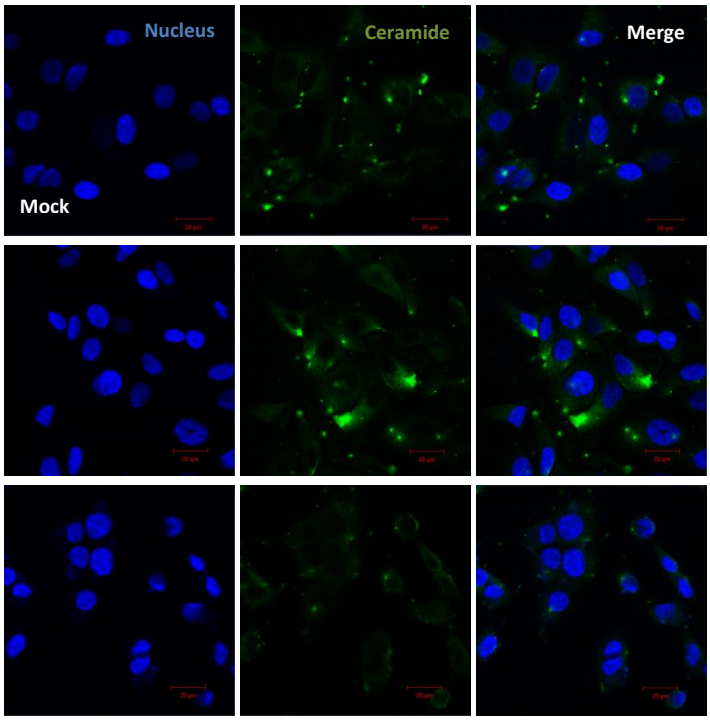
Our results indicated that ceramide level significantly increased in IAV-infected cells at 8 hpi (p-value= 0.001377) (Figure 11A) and 24 hpi (p-value= 0.001753) (Figure 11B). When cells were treated with GW4869, ceramide levels returned to the baseline line (ceramide level in un-infected cells) at 8 hpi (Figure 11A). At 24 hpi (Figure 11B), inhibition of nSMase with GW4869 resulted in a significant drop in ceramide signal compared to IAV-infected cells, although it did not return to the baseline level. Therefore, our data suggests that IAV induces cellular ceramide accumulation that is partially mediated by nSMase.

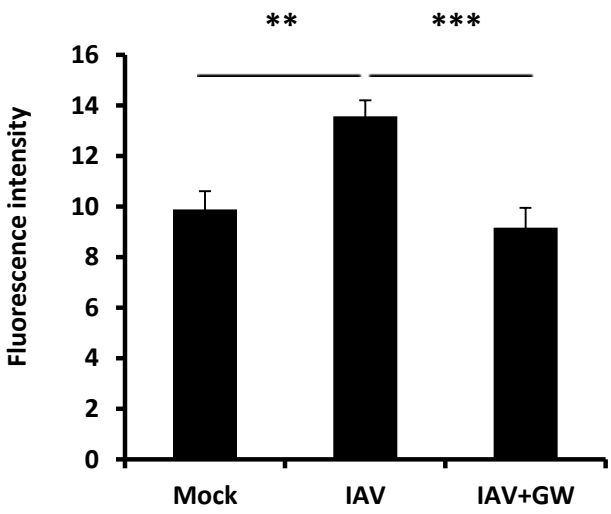


*

Figure 10. Activity of nSMase in cells treated with 20 µM GW4869. A549 cells were either left untreated or treated with GW4869 (20 µM). At 1 and 24 h post-treatment, cells were harvested, and proteins were extracted and quantified. nSMase activity was then determined using Amplex® Red SMase assay kit. Data are represented as the mean ± SD. Statistical significance between the untreated and the indicated concentration was tested using the Student's *t-test; *p < 0.0.*

A





B

Nucleus Ceramide

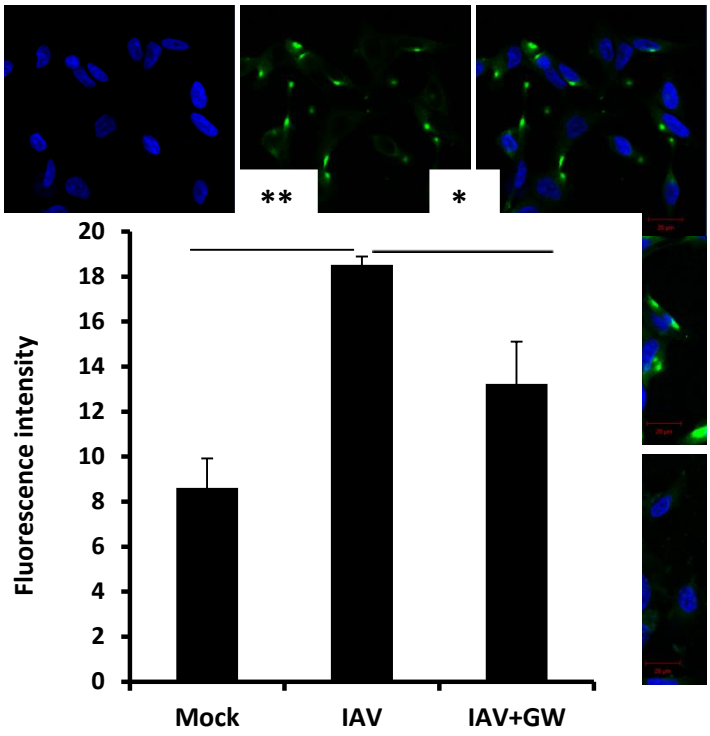

**
*
Fluorescence intensity
20
18
16
14
12
10
8
6
4
2
0
Mock
IAV
IAV+GW

Figure 11. IAV induces ceramide accumulation in A549 cells through nSMase pathway. Cells were pretreated with GW4869 (20 µM) 1 h before, during and after inoculation with IAV at an MOI of 1. Ceramide accumulation at the plasma membrane 8 (A) and 24 (B) hpi was detected by immunofluorescence staining and confocal microscopy (green). Nuclei were stained with DAPI (blue). Images were quantified using ZEN software (scale bar= 20 µm). Data are represented as the mean ± SD. Statistical significance between the untreated and the indicated concentration was tested using the Student's *t-test; *p < 0.05; **p < 0.01; ***p < 0.001*.

C. nSMase is required for efficient IAV replication

We next tested whether nSMase activity and the subsequent accumulation of ceramide are required for efficient IAV replication. To do so, we measured virus replication in A549 cells upon inhibition of nSMase. Briefly, A549 cells were mock-treated or pretreated with increasing concentrations (0.625-20 µM) of GW4869 for 1 h and inoculated with IAV at an MOI of 0.01 in the presence of the inhibitor. Virus production was then quantified at 24 hpi using plaque assay. Treatment of cells with GW4869 at concentrations of 10 and 20 µM significantly attenuated the replication of IAV, resulting in 1.5 $\log_{10}$pfu/ml (p-value= 0.028995) and 3.3 $\log_{10}$pfu/ml (p-value= 0.008041) decrease, respectively (Figure 12A). No significant effect on virus replication was observed at concentration of 5µM and below.

We next determined whether inhibition of nSMase can still affect virus replication when cells are inoculated with a higher inoculum of MOI=1. Similar to our results with MOI=0.01, IAV replication in A549 cells was significantly attenuated by 1-2.2 $\log_{10}$pfu/ml when cells were treated with GW4869 at concentrations ranging between 5-20 µM (Figure 12B). Therefore, our data suggests that inhibition of nSMase impairs IAV replication in A549 cells. Therefore, nSMase is required for efficient IAV replication.

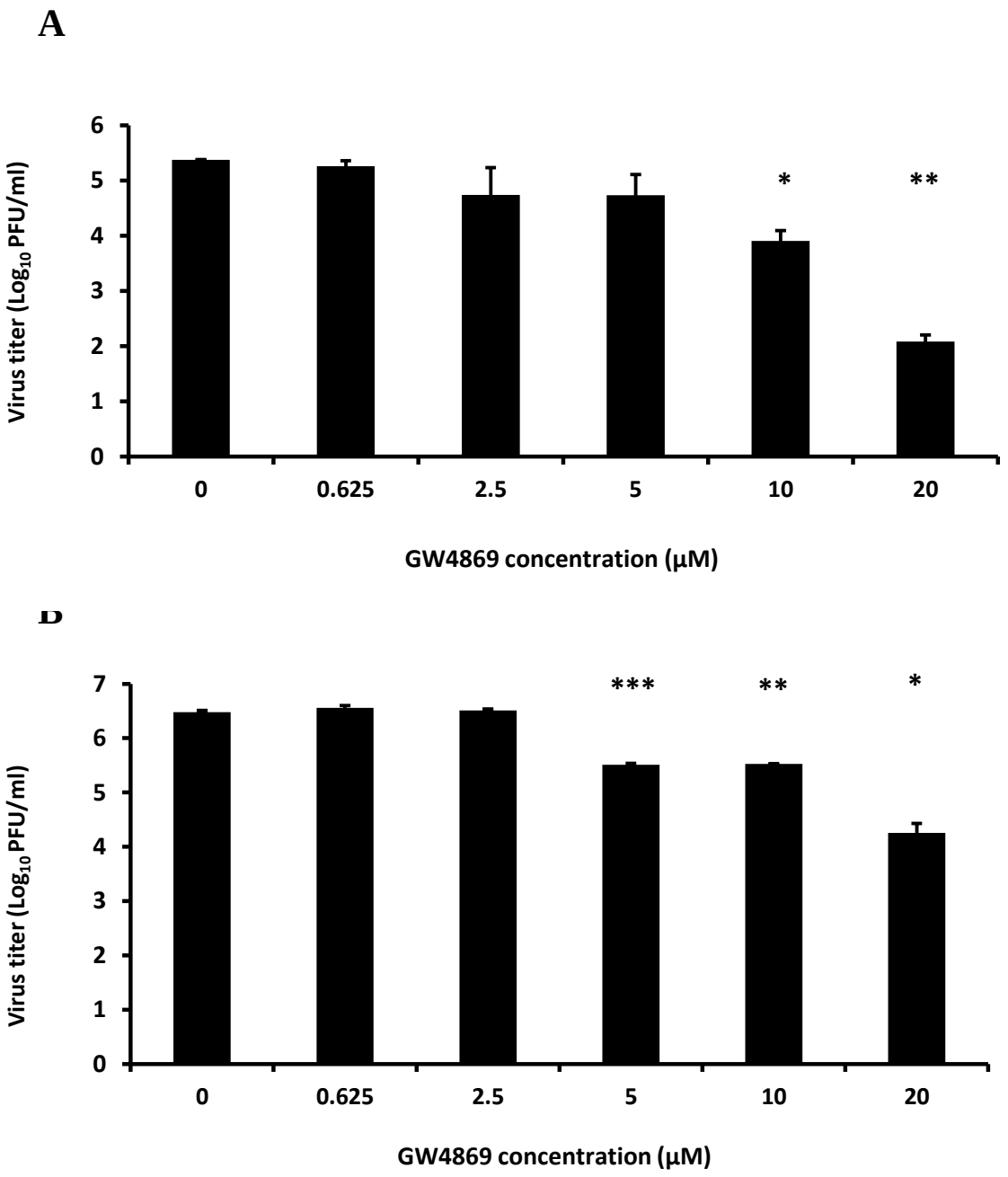


Figure 12. Inhibition of nSMase attenuates IAV replication in A549 cells. Cells were pretreated with the indicated drug concentrations of GW4869 1 h before, during and after inoculation with IAV at an MOI of 0.01 (A) or 1 (B). The amount of infectious virus released into the culture medium at 24 hpi was determined by plaque assay on MDCK cells. Data are represented as the mean ± SD. Statistical significance between the mock (0 μM) and the indicated concentrations was tested using the Student's *t-test; *p < 0.05; **p < 0.01; ***p < 0.001.*

D. Prolonged inhibition of nSMase reduces IAV production

As acute inhibition of nSMase function was shown to attenuate IAV replication and virus production, we were interested to explore whether prolonged inhibition of nSMase result in total depletion of ceramide and thus a more pronounced inhibitory effect on virus replication. To this end, A549 cells were mock-treated or pretreated with increasing concentrations of GW4869 for 24 h and then inoculated with IAV at an MOI of 1 in the presence of the inhibitor. Virus production was then quantified at 24 hpi using plaque assay. IAV replication in A549 cells was significantly attenuated by 0.9-1.4 log_{10}pfu/ml when cells were treated with GW4869 at concentrations ranging between 5-20 µM (Figure 13). Therefore, prolonged inhibition of nSMase does not further inhibit IAV replication in comparison with acute inhibition.

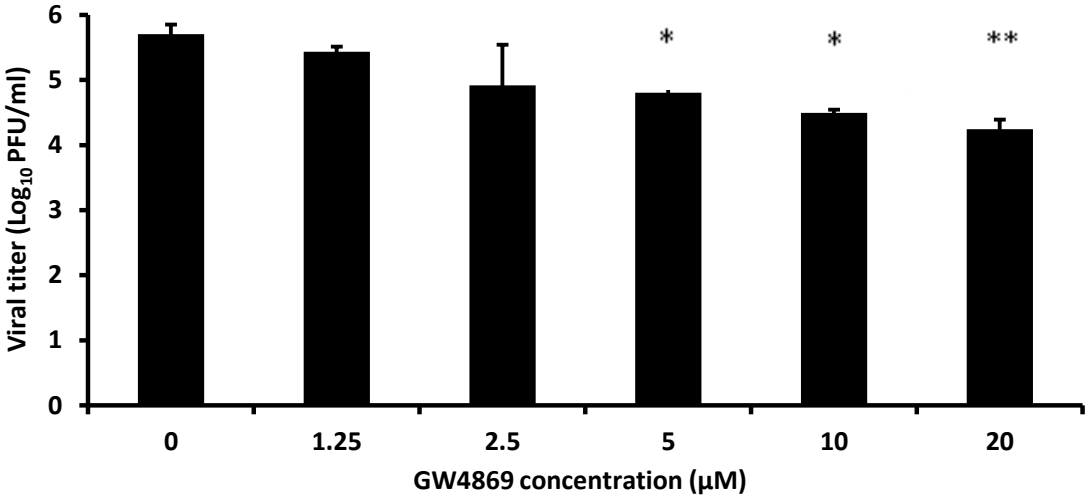


Figure 13. Prolonged inhibition of nSMase does not fully inhibit IAV replication in vitro. A549 cells were pretreated with the indicated drug concentrations of GW4869 24 h before, during and after inoculation with IAV at an MOI of 1.The amount of infectious virus released into the culture medium after 24 h was determined by plaque assay on

MDCK cells. Data are represented as the mean ± SD. Statistical significance between the mock (0 µM) and the indicated concentrations was tested using the Student's *t-test; *p < 0.05; **p < 0.01*.

E. Basal nSMase activity may be sufficient for IAV replication

Having established that nSMase activity is important for IAV infection, we next asked whether IAV induces nSMase activation in A549 cell. Because we hypothesized that accumulation of ceramide at the plasma membrane would be essential during the early steps of binding and entry of the IAV replication cycles, nSMase activity was measured at 0-6 hpi. Surprisingly, no significant change in nSMase activity in IAV-infected cells relative to that in control, uninfected cells was noted between 0-360 mins (Figure 14). This observation suggests that nSMase was not further activated due to IAV infection with IAV and that basal activity may be sufficient and required for efficient IAV replication.

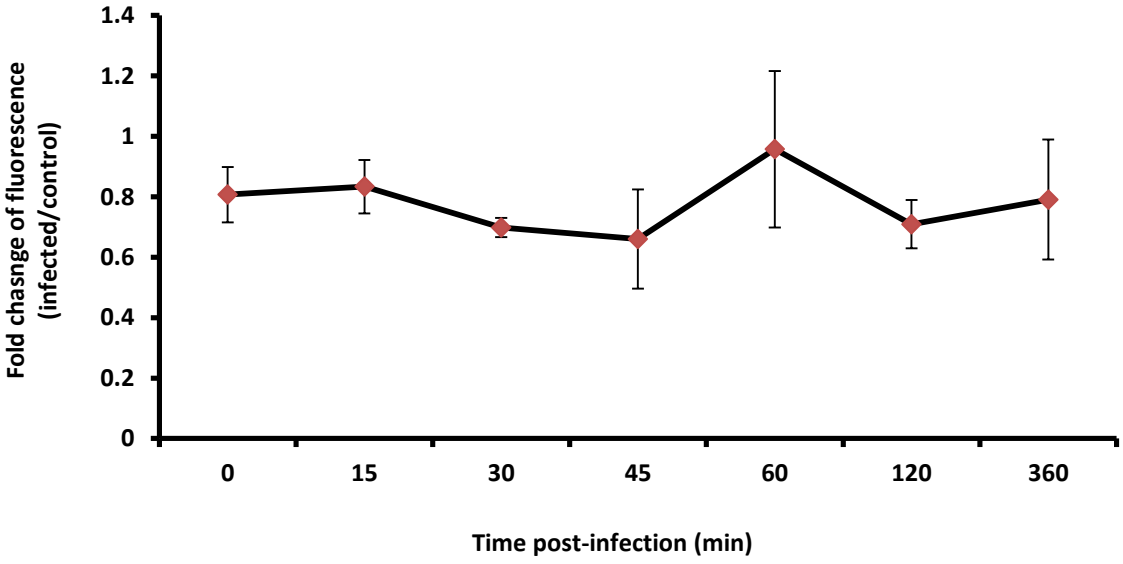


Figure 14. Infection with IAV does not further activate nSMase. A549 cells were either left uninfected (control) or infected with IAV (MOI=1). At the indicated time points, cells were harvested, and proteins were extracted and quantified. nSMase activity was

then determined using Amplex® Red SMase Assay Kit. Fold change of fluorescence representing nSMase activity was calculated relative to control, uninfected cells. Data are represented as the mean ± SD.

CHAPTER IV

DISCUSSION

IAV is a major respiratory pathogen with serious disease burden (Rothberg, Haessler, & Brown, 2008). Current influenza antiviral drugs target viral proteins (Kamali & Holodniy, 2013), which are prone to frequent mutations and resistance. Therefore, the identification and targeting host cell factors that are required for efficient IAV replication may provide promising antiviral approaches. Lipid rafts are membrane domains that are rich in sterols and sphingolipids (Pike, 2006). They have been demonstrated to play an important role in the replication of many viruses including IAV (Lorizate & Kräusslich, 2011). nSMase breaks down SM into ceramide and phosphorylcholine, causing the formation of ceramide-enriched membrane platforms, which in turn play important roles in infections with pathogens associated with rafts (Faulstich et al., 2015; Gassert et al., 2009). However, the role of this enzyme during IAV replication has not yet been assessed. In this study, we showed that IAV induces accumulation of ceramide at the plasma membrane. Inhibition of nSMase resulted in attenuated IAV replication and decreased ceramide production. Nonetheless, nSMase activity in infected cells was similar to its baseline activity in uninfected cells. Therefore, our data demonstrate for the first time a novel role for nSMase during IAV replication.

Previous studies have shown that nSMase inhibition attenuated the replication of viruses including WNV and herpes simplex virus-1 (HSV-1) (Amtmann, Zöller, & Schilling, 2000; Martín-Acebes et al., 2014). The suppression of WNV replication was attributed to the reduction in the level of ceramide resulting in an impaired budding process (Martín-Acebes et al., 2014). In contrast, pharmacologic inhibition of nSMase function enhanced the replication of Sindbis virus (Jan et al., 2000). Our data indicated attenuation of IAV replication upon inhibition of nSMase, consistent with the studies on WNV. Worth noting, ceramide segregation into highly ordered domains has been associated with membrane modification or bending triggering budding processes (López-Montero, Monroy, Vélez, & Devaux, 2010; Trajkovic et al., 2008). IAV utilizes the membrane lipid rafts during its entry and budding steps (Barman & Nayak, 2007; Scheiffele, Roth, & Simons, 1997). Altering the composition of lipid rafts can influence the efficiency of IAV replication (Barman & Nayak, 2007). Moreover, the HA protein of IAV virus was found to associate with and cluster in sphingolipid-cholesterol membrane domains or lipid rafts to provide adequate amounts for HA for the budding virus for efficient virus to cell fusion (Scheiffele et al., 1997). IAV envelope (Blom et al., 2001) has been also found to be enriched with glycosphingolipids. Therefore, inhibition of nSMase and the subsequent decrease in ceramide accumulation might alter the properties of the plasma membrane and interfere with two critical steps in IAV replication: i.e. fusion and budding. Moreover, certain receptors have been shown to cluster in nSMase-induced ceramide platforms to facilitate the entry of bacteria like *Neisseria gonorrhoeae* (Faulstich et al., 2015). Thus, the suppression of ceramide-enriched platforms could interfere with the availability of receptors that are engaged by IAV for efficient cell entry.

Another possible mechanism for IAV attenuation upon pharmacological inhibition of nSMase is through the suppression of Raf/MEK/ERK system. Studies have shown that signaling through the Raf/MEK/ERK cascade is crucial for IAV replication (Pleschka et al., 2001). Blockade of Raf-1 signaling led to the retention of IAV RNPs in the nucleus and subsequent inhibition of IAV production. nSMase was demonstrated to be an important factor in activating Raf-1 and ERK (Avota, Gulbins, & Schneider-Schaulies, 2011), serine/threonine protein kinases participating in the Raf/MEK/ERK signal transduction cascade. This was directly linked to the subsequent accumulation of ceramide in response to DC-SIGN receptor ligation. Therefore, the inhibition of nSMase might result in suppression of the Raf/MEK/ERK cascade and subsequently inhibit the export of IAV RNPs to the cytoplasm resulting in the observed attenuated virus replication.

The finding that ceramide is important for infection is compatible with previous findings regarding adenovirus infection (Kanj et al., 2006). Adenovirus induction of *de novo* synthesis-derived ceramide accumulation has been shown to be important for the lytic phase of infection, as blockade of ceramide synthesis significantly delayed cell lysis. Precisely, ceramide pathway has been found to be required for the regulation of serine/arginine-rich (SR) proteins. These proteins are part of the cellular RNA splicing machinery and are dephosphorylated during the late phase of the viral life cycle (Kanj et al., 2006). Worth noting, splicing is a necessary step for influenza virus replication (Dubois, Terrier, & Rosa-Calatrava, 2014). Influenza virus has been shown to utilize the cellular splicing machinery to process its M and NS gene RNA, a necessary step for virus

replication (Dubois et al., 2014). Therefore, the inhibition of ceramide production might result in inhibition of IAV mRNA splicing and subsequently inhibit virus replication.

Several studies have described the activation of nSMase due to infection with bacterial and viral pathogens (Faulstich et al., 2015; Jan et al., 2000; Jana & Pahan, 2004; Mueller et al., 2014). In contradiction, our study revealed no significant change in nSMase activity in IAV-infected cells relative to that in uninfected cells. This suggests that while nSMase activity is crucial for IAV replication, nSMase is not further activated due to IAV infection and that basal activity is sufficient for efficient IAV replication.

In conclusion, our study shows evidence of a potentially novel role for nSMase during IAV infection that warrants further investigations. Pharmacological inhibition of nSMase attenuates the production of IAV. This may be attributed to the subsequent inhibition of nSMase-induced ceramide accumulation at the plasma membrane. Therefore, nSMase and ceramide-enriched membrane platforms can be potential targets to prevent and treat IAV infections.

Follow-up studies corroborating our findings using a genetic approach whereby nSMase is silenced by RNA interference are essential. Furthermore, A549 cell line is established from lung adenocarcinoma tissue. Despite that A549 cell line is a popular model to study influenza A virus pathogenesis and cell signaling, confirming the results in a normal primary cell line would be essential. Moreover, as outlined above, multiple pathways might be involved in the ceramide accumulation during IAV infection. Indeed, we have previously shown that the de novo pathway is also responsible for ceramide

generation. Therefore, further studies are needed to fully elucidate the dynamics of activation of ceramide synthesis pathways and their role during IAV infection.

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