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Role of *Ixodes scapularis* Sphingomyelinase-Like Protein (IsSMase) in Tick Pathogen Interactions

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ROLE OF *IXODES SCAPULARIS* SPHINGOMYELINASE-LIKE PROTEIN (*IS*SMASE) IN TICK PATHOGEN INTERACTIONS

by

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ABSTRACT

ROLE OF *IXODES SCAPULARIS* SPHINGOMYELINASE-LIKE PROTEIN (*IS*SMASE) IN TICK PATHOGEN INTERACTIONS

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Old Dominion University, 2020
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Arthropod-borne diseases are one of the major concerns throughout the world. *Ixodes scapularis* (hard tick) is one of the major vectors that is involved in arthropod-borne disease transmission. Langat virus (LGTV) is a model pathogen that is very similar to other medically important flaviviruses such as Tick-Borne Encephalitis virus (TBEV) and Powassan virus (POWV). Sphingomyelinase-like protein (*Is*SMase, a Sphingomyelinase D or SMase D, a venomous protein ortholog of spiders) is an enzyme present in ticks that helps to catalyze the hydrolysis of the sphingomyelin (cell membrane lipid) into phosphocholine and ceramide. The objective of our study is to delineate the role of *Is*SMase in exosome biogenesis upon LGTV infection in ticks. Our previous study showed that LGTV-infection enhanced the production and release of exosomes to mediate the transmission of flavi-viral proteins and infectious RNA genomes from the arthropod to the vertebrate host. Understanding the mechanism(s) of arthropod-borne flavivirus transmission via exosome biogenesis is very important. My MS thesis project explored the detailed role of *Is*SMase in tickborne viral replication and pathogenesis and provided molecular insights of viral modulated survival strategies in ticks. Our data, in specific, suggests an important role for *Is*SMase in regulating viral replication in ticks, and in general a mechanism for anti-viral pathways in medically important vectors.

This manuscript is dedicated to my parents Late. Govind Prasad Regmi and Anjana Regmi.

To my relatives, teachers and friends.

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A debt of gratitude is also owed to my father, late Govind Prasad Regmi, my mother, Anjana Regmi, my family and relatives for influencing me to be a good person and constantly loving and supporting me unconditionally. I would like to thank all the members of Nepalese community from Old Dominion University for providing support, happiness and unforgettable memories especially during the festivals. They made me feel like I am in home even though I am far away.

ABBREVIATIONS

ATCC	American Type Culture Collection
BSL-2	Biosafety Level - 2
CDC	Centers for Disease Control and Prevention
DENV	Dengue virus
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribose nucleic acid
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
GDPD	Glycerophosphoryl diester-phosphodiesterase
GW4869	Dihydrochloride hydrate 4869
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSP70	Heat shock protein 70
IAFGP	Antifreeze glycoprotein
IL-4	Interleukin-4
ILV	Intraluminal vesicles
ISE6 Ticks	*Ixodes scapularis* ticks
*Is*SMase	*Ixodes scapularis* Sphingomyelinase
LGTV	Langat virus
MOI	Multiplicity of infection
MVBs	Multivesicular bodies
NIH	National Institute of Health
NNDSS	Notifiable disease surveillance system
OCP	Onchocerciasis control program
p.i.	Post-infection
PFU/ML	Plaque forming units per milliliter
PKC	Protein kinase C
POWV	Powassan virus
QRT-PCR	Quantitative real-time polymerase chain reaction

RNA	Ribose nucleic acid
SD	Standard deviation
SM	Sphingomyelin
SMase D	Sphingomyelinase D
SMPD3	Sphingomyelin phosphodiesterase 3
TBD	Tick borne disease
TBEV	Tick-borne encephalitis virus
TLR	Toll like receptor
USA	United States of America
VBD	Vector-borne diseases
WHO	World Health Organization
WNV	West Nile virus

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1. INTRODUCTION AND BACKGROUND

1.1 Vector and Vector-borne Diseases

Vectors, in biological sciences, have several definitions. A vector is an organism (invertebrate or vertebrate) which acts as a carrier of an infectious agent among organisms of different species (Kuno and Chang, 2005). In a broader sense, different agents can be considered as a vector which includes living organisms like microbes and intermediate parasites, or an inanimate medium of infection like syringe and dust particles. In this chapter, we are more focused on hematophagous arthropod vectors (that feed on blood at either some or all stages of their lives) like ticks and mosquitoes. During blood feeding by these vectors, there is an entry of pathogen in the bloodstream of the host by various means.

Vector-borne disease (VBD) is an important communicable disease with a specific feature of requiring a blood-feeding arthropod vector (an intermediate host) like tick or mosquito to transmit infectious agents between humans or from animals to humans. These arthropods transmit infectious agen ts like bacteria, virus, protozoa and filarial nematodes that cause disease in humans resulting in severe morbidity and mortality to cause global burden (Hill et al., 2005). Depending upon the type of etiological agents/pathogens, VBDs in humans can be classified as protozoan diseases (malaria, leishmaniasis, trypanosomiasis, Chagas disease), viral diseases (Japanese encephalitis, dengue hemorrhagic fever, yellow fever), filarial nematode diseases (onchocerciasis, lymphatic filariasis) and bacterial diseases (tularemia, Lyme disease) (Hill et al., 2005; WHO, 2017).

According to World Health Organization (WHO, 2017), VBDs cause annual deaths of more than 700,000 which is more than 17% of all infectious diseases. Malaria is one of the most significant VBDs in the world causing 400,000 deaths every year. Most of the VBDs are preventable disease if informed protective measures are taken (WHO, 2017). However, it is unfortunate that the available measures for alleviating the impact of these VBDs are not sufficient and there is an increase in public health burden rate of some VBDs like dengue, malaria and leishmaniasis. Developing countries are

generally experiencing higher burden of VBDs (Hill et al., 2005). The reason behind it may be because some of them are under-reported and some disease burden could be difficult to be determined. The incidence of many VBDs are expected to rise over the next decade. Also, the emergence of new pathogenic strains and unidentified agents have resulted in an increasing public health concern.(Hill et al., 2005).

1.2 Vector Control

Controlling vector is one of the important strategies to prevent VBDs. Also, looking at the history, vector control has proven successful to eliminate diseases. For example, Onchocerciasis Control Program (OCP, 1974-2002) was able to eliminate onchocerciasis in 10 of 11 countries (in which it was operated) as a public health problem. In this program, vector control was done by spraying insecticides weekly in fast flowing rivers (that are the breeding sites of vector) to kill the larvae of the black fly (a vector) and break the life cycle of the parasite (WHO, 2020). Also, in most of the countries with temperate climates in the northern hemispheres, this strategy of vector control programs has led to the eradication of malaria. Despite the continuous effort made in this field, there are still a lack of effective vector-control programs for numerous VBDs. There are numerous reasons that limit the discovery and implementation of successful vector-control programs which include neglect of these researches in the past, reduction of the effectiveness of vector-control agents due to the emergence of resistance in vectors, poor understanding of the complex population and ecological structures of vectors due to their highly diverse species (Hill et al., 2005).

1.3 Tick-borne Disease

Some clinically important emerging tick-borne diseases (TBDs) in the United States are Lyme disease, babesiosis, anaplasmosis, Powassan virus disease, tularemia and spotted fever rickettsiosis (also includes Rocky Mountain spotted fever). State and local health departments (in 2017) have reported that there is a record number of above-mentioned TBD cases, which is increased from 48,610 cases in 2016 to 59,349 cases in 2017 (CDC, 2018c). Data from Notifiable Disease Surveillance System (NNDSS) have shown that the number of tick-borne diseases have doubled in between

2004 and 2016, and also researchers have discovered seven new human infecting tick-borne pathogens during this period (CDC, 2018b, c). According to CDC, prevention to these threats are not fully under control in the United States. In order to respond ticks and tick-borne diseases, vector control organizations are facing increasing demands (CDC, 2018a).

1.4 *Ixodes scapularis* ticks

Ixodidae is economically important and the largest family which consists of 13 genera and 650 species approximately. The characteristic feature of this family is that there is a presence of a plain sclerotized scutum or shield on the dorsal side (mostly ornated in white or gold patterns against a gray or brown background). They feed on their hosts for long period of time (from several days to weeks depending on various factors like species, life stage or host type). All the Ixodid ticks contain 4 segments in the palp and paired chelicerae in their mouth parts with toothed hypostome situated ventrally (Sonenshine and Reo, 1991).

1.5 Developmental cycle of *I. scapularis*

Blood meal is not a prerequisite for mating of male and female *I. scapularis*. Male inserts its hypostome and chelicerae into female's genital pore (while palps being spread on sides) during mating to transfer the spermatophore. Female then engorges the blood meal and finally drop off the host. After 14 days, female ticks lay multicellular eggs coated with wax from the genital pore. The eggs finally embryonate within 35 days and hatch into the larvae. These larvae start seeking for hosts in groups to feed. After finding an appropriate host, they feed for four days, engorge and drop off of the host. In approximately 28 days, larvae molt to the nymphal stage. After 14 days of molting, nymphal ticks become matured and start seeking the host to feed. Nymphs feed on host for 4-6 days, drop off of the host and undergo molting to emerge as adult male/female in 4-5 weeks. It takes adults 14 days for maturation after which their cuticle stiffens and sclerotin forms, and they are ready to mate either during feeding on large mammals or off host (Kocan et al., 2015).

1.6 Development of tick cell culture

After the first report for the establishment of continuous tick cell lines (Bell-Sakyi et al., 2003), there are several ticks cell lines currently being reported. This discovery of tick cell lines has proven to be an important breakthrough because they opened a door for researchers for the study of tick biology and tick-pathogen interaction *in vitro*. Beside this, it has also decreased an animal dependence for research of ticks and tick-borne diseases. It was the *I. scapularis* derived cell lines that was used for the first time for the propagation of clinically important tick-borne pathogens like *Borrelia, Rickettsia, Anaplasma, Ehrlichia* and many viruses (Zivkovic et al., 2009). Beside this, ticks cell culture have recently been applied to genetic transformation studies and gene silencing (de la Fuente and Contreras, 2015).

1.7 Langat Virus

Langat virus (LGTV) is a virus of the genus flavivirus. It was isolated for the first time in Malaysia and neighboring Thailand from pools of *Haemaphysalis* and *Ixodes granulatus* ticks (Smith, 1956). The genus flavivirus contains approximately 70 viruses, and they can cause several diseases in humans like febrile illness, hemorrhagic fever, biphasic fever and encephalitis. The transmission of these flaviviruses into humans takes place by either bite from infectious ticks or mosquitoes or through blood transfusions (Gould and Solomon, 2008; Gritsun et al., 2003). Tick-borne flaviviruses are found in different parts of the world in Asia, Europe and in America which includes Powassan virus (POWV), Langat virus (LGTV), Tick borne encephalitis virus (TBEV), Louping ill virus, Kyasanur Forest disease virus and Omsk hemorrhagic fever virus. Most of the above mentioned viruses are closely related both genetically and antigenically and they are all the members of mammalian tick borne flaviviruses (Calisher et al., 1989; Gritsun et al., 2003).

LGTV is low-pathogenic or non-pathogenic tick-borne flavivirus under natural conditions for humans. However, the virus is known to infect rodents like ground rats and the noisy, long-tailed rat. LGTV when inoculated intracerebrally in young laboratory mice causes encephalitis but it has not been found to cause an overt disease in adult rodents and non-human primates in their natural environment (Gritsun et al., 2003).

Since LGTV resembles both genetically and phylogenetically with TBEV (a deadly human pathogen) and does not cause disease in human, it is widely used as a model virus in the laboratory for the research related to TBEV (Marin et al., 1995; Price et al., 1970). Also, LGTV was used as a live-attenuated vaccine to prevent TBEV infection but was discontinued because encephalitis was observed among few humans administered with this vaccine (Price et al., 1970).

1.8 Exosome

Exosomes are small secretory vesicles whose size range from 30 to 100 nm and are derived from cell's endosomal pathway during the maturation of endosomes. The membranes of exosomes are rich in lipids like cholesterol, sphingolipids and ceramide (De Toro et al., 2015) as they are released from almost all cell types. Late endosomes are developed after the maturation of early endosomes which characteristically form Multivesicular Bodies (MVB) or Intraluminal Vesicles (ILV) inside endosome's lumen. These MVBs/ILVs finally fuse with lysosomes and plasma membrane to degrade the contents within cells. This results to release those contents into the extracellular environment in the form of exosome (Beach et al., 2014). After the discovery of exosome, the biogenesis, composition and secretion of exosomes have been extensively studied. Exosomes were previously thought to be cell's garbage bags but now they are considered as important nano-vehicles that transport specific cargo in and out of the cells (Villanueva, 2014). The mechanism of packaging these cargos into the vesicles, their biogenesis and excretion are poorly understood. However, these mechanisms are believed to be facilitated either through ESCRT (Endosomal sorting complex required for transport)-dependent pathway (Colombo et al., 2013) or ceramide-dependent pathway (Trajkovic et al., 2008).

In recent years, exosomes are proven to be a key facilitator of intercellular communications to perform various cellular processes like cell growth, differentiation, migration, immune cell modulation and neuronal signaling (Meckes and Raab-Traub, 2011). Beside these, exosomes are considered to be a key player in spreading human diseases. For example, exosomes have been linked to tumor growth, progression and metastasis in cancer. Exosomes are present abundantly in circulating biological fluids

such as blood, urine and Cerebrospinal fluid (CSF) and it has already been discovered that exosomes contain specific RNAs, DNAs, proteins and lipids from their cells of origin (Meckes and Raab-Traub, 2011).

Viruses are intracellular obligate parasites which hijack cellular pathways so that they can complete their replication and life cycle and some viruses are able to manipulate the host vesicular trafficking machinery pathway for their assembly and transmission. Some viruses such as human immunodeficiency viruses (HIV) are considered to hijack the exosome pathway by directly manipulating machinery involved in the biogenesis of exosomes, like the ESCRT proteins-dependent pathway (Votteler and Sundquist, 2013). These characteristics of exosomes have generated tremendous interest in the scientific community working in this field and it is being considered that exosomes function as delivery vehicles and they may be used in diagnostics and therapeutics.

1.9 *Is*SMase

Ticks are small arthropods which rely on host's blood for its survival. So, ticks develop complex strategies to facilitate blood feeding for long period of time. These strategies include the production of several pharmacological agents in tick saliva that modulate the itch, pain, wound healing, blood clotting, immune responses and inflammation in the host (Alarcon-Chaidez et al., 2009). They make easier for ticks to feed for extended time period within the host and allowing ticks a sufficient amount of time to transmit pathogens into the host (Nuttall and Labuda, 2004). Among several pharmacological agents, a novel sphingomyelinase-like enzyme (*Is*SMase) in *Ixodes scapularis* tick is secreted in saliva which modulates the host adaptive immune response by inclining the host CD4+ T-cells to shift from a neutralizing Th1 cytokine response towards a Th2 cytokine profile. This *Is*SMase directly programs the CD4 T-cells to express IL-4 (Interleukin-4), a hallmark of Th2 effects (Alarcon-Chaidez et al., 2009). *Is*SMase is a neutral form of sphingomyelinase and it is Mg2+ dependent enzyme. The bioinformatics analysis showed that *Is*SMase gene in ticks is highly homologous to the sphingomyelinase D (SMase D) protein of *Loxosceles* venomous spider (Alarcon-Chaidez et al., 2009). Similarly, a bioinformatics sequence similarity

search done by a research group (Dias-Lopes et al., 2013) has identified several novel SMases D in different pathogenic organisms like bacteria, fungi, mites, ticks and spiders.

Naked viruses are released by disruption of the plasma membrane from the infected host's cells, however, in enveloped viruses, there is a presence of host cell-derived lipid bilayer acquired during budding that surrounds the virus nucleocapsid (Kiyokawa et al., 2004). Membrane lipids are not randomly incorporated into viral envelope during the release of enveloped viruses. Virions may have different lipid composition than that of the host cell membrane (Kiyokawa et al., 2004).

The flavivirus lifecycle is intimately associated to cellular lipids. RNA's replication and acquisition of lipid envelope are associated not only with host cell membrane but also with specialized membranous structures derived from the endoplasmic reticulum (ER) (Gillespie et al., 2010). Flaviviruses possess a feature to selectively manipulate host cell lipid metabolism during infection and promote the synthesis and accumulation of specific lipids (glycerol-phospholipids, cholesterol, sphingolipids and fatty acids) within infected cells (Martin-Acebes et al., 2011). Among the cellular lipids, sphingolipids merit special attention since it is a major target tissue during flavivirus infection. These viruses can take advantages of sphingolipid content present in biological membranes to develop specialized membrane sites for RNA replication and particle biogenesis (Chukkapalli et al., 2012). Lipidomic analyses have shown that there is an increase in the content of both sphingomyelin (SM) and ceramide in flavivirus-infected cells. Ceramide is specifically associated with West Nile Virus (WNV, a flavivirus) replication and viral particle biogenesis (Aktepe et al., 2015; Martín-Acebes et al., 2014; Perera et al., 2012). For the first time, we showed that SM levels modulate LGTV infection or vice-versa *in vivo* and *in vitro*, thus identifying this sphingolipid as a key cellular factor for LGTV replication.

SMase D (also called as sphingomyelin phosphodiesterase D or phospholipase D) is an important toxin found in *Loxosceles spp* spiders' venom and it is responsible for dermal necrosis (Forrester et al., 1978; Tambourgi et al., 1998). SMase D in spider results in the catalytic hydrolysis of SM lipid to form ceramide 1-phosphate (acyl sphingosine 1- phosphate) and choline, however the mammalian sphingomyelinase

converts SM into ceramide and phosphocholine, SM being one of the major constituent that is present in the outer surface of the lipid bilayer in the plasma membrane of most eukaryotic cells (De Andrade et al., 2006; Forrester et al., 1978).

The presence of this toxic enzyme in spiders, ticks and bacteria (which are all medically important but are distantly related organisms) made it more interesting to study. Bioinformatics analysis on SMase D identified a common motif at the C-terminal end (with unknown function) supports the inference that these enzymes are originated from a broadly conserved glycerophosphoryl diester phosphodiesterase (GDPD) family, even though this motif is absent in this family (Cordes and Binford, 2006). These conserved C-terminal motifs (SMD-tail) help to make the entire internal structure of SMase D TIM barrel stable. The above mentioned works suggest that the enzyme SMase D are present widely in several genera, and they possibly act as a common pathogenic effector for a significant diversity of organisms (Dias-Lopes et al., 2013).

2. RESULTS

2.1 Bioinformatics analysis showed *Is*SMase closely related to spider's venomous protein ortholog

As discussed earlier, the bioinformatics analysis of *Is*SMase gene in ticks showed homology with the SMase D protein of *Loxosceles* venomous spider (Alarcon-Chaidez et al., 2009). Along with this, it was also identified that several novel SMases D were present in different clinically important pathogens like bacteria, ticks, fungi, spiders and mites (Dias-Lopes et al., 2013). The identity alignment of a novel sphingomyelinase-like enzyme, *Is*SMase (Q202J4) in *Ixodes scapularis* tick saliva gives an idea about this tick molecule to be an ortholog of venomous protein (Alarcon-Chaidez et al., 2009). The *Is*SMase gene transcripts (194 bp product) were amplified in unfed nymphs, 24 hours partially fed nymphs and uninfected-tick cells (Figure 1).

We performed detailed bioinformatics along with the comparative and feature prediction analysis of *Is*SMase protein (accession number ABD73957) with its orthologs from other ticks like *Amblyomma maculatum* (Hypothetical protein; AEO33547) and *Rhipicephalus pulchellus* (sphingomyelin phosphodiesterase; JAA56531), and other orthologs from spider like *Loxosceles similis* (*lo*xtox protein; ANY30961), *Hemiscorpius lepturus* (venom toxin; API81381) and *Scicarius patagonicus* (Sphingomyelinase D; COJB69). ClustalW alignment of *I. scapularis* SMase D amino acid sequence showed 40.7% and 43.1% identity with *A. maculatum* and *R. pulchellus* ticks respectively, and 41.1%, 39.4% and 37.2% identity with *S. patagonicus*, *H. lepturus* and *L. similis* spider orthologs respectively (Figure 2A). A conserved motif/domain glycerophosphoryl diester-phosphodiesterase (GDPD-like SMase D-PLD) from SMase D family was found by the comparative sequence analysis of *Is*SMase protein with tick or spider orthologs (highlighted with a black box), and the underlined one being the predicted leader peptide for *Is*SMase (Figure 2A and 2B). Domain analysis showed that *Is*SMase and other SMase D orthologs share the catalytic sites. The residues (H34, H70, C76, and C80) highlighted with arrows are important for catalytic activity (Figure 2A). The catalytic

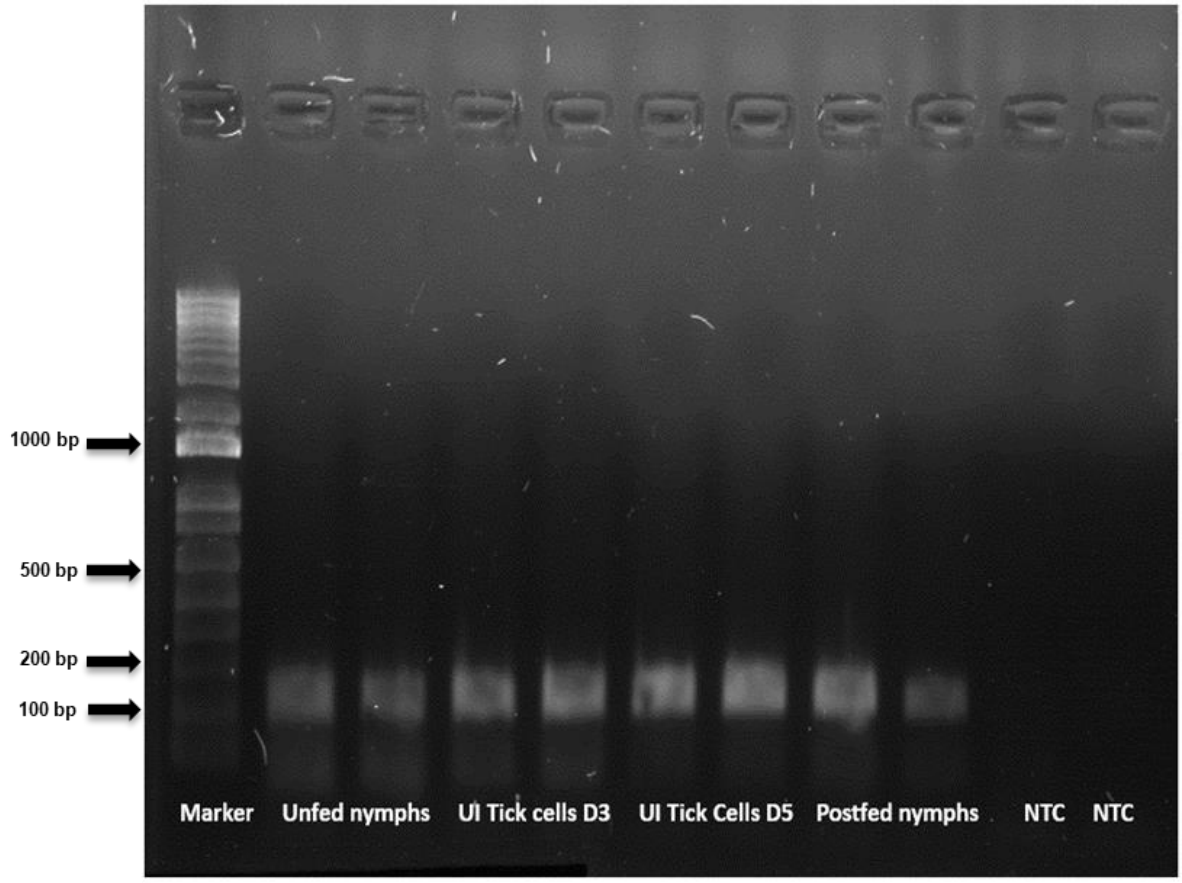

Figure 1. Amplification of *I. scapularis* Sphingomyelinase-like protein (*Is*SMase) from ticks and tick cells.

PCR amplification of *Is*SMase from I. scapularis unfed or post-fed nymphal ticks or uninfected ISE6 tick cell line cDNA is shown. Similar size fragments were amplified from all three tested groups, and band of approximately 194 bp was detected on 1% agarose gel. Marker indicates size of the product amplified and NTC denotes no template control. M represents DNA ladder.

site lies in between 29-65 residues with two catalytic loops being identified (present in between 64-71 and 74-78 residues) (Figure 2B). There was an overlap observed in the magnesium binding site with the catalytic site which lied in between the residues 49/51-109 (Figure 2B).

The phylogenetic analysis revealed that *Is*SMase has a close relation and forms a clade that cluster with venomous spider ortholog SMase D. Both *A. maculatum* and *R. pulchellus* tick orthologs formed different sub-clades within the main clade, which suggests that there are differences in new genera ticks (Figure 3A). Also, Tick SMase D orthologs from *A. maculatum* and *R. pulchellus* showed that there is a high degree of divergence to *Is*SMase and the spider orthologs (Figure 3B). Apart from this, protein feature prediction analysis showed that there is a presence of twelve Protein Kinase C (PKC) phospho-sites (with 8 Threonine and 4 Serine residues), three Tyrosine Kinase phospho-sites (with 3 Tyrosine residues), two cAMP/cGMP dependent phospho-sites (with 1 each of Serine and Threonine residues), six Casein Kinase II phospho-sites (with 5 Serine and 1 Threonine residue), three N-myristoylation sites (with 3 Glycine residues) and two N-glycosylation sites (with 2 N-Linked (GlcNAc...) Asparagine residues) (Fig. 3C). The presence of these phosphor and protein modification sites in *Is*SMase suggest that the enzyme may be highly functional in ticks.

2.2 *Is*SMase is not influenced by developmental changes in *Ixodes scapularis* tick's life cycle

To confirm the expression of *Is*SMase in ticks and support the bioinformatics analysis done above, we first performed Quantitative Real Time-PCR (QRT-PCR) analysis. It showed that *Is*SMase is expressed in all developmental stages of ticks (larvae, nymphs, adult males and adult females). This also gives an idea about the importance of this molecule in different developmental stages of ticks. The analysis showed that mRNA expression levels of *Is*SMase appeared to be lower in larval and nymphal ticks, and it was higher in adult male and adult female ticks (Figure 4A). However, there were not any significant differences ($P > 0.05$) in expression of *Is*SMase among different developmental life cycle stages (Figure 4A). This result suggests that *Is*SMase transcripts are not influenced by developmental changes in ticks.

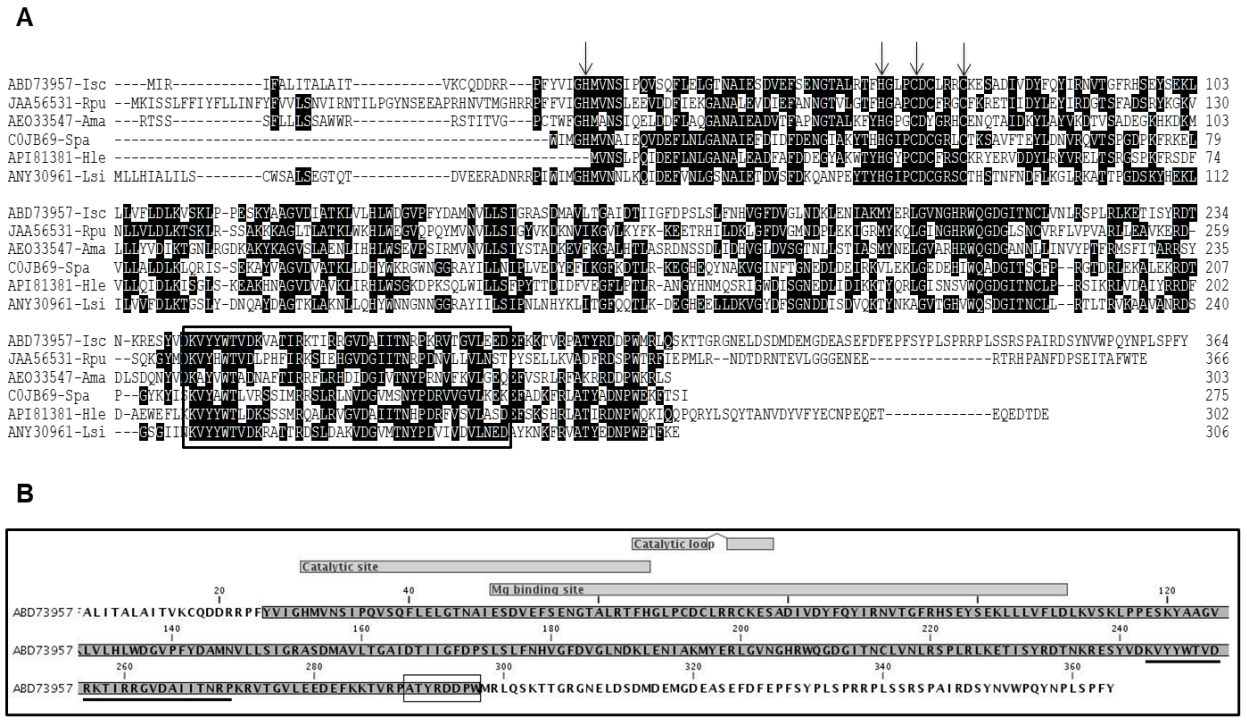

Figure 2. Sequence alignments and prediction analysis of *Is*SMase with tick and spider orthologs. (A) The deduced *I. scapularis* (*Isc*) SMase (*Is*SMase) amino acid sequence alignment (with other orthologs) using ClustalW program in DNASTAR Lasergene is shown. Matching residues are shaded in black color. GenBank accession numbers for *Rhipicephalus pulchellus* (*Rpu*) sphingomyelin phosphodiesterase, *Amblyomma maculatum* (*Ama*) hypothetical protein, *Scicarius patagonicus* (*Spa*) Sphingomyelinase D, *Hemiscorpius lepturus* (*Hle*) venom toxin, *Loxosceles similis* (*Lsi*) *l*oxtox protein sequence is shown. VectorBase accession numbers for *Isc*, *Rpu*, *Ama* are provided. Total length of the amino acid sequence is provided at right end of each sequence. (B) Annotation/prediction analysis performed in DNASTAR, for *Is*SMase protein sequence is shown. The catalytic site and Magnesium binding sites and their overlap is shown. The underlined sequence indicates the glycerol-phosphodiester phosphodiesterase-like motif and the SMaseD consensus motif is shown as boxed amino acid sequence.

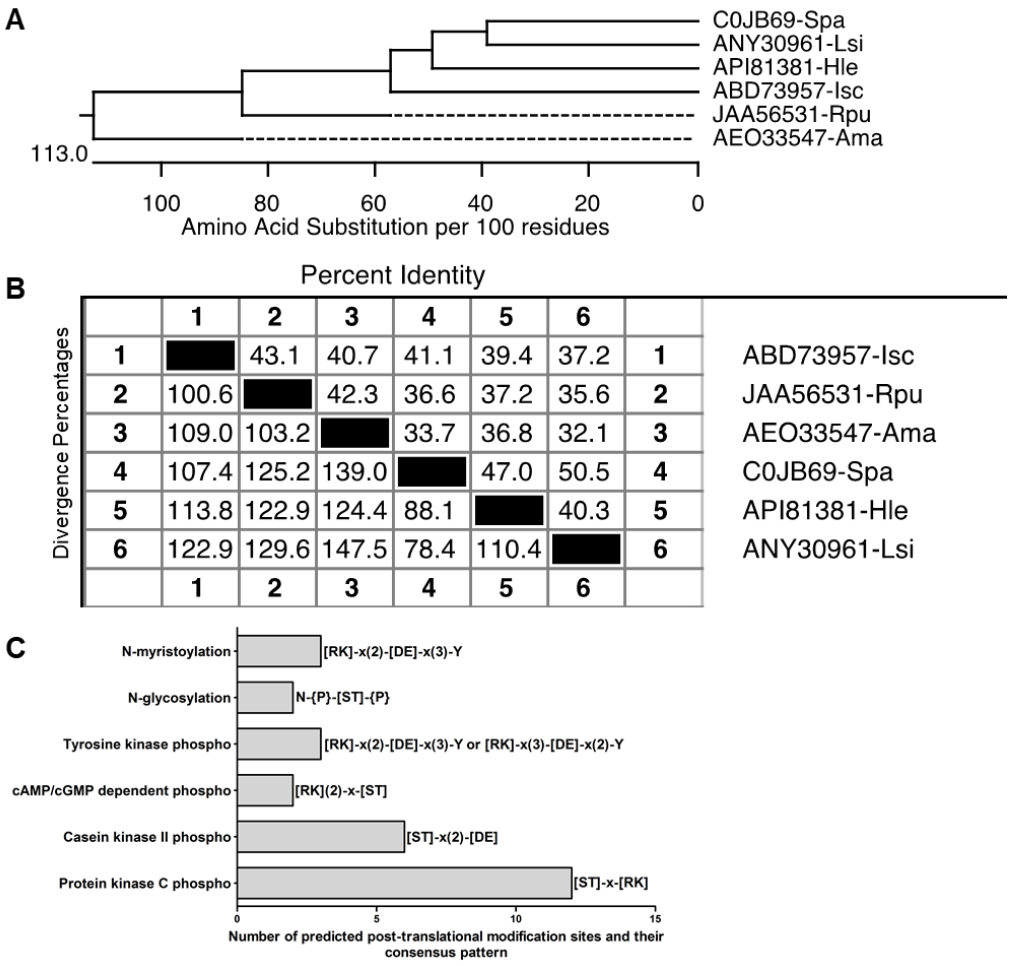

Figure 3. Bioinformatics comparison of *Is*SMase to other orthologs by phylogenetic analysis, percent identity, and protein feature prediction analysis.

(A) Phylogenetic analysis comparing the *Is*SMase and other orthologs was performed in DNASTAR by ClustalW slow/accurate alignment method using Gonnet as default value for protein weight matrix. Scale at the bottom denotes amino acid substitutions per 100 amino acid residues. (B) Percent identity (horizontally above black boxed diagonal) and divergence (vertically below black boxed diagonal) of *Is*SMase nucleotide sequence in comparison to *Rhipicephalus pulchellus* (*Rpu*) sphingomyelin phosphodiesterase, *Amblyomma maculatum* (*Ama*) hypothetical protein, *Scicarius patagonicus* (*Spa*) Sphingomyelinase D, *Hemiscorpius lepturus* (*Hle*) venom toxin, *Loxosceles similis* (*Lsi*) loxtox protein sequence is shown. (C) Protein feature prediction analysis showing *Is*SMase protein modification sites and relevant amino acids as functionally active residues. *Is*SMase contains twelve Protein Kinase C (PKC) phospho-sites, six Casein Kinase II phospho-sites, two cAMP/cGMP dependent phospho-sites, three Tyrosine Kinase phospho-sites, two N-glycosylation sites, and three N-myristoylation sites.

2.3 *Is*SMase expression is reduced upon LGTV infection in both *in vitro* (tick cells) and *in vivo* (fed/unfed nymphs)

It has been already shown that LGTV can readily infect ISE6 tick cells resulting in an increased viral load at 72 hours post-infection (p.i.) (Zhou et al., 2018). In this study, QRT-PCR analysis showed that unfed nymphal ticks generated by synchronous infection were positive for LGTV loads (Figure 4B). Viral loads were detected in all synchronously infected ticks; however the *Is*SMase transcript levels were reduced significantly ($P<0.05$) in LGTV-infected unfed nymphs (Figure 4C). We performed one more similar experiment in fed nymphs. This is a transmission experiment where LGTV-infected *I. scapularis* ticks (generated by synchronous infection) was fed on uninfected mice, with ticks being collected at 24 hours during feeding. QRT-PCR analysis of LGTV-PrM transcript levels showed that viral burden was significantly higher ($P<0.05$) in the infected fed nymphs in comparison to uninfected fed nymphs. We also found that *Is*SMase transcript levels were reduced significantly ($P<0.05$) in LGTV-infected fed nymphs as compared to uninfected ones (Figure 4E), and very similar to infected unfed-nymphs.

We performed another experiment to determine *Is*SMase levels in ISE6 tick cells (*in vitro*). For this, we first performed time dependent LGTV-infection in tick cells (with Multiplication of Infection; MOI=1) with two different time-points; early (24 hours p.i.) and late time points (72 hours p.i.). QRT-PCR analysis showed that there was a significant increase ($P<0.05$) in LGTV-infection over the time points of both 24 and 72 hours p.i. (Fig. 5A). And in these same samples, we found that *Is*SMase transcripts were significantly reduced ($P<0.05$) at both tested time points in infected groups when compared to the uninfected control groups (Figure 5B). We also performed a dose-dependent experiment by infecting similar number of ISE6 tick cells for 72 hours p.i. with different MOIs (MOIs of 1, 2, and 3; uninfected group (MOI=0) considered as control). We observed that ISE6 tick cells were susceptible to LGTV infection at MOI 3, but they were not at MOI 1 and 2. Nearly 20-25% of tick cells showed cell death at MOI 3 of LGTV-infection. LGTV-PrM transcript levels were found to be upregulated in all infected (MOI 1, 2 and 3) tick cells as compared to uninfected control (Figure 5C).

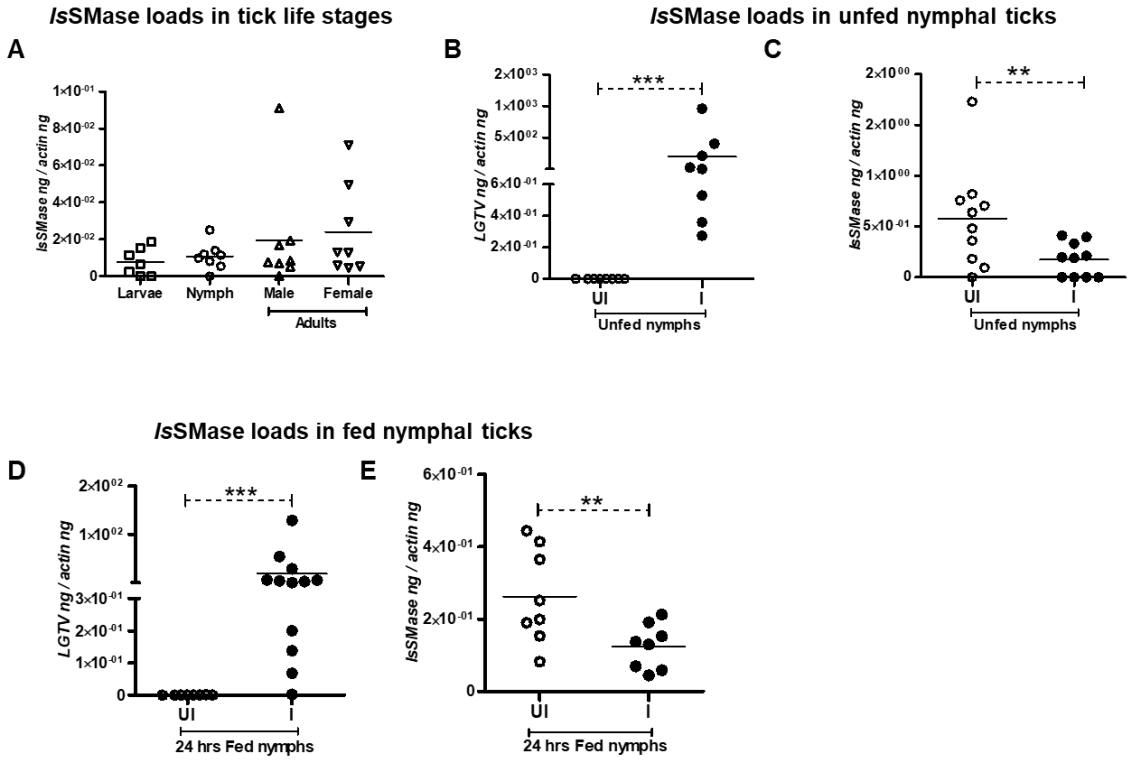

Figure 4. *Is*SMase expression is reduced upon LGTV infection in ticks.

QRT-PCR analysis showing *Issmase* gene expression levels (A) in different developmental stages of ticks. Viral loads (B, D) or *Is*SMase transcripts levels (C, E) were shown in uninfected (UI) or LGTV-infected (I) unfed (B, C) or partially fed (24 h post) (D, E) nymphs. Each square, circle, triangle, or inverted triangle indicates one tick (A-E). Open circles represent an uninfected (UI) group, whereas closed circles denote LGTV-infected (I) group (B-E). LGTV prM-E or *Is*SMase mRNA levels were normalized to tick beta-actin levels. P-value determined by Student's two-tail t test is shown. The asterisk indicates significance, and ** or *** denotes a P value of less than 0.01 or 0.001, respectively.

*Is*SMase transcripts were also found to be significantly downregulated (P<0.05) at all tested MOIs in comparison to the uninfected control (Figure 5D).

We also found that LGTV loads were abundantly present in tick cell-derived LGTV-infected exosomes (Figure 6A) which reproduced our previous published study (Zhou et al., 2018). However, we were unable to detect *Is*SMase transcript levels in tick cell-derived exosomes (Figure 6C). We did detect one of the exosomal marker (HSP70) transcript levels in the same exosome sample (Figure 6B). These data suggested that the levels of *Is*SMase were reduced/undetectable with the increase in LGTV-infection in unfed, fed (24 h) *I. scapularis* ticks and in ISE6 tick cells, and also in time and dose dependent manner.

2.4 LGTV loads were reduced and *Is*SMase transcript levels were restored upon GW4869 inhibitor treatment

Dihydrochloride hydrate (GW4869) is a selective inhibitor for neutral sphingomyelinase(s) (nSMase) which is permeable to cell membrane and affects the exosome release and production in cells. We performed an experiment to determine whether treatment with GW4869 (1 µM) with two different time points of 4 and 24 hours reduce the viral (LGTV) load in tick cells or not. QRT-PCR analysis revealed that the loads of LGTV were significantly reduced (P<0.05) at both 4 and 24 hours of GW4869 treatment (Figure 7A and 7C) and this downregulation of LGTV loads/replication correlated with a significant upregulated expression (P<0.05) of *Is*SMase in these tick cells at both tested time points (Figure 7B and 7 D). Experimental groups were compared to the mock group treated with vehicle DMSO that served as control. Equal volume of DMSO solvent that represented GW4869 inhibitor was used in our analysis.

These data showed that there is a direct association of LGTV loads/replication in suppression of the levels of *Is*SMase in tick cells. This inhibition of LGTV loads due to the treatment of GW4869 suggest that the activity of *Is*SMase enzyme may also be affected in ticks.

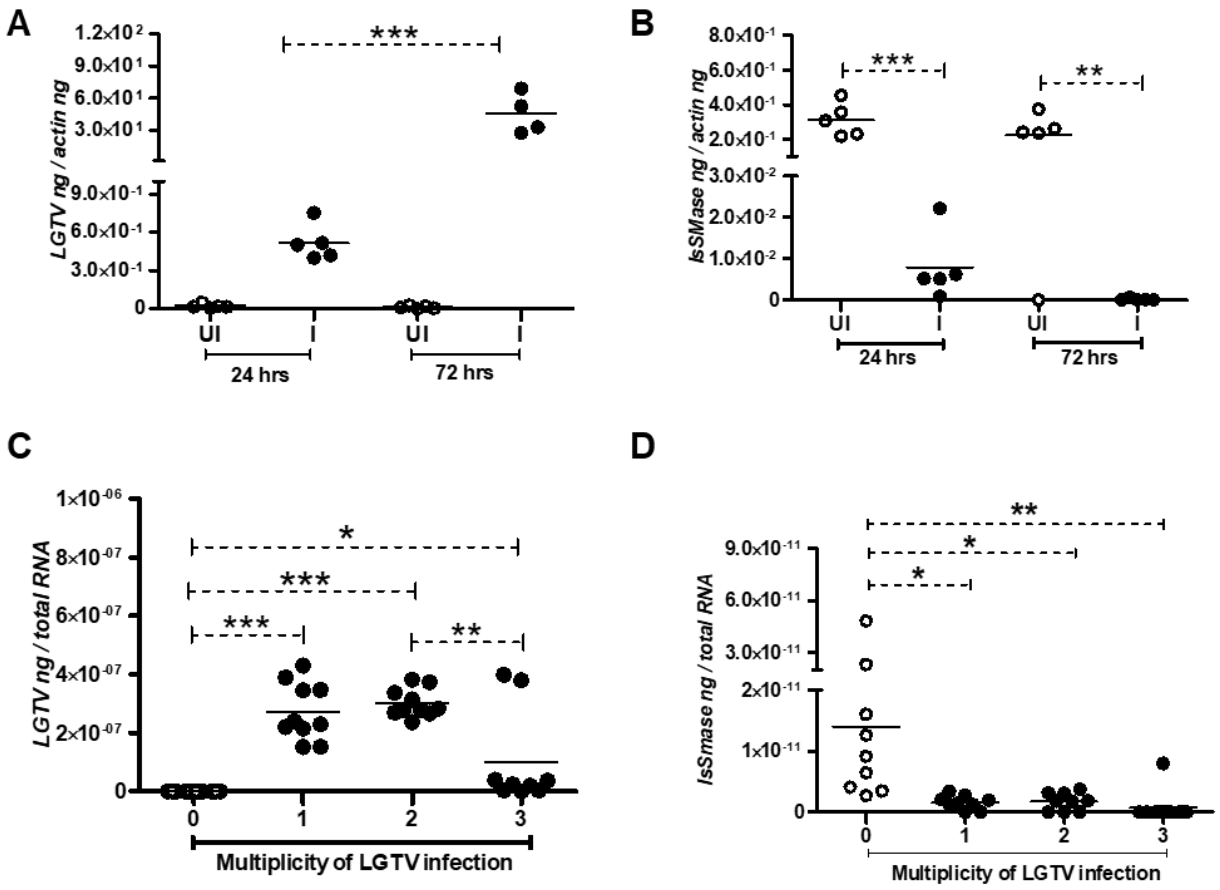

Figure 5. Reduced *Is*SMase expression upon LGTV infection in time and dose dependent.
QRT-PCR analysis showing LGTV viral loads (A, C) or *Issmase* gene expression levels (B, D) in time (A, B), or dose (C, D) response in ISE6 tick cells. LGTV loads (A) and *Is*SMase transcript levels (B) were determined at two different time points of 24 and 72 h p.i. In doses response panels (C, D), numbers (0, 1, 2, 3) indicates multiplication of infection (MOI) corresponding to LGTV loads (C) or levels of *Is*SMase transcripts (D) in uninfected (0) or LGTV-infected (1, 2, 3 MOI) tick cells. Each circle indicates one sample. Open circles represent an uninfected (UI or 0) group, whereas closed circles denote LGTV-infected (1, 2, and 3 in dose or I in time points), group. LGTV prM-E or *Is*SMase mRNA levels were normalized to tick beta-actin levels. P value determined by Student's two-tail t test is shown. The asterisk indicates significance, and *, ** or *** denotes a P value of less than 0.05, 0.01, 0.001, respectively.

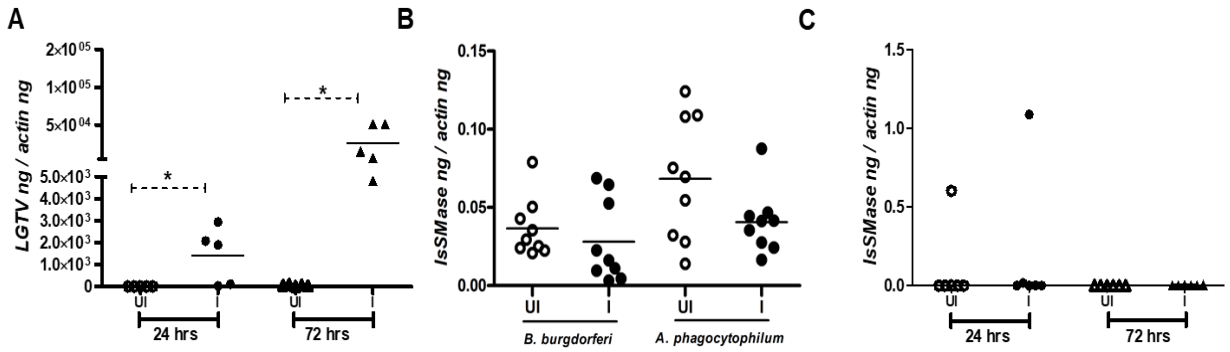

Figure 6. Exosomes derived from tick cells had low or undetectable levels of *Is*SMase.
QRT-PCR analysis showing (A) detectable LGTV loads, (B) HSP70 transcript levels and (C) low or undetectable levels of *Is*SMase transcripts in tick cells that are uninfected (UI) or LGTV-infected (I) from two time points (24 and 72 h p.i.). Each circle, triangle or inverted triangle, or square represent one replicate. Open circles indicate uninfected (UI) and closed circles denotes LGTV-infected group of tick cell-derived exosomes. LGTV prM-E, *hsp70* or *Is*SMase mRNA levels were normalized to tick beta-actin levels. P value determined by Student's two-tail t test is shown. The asterisk * indicates significance and denotes a P value of less than 0.05.

2.5 LGTV infection decreases *Is*SMase enzyme activity and build-up SM lipid

Because LGTV infection in ticks reduced *Is*SMase transcript levels, we performed *Is*SMase enzymatic activity assay (at two different time points of 24 and 72 hours p.i., with MOI=1 LGTV-infection) to address whether the increased replications of LGTV also affect the *Is*SMase enzymatic activity and its function. The enzyme activity assay revealed that LGTV infection in tick cells significantly reduced ($P<0.05$) *Is*SMase activity at an early tested time point (24 hours p.i.) however there was no difference observed at later tested time point (72 hours p.i.) (Figure 8A).

Sphingolipids are the ubiquitous constituents of almost all the cellular membranes, including plasma membranes and membrane-bound organelles. SM plays an important role in signal transduction (Bartke and Hannun, 2009). The enzyme sphingomyelinase hydrolyzes SM to release phosphocholine into the aqueous environment and ceramide that diffuse through the plasma membrane (Bartke and

Hannun, 2009; Clarke et al., 2006). Hence, the reduced activity of *Is*SMase upon LGTV-infection suggested accumulating lipid metabolism. We performed sphingomyelin quantification assay (at two time points p.i. with LGTV, 24 and 72 hours) to determine the SM levels in tick cells. This assay showed that the level of SM significantly increased ($P<0.05$) upon LGTV infection at both tested time points (24 and 72 h of p.i.) when compared with the respective uninfected controls (Figure 8B). This data suggests that there is an increase in SM levels with the decrease in *Is*SMase enzymatic activity upon LGTV infection in ticks.

2.6 *Is*SMase activity is restored upon GW4869 treatment by suppressing LGTV induced SM lipid levels

Since, the results from Figure 7 gave us an idea that the treatment of GW4869 inhibitor (at 1 µM) lowered the LGTV loads and restored the *Is*SMase transcript levels significantly, we designed an additional experiment to analyze *Is*SMase enzymatic activity and the SM levels. We treated tick cells with GW4869 inhibitor (1 µM) for 4 hours and infected these cells with LGTV (MOI 1) and collected these cells for two different time points (24 and 72 hours p.i.). We found that upon GW4869 treatment, *Is*SMase enzyme activity was significantly ($P<0.05$) increased at an early tested time-point (of 24 hours p.i.) compared to mock control (Figure 8C) suggesting that the *Is*SMase level has been restored by GW4869 treatment. No significant increase of *Is*SMase activity was observed in latter time points (72 hours p.i.). In contrast to the above result for *Is*SMase activity, SM lipid build-up was significantly inhibited ($P<0.05$) upon GW4869 treatment when compared to the LGTV-infected mock control group at both tested time points (of 24 and 72 hours p.i.) (Figure 8D). This data suggests that LGTV load is suppressed by GW4869 inhibitor in tick cells which in turn inhibited the SM lipid levels. This finally leads to the restoration of *Is*SMase enzyme activity and its function.

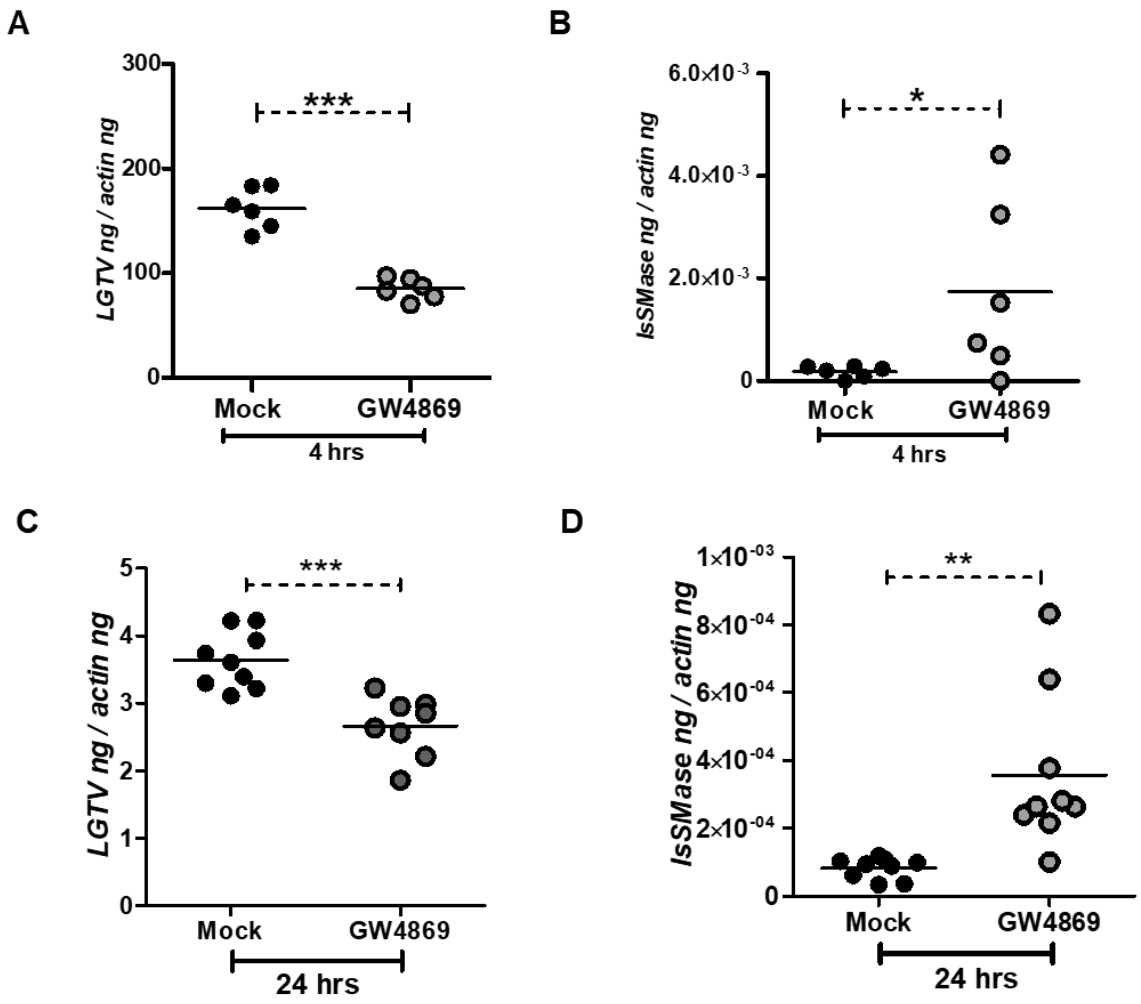

Figure 7. GW4869 treatment reduced LGTV loads and restored the *Is*SMase expression.
QRT-PCR analysis showing reduced LGTV loads (A, C) or restored *Issmase* expression (B, D) upon GW4869 pre-treatment (1 µM) of ISE6 tick cells for either 4 h (A, B) or 24 h (C, D) followed by LGTV infection (for 72 h p.i.). Both mock and GW4869-inhibitor treated groups were infected with 1 MOI of LGTV. Mock represents the group treated with vehicle DMSO. Each circle indicates one sample replicate. Black circles represent LGTV-infected Mock group, whereas grey circles denote LGTV-infected GW4869 inhibitor treated group. LGTV prM-E or *Is*SMase mRNA levels were normalized to tick beta-actin levels. P value determined by Student's two-tail t test is shown. The asterisk indicates significance, and *, ** or *** denotes a P value of less than 0.05, 0.01, 0.001, respectively.

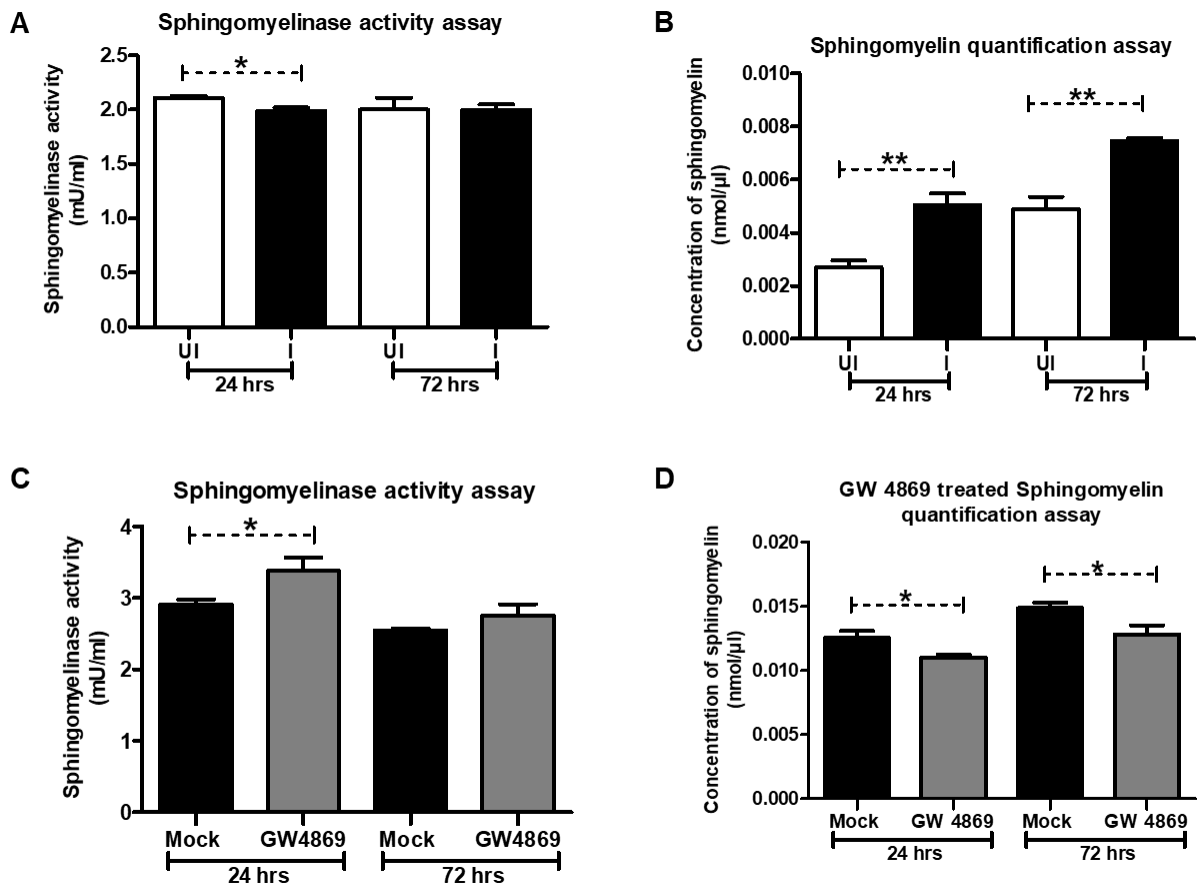

Figure 8. LGTV infection reduced *Is*SMase activity resulting in induced levels of sphingomyelin lipids but GW4869 treatment restored the infection-mediated effects.

(A) Sphingomyelinase activity measurement assay showing reduction upon LGTV-infection at 24 h (p.i.). No differences were observed at 72 h p.i.. (B) Sphingomyelin quantification assay showing induction or accumulation of lipid upon LGTV-infection at both 24 and, 72 h post-infection. (C, D) Treatment with GW4869 (1 µM) inhibitor showing restoration of the LGTV-mediated reduced *Is*SMase activity and inhibition of the accumulated levels of sphingomyelin lipids. White bars represent uninfected (UI) and black bars denote LGTV-infected (I). Mock represent group treated with vehicle DMSO. Both mock and GW4869 groups were infected with LGTV (MOI 1) for indicated time points. *Is*SMase activity and lipid levels were measured in milliunits/ml. P value determined by Student's two-tail t test is shown. The asterisk indicates significance, and *, ** denotes a P value of less than 0.05 or 0.01, respectively.

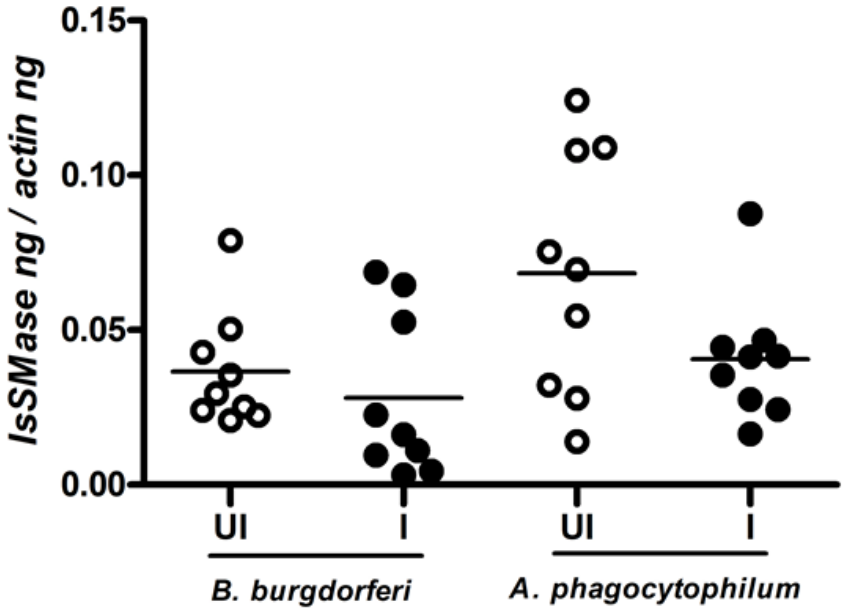

Figure 9. *Is*SMase expression is unaffected in presence of other pathogens.
QRT-PCR analysis showing levels of *Is*SMase transcripts in unfed *I. scapularis* ticks infected with either an extracellular bacterium *Borrelia burgdorferi* or an intracellular bacterium *Anaplasma phagocytophilum*. Open circles indicate uninfected (UI) and closed circles denote infected (I) groups. *Is*SMase mRNA levels were normalized to tick beta-actin levels. No significance differences were noted between the uninfected and infected groups, respectively.

The overall results from our study suggested that tick-borne LGTV suppress a spider venomous ortholog *Is*SMase enzyme levels and its activity in ticks which then induce SM lipid levels and may facilitate the replication of virus and their budding into exosomes for transmission.

3. DISCUSSION

The molecular mechanisms that support the survival strategies of many of the vector-borne pathogens have not been clearly understood. *Is*SMase, a novel Sphingomyelinase-like enzyme in *Ixodes scapularis* tick saliva showed high homology with SMase D, which is sphingomyelinase D protein in *Loxosceles* venomous spider (Alarcon-Chaidez et al., 2009). The same study showed that *Is*SMase modulated the host adaptive immune response by inclining the host CD4+ T-cells and thus shift a neutralizing Th1 cytokines response towards a Th2 cytokines profile. *Is*SMase directly programmed CD4+ T cells in order to express Interleukin 4 (IL-4; a hallmark of Th2 effect), which means that *Is*SMase can regulate the expression and programming of IL-4. Same group of researchers has proposed that a freeze-thaw stable structure within this molecule may bind to a Toll-like receptors (TLRs) or receptors on innate immune cells or antigen presenting cells (like monocytes or dendritic cells) due to which Th2 differentiation may switch on (Alarcon-Chaidez et al., 2009).

We determined the role of *Is*SMase in tick-borne flavi-viral (LGTV) infection in this study. After the detailed bioinformatics and comparative analysis, it was confirmed that *Is*SMase in ticks and SMase D in venomous spider are protein orthologs. Sphingomyelinase D (SMase D) protein in venomous spider were known to have several isoforms. These isoforms are broadly classified into two major groups, class I and class II SMase D proteins. In class I SMase D proteins, a single disulphide bridge and variable loops are present, however, in class II proteins, an additional intra-chain disulphide bridge is present which connects a flexible loop with a catalytic loop. Class I proteins is relatively more toxic than class II (Binford et al., 2009; Dias-Lopes et al., 2013; Pedroso et al., 2015; Zobel-Thropp et al., 2010). ClustalW alignment of SMase D amino acid sequence of *I. scapularis* (*Is*SMase) showed higher identity with *A. maculatum* and *R. pulchellus* ticks. However, the phylogenetic analysis showed that *Is*SMase formed a clade closer to the spider venom protein orthologs from *H. lepturus, S. patagonicus*, and *L. similis*. The protein feature prediction analysis showed *Is*SMase to be a multifunctional protein that have various active sites and motifs. Because there

are several phospho-sites present, *IsSMase* is perhaps regulated by the process of phosphorylation.

*Is*SMase role in infections with tick-borne pathogen transmission has not been studied. Previous study has shown that some medically important arthropods (ticks and mosquitoes) secrete extracellular vesicles (EVs) containing exosomes which help to mediate the transmission of flavi-viral RNA and proteins/polyproteins into human cells (Zhou et al., 2018). The same study showed that infection of tick-borne LGTV was possible in *I. scapularis* (ISE6) tick cells leading to increased viremia at 72 hours post-infection with the dissemination of viral proteins, both negative and positive strands of LGTV-RNA via secured exosomes. This study (Zhou et al., 2018) leaded us to further analyze the importance of neutral sphingomyelinase(s) in *I. scapularis* ticks upon LGTV infection.

These findings from our study showed that LGTV infection reduced *Is*SMase levels in tick cells and unfed/fed ticks dramatically. The reduced *Is*SMase levels resulted to build SM lipid levels up which in turn might facilitate budding and release of exosomes containing LGTV viral RNA and proteins. Furthermore, upon GW4869 inhibitor treatment, the reverse trend was seen where *Is*SMase expression was restored. This suggests that an involvement of GW4869 molecule results in inhibiting the replication and transmission of LGTV via blocking/inhibition of exosome release and dissemination. On the basis of results we observed, we assume that the viral replication is perhaps being interfered by the venomous properties of *Is*SMase and hence suggests that there might be an important role of this molecule in anti-viral pathways. The increased expression of *Is*SMase and its enzymatic activity after GW4869 treatment perhaps suggests a negative role for this enzyme in the biogenesis of exosome.

Also, a report suggests that genome replication of positive-strand RNA viruses is facilitated by host lipids (Zhang et al., 2019). West Nile Virus (WNV) infection increased sphingomyelin levels both *in vivo* and *in vitro* due to which SM have been considered as an antiviral target against WNV pathogenesis (Martin-Acebes et al., 2016). Sphingolipids are considered as “choke points” for targeting the blockage of virus transmission during dengue virus infection and replication (Chotiwan et al., 2018). Several studies have shown that cell membrane components like sphingolipids facilitate

in almost every step of virus life cycles that include attachment and fusion of membrane, replication, protein sorting, intracellular transport and budding/exogenesis of viral particles and virions (Bartke and Hannun, 2009; Hannun and Obeid, 2018; Schneider-Schaulies and Schneider-Schaulies, 2015). During viral genome replication, Influenza A virus (IAV) has been found to manipulate the cellular signaling and thus the sphingosine metabolism by activating the transcription factor NF-kB and the sphingosine kinase (Vijayan and Hahm, 2014). Also, during viral assembly and budding, human immunodeficiency virus (HIV) interacts directly with glycosphingolipids, however hepatitis C virus (HCV) uses the lipid components (Hirata et al., 2012; Schneider-Schaulies and Schneider-Schaulies, 2015). Sindbis virus replicates better in the absence of acid sphingomyelinases (Jan et al., 2000). Measles virus activates the sphingomyelinases (SMases) whereas rhinovirus stimulates the ceramide-enrichment and endocytosis (Avota and Schneider-Schaulies, 2014; Dreschers et al., 2007). Our study suggested that the expression and activity of *Is*SMase is inhibited by LGTV and thus induced SM lipids' production and accumulation. However, GW4869 (1 μM) treatment inhibited virus induced SM lipid production but restored *Is*SMase expression and activity as a feedback loop. This suggests *Is*SMase (venomous ortholog) specific role in inhibition of tick-borne viral replication given that *Anaplasma phagocytophilum* and *Borrelia burgdorferi* bacteria showed no significant reduction in *Is*SMase expression (Figure. 9). This specific viral-mediated *Is*SMase inhibition clearly indicates this enzyme functions in antiviral activity and indicates a new role of this venomous protein in tick defense mechanism(s).

4. MATERIALS AND METHODS

4.1 Ticks, synchronous infections, and tick feeding on mice

Unfed *Ixodes scapularis* ticks were obtained from BEI resources (ATCC)/CDC and were maintained in our laboratory. Ticks were kept with approximately 98% relative humidity at room temperature under a photo period of 10 h of darkness and 14 h of light. We followed published protocols for synchronous infection of *Ixodes scapularis* ticks (Mitzel et al., 2007; Taank et al., 2018). Unfed nymphs were kept in sterile 1.5 ml eppendorf tubes. Out of 48 nymphs (used in total), 24 nymphs (12 in each tube) were maintained as uninfected (controls) and remaining 24 (12 in each tube) were infected with LGTV by synchronous infection. For synchronous infection, nymphs were immersed into 0.5 ml of complete Dulbecco's modified eagle's medium (DMEM) containing 1×10^7 pfu/ml of LGTV. For the uninfected group (control), nymphs were immersed into DMEM media with no virus in it. These tubes were incubated at 34º C for 45 minutes (tubes vortexed in every 10 minutes interval to redistribute ticks into the media). These tubes were then chilled on ice (for 2 minutes) and centrifuged at 200x g for 30 seconds. Nymphs were then taken out of media and washed twice with cold 1x PBS (by centrifugation) to get rid of virus and media being attached on the tick's body surface. Ticks were then dried with Whatman paper and transferred into sterile collection tubes that had holed caps covered with sterile nylon mesh cloth. The uninfected and infected nymphs were kept in different tubes and labelled properly. Tubes containing ticks were kept in an incubator for 17 days maintained at room temperature and a relative humidity of 98%. LGTV-infected ticks generated by this method were used as LGTV-infected unfed ticks in the study. Remaining LGTV-infected ticks were partially fed (24 hours during feeding- DF) on wild type C57BL/6 mice (purchased from Charles River Laboratories, Inc.) and were pulled with forceps. Uninfected ticks being fed on naïve C57BL/6 mice, were used as controls. All animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH, USA. Institutional Animal

Care and Use Committee (IACUC; protocol # 18-011) approved the protocol for blood feeding of *I. scapularis* ticks on mice.

4.2 *In vitro* tick cell culture, infection and exosome isolation from cell culture supernatants

Ixodes scapularis ISE6 tick cell line was used in our study. Tick cells were grown and maintained in the same way as done by Zhou et al., 2018. LGTV virus used in this experiment was laboratory virus stocks of Wild type LGTV (LGT-TP21) strain maintained in Vero monkey kidney cells. 5×10^5 cells were seeded in 12-well plates, and infected with LGTV (with MOI 1, for two different time points (24 and 72 hours p.i.), or with three different MOIs of 1-3 for dose response collection at 72 hours p.i.). Exosomes were isolated from tick cell culture supernatants by differential ultracentrifugation method as described in recent studies (Théry et al., 2006). We used concentrated cell culture supernatants to isolate exosomes. Purified exosome were stored at -80 °C in RNA lysis buffer and used for RNA extractions.

4.3 RNA extraction, cDNA synthesis, and QRT-PCR Analysis

Total RNA extraction was done from both uninfected and LGTV-infected-nymphal ticks (unfed or 24 hours during feeding), ISE6 tick cells or exosomes derived from tick cells, by using Aurum Total RNA Mini kit (BioRad) and following company's instructions. 1 µg of RNA was converted into cDNA using iScript cDNA synthesis kit (BioRad) by following company's instructions. The generated cDNA was used as template for the amplification and determination of viral loads and *Is*SMase levels by performing QRT-PCR using the iQ-SYBR Green Supermix kit (BioRad, USA), following manufacturer's instructions. Published forward and reverse primers were used to detect LGTV PrM-E transcripts (Zhou et al., 2018). *Is*SMase transcript detection was done using our primers pairs 5' CGCCGCTGGAGTAGACATC 3' and 5' GACCCACATCGAATCCCACA 3'. The *hsp70* transcripts were amplified using published primers (Vora et al., 2018). Tick beta-actin is quantified with published primers, (Taank et al., 2018) to normalize the number of templates in all analysis. Equal volumes of cDNA samples were used in parallel for beta-actin, LGTV prM-E, *Is*SMase

and hsp70 primers. The preparation of standard curves was done by 10-fold serial dilutions starting from standard 1 to 6 of known quantities of other primers. For the internal controls, untreated samples were considered.

4.4 Sphingomyelinase and sphingomyelin Quantification assays

We used sphingomyelin quantification and colorimetric sphingomyelinase assay kits from SIGMA-Aldrich and followed all manufacturer's instructions. We plated 5×10^5 tick cells in 12-well cell culture plates and after overnight incubation; cells were infected with LGTV (MOI 1). We collected tick cells for two time points (24 and 72 hours p.i.) for both assays. Cell lysates were resuspended in 1 x PBS and processed for both sphingomyelin lipid levels and sphingomyelinase activity immediately. For each time point and reaction wells, 50 µl of samples (uninfected or LGTV-infected) were used as 6 replicates. Respective zero (blank) and sphingomyelinase/sphingomyelin standards were considered as background in both assays. Samples from both sphingomyelin and sphingomyelinase assays were measured at 655 nm or 570 nm absorbance. Using the standard values, curves were plotted, and enzymatic activity assay or SM lipid quantity was determined from the standard curve.

4.5 Ethics Statement

The Biosafety Level — 2 (BSL-2) infectious experiments that included Langat virus (*in vitro* infection of ISE6 tick cells and synchronous infection of nymphs with Langat virus) was done under the IACUC; protocol # 15-014.

4.6 Statistics

Statistical difference observed in data sets were analyzed using GraphPad Prism6 software and Microsoft Excel 2016. The non-paired, two-tail Student *t* test was performed (for data to compare two means) for the entire analysis. Error bars represent mean (+SD) values, and P values of <0.05 were considered to be significant in all analysis. Statistical test and P values are indicated for significance. Also, each figure legend describes the asterisk that indicate significance.

5. CONCLUSION

Vector-borne disease (VBD) are important communicable diseases with a specific feature of requiring a blood-feeding arthropod vector (an intermediate host) like ticks and mosquitoes to transmit between humans or from animals to humans. Vector control is one of the important strategies to prevent VBDs. Some clinically important emerging tick-borne diseases (TBDs) are Lyme disease, babesiosis, anaplasmosis, Powassan virus disease, tularemia and spotted fever rickettsiosis (that also include Rocky Mountain spotted fever) (CDC, 2018c). Since LGTV resembles both genetically and phylogenetically with TBEV and POWV (deadly human pathogens) and does not cause disease in human, LGTV is widely used as a model virus in the laboratory (Marin et al., 1995). The bioinformatics analysis of *Is*SMase gene in ticks showed homology with the SMase D protein of Loxosceles venomous spider (Alarcon-Chaidez et al., 2009). SMase D in spider results in the catalytic hydrolysis of SM lipid to form ceramide 1-phosphate (acyl sphingosine 1- phosphate) and choline. However, the mammalian sphingomyelinase converts SM into ceramide and phosphocholine (De Andrade et al., 2006; Forrester et al., 1978).

In this study, we showed that in the presence of tick-borne LGTV, *Is*SMase is significantly reduced in both tick cells (*in vitro*) and ticks (*in vivo*). *Is*SMase levels and its activity were also affected upon viral replication. Our data showed LGTV-mediated suppression of *Is*SMase allowed accumulation of SM lipid levels and supported membrane associated viral replication and exogenesis. Inhibition of viral loads and SM lipid build up via GW4869 inhibitor reversed the *Is*SMase levels and restored its activity, thereby proposing an important role for this spider venomous ortholog *Is*SMase in regulating viral replication in association with membrane-bound SM lipids in ticks. Our study suggests a novel role for *Is*SMase, in vector defense mechanism(s) against tick-borne virus infection, and perhaps its important function in anti-viral pathway(s).

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