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RESEARCH ARTICLE

Aedes aegypti lachesin protein binds to the domain III of envelop protein of Dengue virus-2 and inhibits viral replication

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Abstract

Dengue virus (DENV) comprises of four serotypes (DENV-1 to -4) and is medically one of the most important arboviruses (arthropod-borne virus). DENV infection is a major human health burden and is transmitted between humans by the insect vector, *Aedes aegypti*. *Ae. aegypti* ingests DENV while feeding on infected humans, which traverses through its gut, haemolymph and salivary glands of the mosquito before being injected into a healthy human. During this process of transmission, DENV must interact with many proteins of the insect vector, which are important for its successful transmission. Our study focused on the identification and characterisation of interacting protein partners in *Ae. aegypti* to DENV. Since domain III (DIII) of envelope protein (E) is exposed on the virion surface and is involved in virus entry into various cells, we performed phage display library screening against domain III of the envelope protein (EDIII) of DENV-2. A peptide sequence showing similarity to lachesin protein was found interacting with EDIII. The lachesin protein was cloned, heterologously expressed, purified and used for in vitro interaction studies. Lachesin protein interacted with EDIII and also with DENV. Further, lachesin protein was localised in neuronal cells of different organs of *Ae. aegypti* by confocal microscopy. Blocking of lachesin protein in *Ae. aegypti* with anti-lachesin antibody resulted in a significant reduction in DENV replication.

KEYWORDS

Aedes aegypti, Dengue virus, interacting protein partners

1 | INTRODUCTION

Dengue virus (DENV) is one of the most important arboviruses, presenting a major global health concern to nearly half of the world population, living in tropical areas. It infects approximately 400 million people every year (Bhatt et al., 2013), resulting in thousands of deaths, especially in children (Guzman, Halstead, et al., 2010; Guzman, Hermida, Bernardo, Ramirez, & Guillén, 2010; Kyle & Harris, 2008). DENV is a member of the *Flaviviridae* family and it comprises of four different antigenic serotypes (DENV-1 to -4). The infection with one of the four serotypes of DENV can cause dengue disease which includes the symptoms varying from none to the life-threatening sequelae, dengue haemorrhagic fever and dengue shock syndrome (Halstead, 2007).

DENV is an arthropod-borne virus, primarily transmitted by an urban-dwelling mosquito species, known as *Aedes aegypti*

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(Gubler, 1998). *Ae. aegypti* acquires DENV during a blood meal from an infected human and after an incubation period of 7–14 days, the mosquito is capable of infecting another person for the rest of her life. During this incubation period, DENV disseminates from midgut and proliferates in the salivary glands of *Ae. aegypti* (Black et al., 2002; Watts, Burke, Harrison, Whitmire, & Nisalak, 1987). DENV genome consists of a positive single-stranded RNA molecule, which encodes 10 proteins; capsid protein (C), membrane protein (M), envelope protein (E) and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach, Thiel, & Rice, 2006). These proteins are translated in the form of a polypeptide, which is cleaved further into individual proteins during maturation; by the host proteases like furin or signalase on the lumenal side of endoplasmic reticulum and by NS2B/NS3 protease complex on the cytoplasmic side. Out of these 10 proteins, the function of NS1, NS2A, NS4A and NS4B, are less understood, whereas other proteins like NS5, C, M and E proteins play a critical role in DENV replication, encapsulation of viral RNA and attachment to cells in both the hosts (Lindenbach et al., 2006). E is the only protein exposed on virion surface and is involved in virus entry into various cells (Kuhn et al., 2002). E is composed of three domains, DI, DII and DIII (Cruz-Oliveira et al., 2015; Modis, Ogata, Clements, & Harrison, 2005). DIII (located at C-terminus of E) is an immunoglobulin-like β-barrel structure, exposed on DENV surface and characterised by the presence of epitope and cellular-binding motifs (Chen, Maguire, & Marks, 1996; Zhang et al., 2004). The interaction of E with target cell receptors of hosts is probably responsible for tissue tropism, infectivity and pathogenesis of DENV (Chen et al., 1996).

The way DENV replicates in the insect vector, *Ae. aegypti* and in the human host using a genome encoding a mere 10 proteins, displays its remarkable capabilities of survival. For a successful life cycle, DENV must be able to manipulate its insect vector and human host at molecular level through the interaction with various proteins that allow DENV to shift host cellular machinery in its favour. Since viruses have limited genetic capacity, therefore they mostly rely on cellular factors to complete their life cycle in both insect vector and human host. Flaviviruses (DENV, Yellow fever and West Nile virus, etc.) use several insect proteins as a key to infect the cells and modulate their expression profile. A study identified 203 and 202 mosquito genes that were upregulated and downregulated respectively during flavivirus infection (Colpitts et al., 2011). Proteomic identification study for DENV binding proteins in *Ae. aegypti* mosquitoes revealed many proteins like cadherin, translational elongation factor EF-1/Tu, beta-adrenergic receptor kinase and enolase (Muñoz et al., 2013). Actin protein has been reported to interact with E protein of DENV-2 and DENV-4. This interaction was suggested to be cell type specific (Jitoboam et al., 2016). A study suggested that laminin protein (LAMR l) interacts with three DENV serotypes (DENV-1, -2 and -3), which may be a common interacting protein for different DENV serotypes (Tio, Jong, & Cardosa, 2005). A putative conserved cysteine-rich venom protein known as CRVP379 was found to be implicated in DENV infection. Finally, the knockdown of CRVP379 using RNAi or antibody blocking leads to the inhibition of DENV infection in *Ae. aegypti* (Londono-Renteria et al., 2015).

Due to the variation in antigenic property among all four serotypes, there is no effective common antiviral drug available till now (Fink & Shi, 2014; Guzman, Halstead, et al., 2010; Guzman, Hermida, et al., 2010; Sabchareon et al., 2012). The degree of antigenic variation among four serotypes leads to the potential risk of severe disease mediated by the presence of sub-neutralising antibodies against DENV particles. These antibodies can promote a severe disease in an individual through a phenomenon known as antibody-dependent enhancement, where virus can use FcR to infect mononuclear cells (Burke & Kliks, 2006; Flipse, Wilschut, & Smit, 2013; Halstead, 2003; Nikin-Beers & Ciupe, 2015). Several DENV vaccines were designed using traditional approaches like live attenuated viruses, recombinant subunits, virus-like particles, viral vectors, etc. but no vaccine was found capable to provide complete and lasting protection against all four serotypes (Fink & Shi, 2014; Villar et al., 2015).

Transmission-blocking vaccines (TBVs) may be an attractive alternative to traditional vaccines, where an elevated immune response of vertebrate host will block the virus infection to mosquito vectors (Kay & Kemp, 1994). Such types of vaccines have already been designed against malaria infection. Another category of TBVs are based on the insect proteins which downregulate or inhibit the pathogen infection in vector tissues (Carter, 2001). Virus ligands or receptors in mosquito vector are highly desirable candidate target for transmission blockage (Liu et al., 2014; Wang, Conrad, & Mohammed, 2001). The antibodies against C-type lectin, mosGCTLI (Liu et al., 2014) and previously mentioned cysteine protein CRVP379 have interrupted the DENV infection to *Ae. aegypti* effectively, supporting their suitability as TBV candidate protein. Proteins, upregulated upon infection also are promising candidates for interrupting infection, due to their importance in pathogen survival (Chisenhall et al., 2014). The knockdown of upregulated tick histamine release factor (tHRF) from *Ixodes scapularis* during *Borrelia burgdorferi* infection leads to a significant reduction in pathogen titre (Dai et al., 2010).

Further, to identify more interacting protein partners for DENV in *Ae. aegypti*, we have performed phage display library screening against EDIII of DENV-2. We found several peptides that bound specifically to EDIII, which were then analysed on NCBI database. Among them, a neuronal protein, lachesin was selected for future work. Lachesin protein was heterologously expressed and purified using bacterial expression system. Recombinant and endogenous lachesin protein exhibited physical interaction with EDIII. Lachesin protein was further localised in neuronal cells of *Ae. aegypti* using anti-lachesin antibodies and its role in DENV infection was studied.

2 | RESULTS

2.1 | Phage display screening

The peptide sequences of EDIII interacting bacteriophages were searched for similarity against *Ae. aegypti* protein collections available on NCBI database using BLASTp 2.6.2 programme. Several proteins of *Ae. aegypti* were found which could act as receptor for DENV-2

(Table 1). Majority of peptides found in the screen were similar to cell surface receptors and some of these identified receptor proteins had been previously reported to interact with other viruses like kinesin (Dodding & Way, 2011), integrin alpha (Chu & Ng, 2004), cadherin (Salim & Ratner, 2008), myosin heavy chain (Mairiang et al., 2013), etc. which strengthened the credibility and specificity of this phage display screening. According to previous literature, lachesin is a neuronal protein which plays an important role in blood brain barrier (BBB) maintenance. The molecular mechanism involved in dengue pathogenesis of the neurons and the ability of *Ae. aegypti* to maintain a productive flavivirus titre in the brain is poorly understood (Xiao et al., 2015). Hence, to understand the dengue neurotropism better, lachesin was selected over other possible interacting protein partners found in study.

2.2 | DENV interacting peptide exists in I-set immunoglobulin domains of lachesin protein

The amino acid sequence of lachesin protein was searched for the presence of conserved domains and other signatures. Screening against the Conserved Domain Database (CDD) on NCBI and InterPro-Scan, predicted the presence of three domains which include two I-set immunoglobulin domains one at N-terminal (29–137 aa) (Figure 1) and another at C-terminal (233–325 aa), whereas the third domain named Immunoglobulin (Ig)-like domain of neural cell adhesion molecule NCAM-1, was found in middle region (140–229 aa) (Figure 1). Additional screening through SignalP server indicated the presence of signal peptide at N-terminal (1–23 aa). Phobius, a tool for transmembrane topology and signal peptide prediction, also predicted the presence of signal peptides (1–23 aa) and non-cytoplasmic nature (24–334 aa) of lachesin protein. The 3D ribbon structure of lachesin protein was constructed using Phyre2 web portal. The template of 5K6Y (crystal structure of Sidekick-2 immunoglobulin domains 1–4), available in protein data bank was used to predict the 3D structure of lachesin. A total of 89% of amino acids were modelled with >90% confidence. Further, structure analysis revealed the presence of 21 beta strands and three alpha helixes (Figure 2a). Thereafter, to validate the molecular evolution of lachesin, a phylogenetic tree was constructed based on the amino acid sequences of lachesin from various organisms using maximum likelihood method. According to the phylogenetic analysis, *Ae. aegypti* firstly clustered with lachesin of *Aedes albopictus* and then formed a sister group with lachesin of *Anopheles darlingi* and *Culex quinquefasciatus*. Subsequently, they clustered with lachesin from other insects (Figure 2b).

2.3 | Anti-lachesin antibody was produced using purified recombinant lachesin protein

The full length *lachesin* gene was cloned in pET32a expression vector and *E. coli* BL-21 was transformed with this construct. The full length lachesin protein along with N-terminal fusion with thioredoxin tag was induced, expressed, solubilised using detergent (SDS) and purified (Figure S1A,B). Similarly, truncated lachesin was also expressed and purified without using SDS detergent (Figure S1B). Anti-lachesin antibodies were successfully produced in mouse using full length SDS solubilised lachesin protein (Figure S1C). A ~34 kDa band of endogenous lachesin protein was detected in freshly prepared mosquito protein extract using the lab generated lachesin specific antibody (Figure S1D), which suggested the specificity of anti-lachesin antibody. Further to conduct in vitro interaction studies, MBP-tagged EDIII of DENV-2 was purified using bacterial expression system (Figure S2A). The quality (purity and molecular size) of commercially available EDIII protein was also assessed on SDS-PAGE (Figure S2B).

2.4 | Lachesin protein abundantly express in neuronal cells present in various organs of *Ae. aegypti*

To localise the lachesin protein, several organs of *Ae. aegypti* adult female like gut, salivary glands, and ovary were dissected and fixed using paraformaldehyde. Samples were incubated with primary anti-lachesin antibody followed by anti-mouse TRITC-labelled secondary antibody. The expression of lachesin protein was specifically observed within neuronal cells located in salivary glands (Figure 3), ovary (Figure 4) and gut (Figure 5). To confirm the lachesin expression in neuronal cells, antibody against neuronal marker protein known as embryonic lethal abnormal vision (ELAV) of Drosophila was used for co-localisation analysis. Results indicted the clear co-localisation of ELAV and lachesin protein, whereas in the case of control experiment no specific signal was observed, suggesting that lachesin expresses specifically in neuronal cells (Figure 4).

2.5 | Lachesin protein interacts with DENV envelope protein

To confirm the interaction of lachesin with DENV-2, commercially available EDIII of DENV-2 bound to Ni-NTA was incubated with freshly prepared *Ae. aegypti* protein extract. Further, proteins bound to EDIII were resolved and blotted on nitrocellulose membrane through western blotting. The presence of lachesin protein in EDIII bound protein fractions was confirmed using anti-lachesin antibodies. Result revealed the clear single band of endogenous lachesin protein whereas no band observed in control experiment (only bead + *Ae. aegypti* protein extract) which further confirmed the specific interaction of EDIII with endogenous lachesin protein of *Ae. aegypti* (Figure 6a). In the next approach, to confirm the interaction of EDIII with domain 3 (I-set immunoglobulin domain) of lachesin protein, truncated thioredoxin-tagged lachesin protein was incubated with MBP-tagged EDIII of DENV-2. Result showed the specific interaction of between both the proteins whereas no interaction was observed either with MBP tag or thioredoxin tag (Figure 6b).

Using VOPBA, in-vitro interaction of DENV envelope protein with recombinant truncated lachesin protein was analysed. To

perform this experiment, as a first step, DENV particles were detected in supernatant of DENV infected C6/36 monolayer using anti-envelope antibodies. A protein band (~54 kDa) of envelope protein was observed which confirmed the presence of DENV-2 particles in supernatants (Figure S3). Further, membrane blotted with DENV particles, MBP tag and MBP-tagged EDIII was incubated with lachesin protein to check their direct interaction. Bound lachesin protein was detected using anti-lachesin antibodies. Result revealed the clear interaction of lachesin protein with both E protein of DENV and MBP-tagged EDIII. In case of control experiments, no such bands were observed (Figure 7).

2.6 | Lachesin protein plays an essential role in DENV infection in mosquito

To assess the role of lachesin protein in DENV infection in mosquito, DENV was injected to mosquito along with anti-lachesin antibody. In separate experiments, 1/10 and 1/100 dilution of anti-lachesin antibody, pre-immune sera and PBS were injected along with DENV-2. After 6 days of microinjection, DENV-2 titre was quantified using FFA, which revealed that mosquito injected with DENV-2 particles along with anti-lachesin antibodies showed nearly 50% reduction in DENV-2 titre compared to the control experiments (Figure 8), while 1/10 and 1/100 dilution of anti-lachesin antibody did not show any significant reduction of viral titre (Figure S4). Statistical significance was checked using one-way ANOVA and unpaired *t* test ($p < .0001$).

3 | DISCUSSION

Mosquitoes are vectors for many pathogenic viruses of medical importance throughout the globe (Guzman, Halstead, et al., 2010; Guzman, Hermida, et al., 2010; Kyle & Harris, 2008). DENV is one among them, attracting more concern as it shares a highly evolved and complex association with both the insect vector and the human host. Till date no suitable vaccine for DENV has been identified. Methods including insect vector control and prevention of pathogen transmission by insect vector have become more promising tools to control DENV spread (Fink & Shi, 2014; Villar et al., 2015). Understanding the molecular mechanism involved in DENV-*Ae. aegypti* association is an essential element to control DENV spread. The success in prevention of pathogen transmission is entirely dependent upon the target protein selected for making virus resistant mosquitoes. In order to contribute more information regarding mosquito proteins which are essential and interact with the virus during dengue infection, we have performed phage-display screening against EDIII of DENV-2. The peptide sequences of EDIII interacting bacteriophages were searched for similarity on NCBI database against mosquito proteins. Several probable interacting protein partners were found (Table 1); of them some myosin heavy chain (Mairiang et al., 2013) proteins were previously reported to interact with DENV.

During the selection of candidate protein for further work, we adopted various criteria which included motifs present in proteins, expression profile, protein function and possible contribution during dengue pathogenesis in mosquito. A protein named lachesin was found as an important protein since it belongs to immunoglobulin superfamily (IgSF) and expresses on the membrane, which are the characteristic features of virus receptor proteins. Further, the amino acid sequence of lachesin protein was analysed using online available web tools like InterProScan and NCBI CDD. Result from both the screening revealed the presence of two immunoglobulin I-set domains at N terminal (29–137 aa) and C terminal (233–325 aa) whereas third immunoglobulin domain (NCAM; 140–229 aa) was present in middle region. The proteins having any domain among V-set, C-set and I-set domains belong to IgSF of molecules. V-set and C-set domains are the most common immunoglobulin domains which are named according to occurrence in the variable and constant region of immunoglobulin respectively (Dermody et al., 2009). The third I-set domain is an intermediate structure between V-set and C-set, found frequently in the cell surface receptors. Several proteins of IgSF have previously been reported to mediate the virus attachment to the host cells (Table 2). Though diverse group of viruses bind to IgSF molecules for cell attachment and entry, but they do not share common mode of binding as indicated by structural analysis of virus-receptor complex. The members of IgSF contain domains with characteristic immunoglobulin fold, which are defined by two opposing antiparallel β-sheets joined in a unique manner (Klatzmann et al., 1984; Kondratowicz et al., 2011; Tatsuo, Ono, Tanaka, & Yanagi, 2000). A similar pattern of two opposing antiparallel β-sheets in each immunoglobulin domains (D1, D2 and D3), was observed in 3D structure of lachesin protein, constructed using Phyre2 web server. Considering the significance of IgSF molecule in virus attachment to cell, lachesin protein was selected for detailed analysis. Phylogenetic analysis also revealed the conserved nature of lachesin protein among invertebrates and vertebrates.

Lachesin gene was cloned and expressed using bacterial heterologous expression system. Purified full length lachesin protein was used to produces anti-lachesin antibody in mouse. IgG fraction of the anti-lachesin antibody was used for localisation of lachesin protein in *Ae. aegypti*. The organs of *Ae. aegypti* were dissected and processed further for lachesin expression. Results revealed the neuron specific expression of lachesin in mid-gut, salivary gland and ovary which were again confirmed by co-localisation of ELAV protein with lachesin. The expression of lachesin in neuron and axons was first reported in grasshopper embryo as a membrane protein (Karlstrom, Wilder, & Bastiani, 1993). A study conducted on lachesin mutant embryo of *Drosophila* suggested that lachesin expresses on the cell surfaces of the specific tissues such as trachea, hindgut, foregut and nervous system which is required for the proper morphogenesis of the tracheal system and cell adhesion (Llimargas, Strigini, Katidou, Karagogeos, & Casanova, 2004). Another study reported the expression of lachesin in glia cells of *Drosophila* as a component of the septate junctions, which form the barrier around both central nervous system (CNS) and peripheral nervous system (Strigini et al., 2006). Further, the knockdown of lachesin leads to development of abnormal structure of BBB and neurons. There is

no additional report regarding lachesin expression and functions in insects.

The neuropathogenesis of DENV infection is poorly understood in human as well as in mosquito. Few receptors proteins like Hsp70, Hsp90, 65 kDa protein of neuroblastoma cells, three membrane proteins (60, 70 and 130 kDa) from grey matter and 70 kDa protein of white matter of CNS are the only known neuronal proteins in human host that interact with DENV particles during neuro-invasion (Salazar et al., 2013). On the other hand, in mosquito, Hikaru genki (AaHig) is the only neuronal protein that interacts with DENV and prevents the lethal DENV infection of the CNS which may facilitate DENV transmission in nature (Xiao et al., 2015). Another study, conducted by Tham, Balasubramaniam, Chew, Ahmad, & Hassan (2015), identified a sensory neuron protein-1 using yeast two hybrid cDNA library screening which interact with pre-membrane and envelope protein of DENV. In our next experiment, purified EDIII exhibited the positive interaction with endogenous as well as recombinant lachesin protein of mosquito which further suggests that lachesin could act as receptor for DENV during neuro-invasion since it is a neuronal surface receptor protein as indicated by domain structures. On the other hand, localisation of lachesin in glia cells which are the fundamental unit of BBB, indicates that direct interaction of lachesin with DENV particles could play a critical role in BBB failure during DENV neuropathogenesis (Strigini et al., 2006).

Anti-lachesin antibody injected mosquitos were able to survive without significant variation in their physiological parameters (mortality and fecundity) compared to normal *Ae. aegypti*. To evaluate the role of lachesin protein in DENV replication, DENV was injected along with the anti-lachesin antibodies and the result reflected its ability to reduce the virus titre (~50% reduction) and hence its replication in insect vector, *Ae. aegypti*. DENV binding to lachesin protein could be an important step during DENV attachment and fusion to neurons. DENV classically has been considered as non-neurotropic virus, however evidences form several studies in both hosts suggested that DENV is able to infect and replicate in neural cells (Domingues et al., 2008; Jackson et al., 2008; Salazar et al., 2013). DENV infection to CNS in human host leads to an acute phase known as dengue encephalitis wherein vascular leakage and BBB rupture are the prevalent symptoms. Unlike human host, the CNS located in the head of mosquito can maintain productive flavivirus infection and it does not lead

TABLE 1 List of possible candidate receptors of dengue virus-2 (DENV-2) found in phage display screening

Protein	Accession No.	Start	Interacting peptide sequence in protein
Kinectin	XP_001661045.1	223	LKALKE
Reticulon/nogo receptor	XP_001661045.1	39	GLKALK
Tyrosine-protein kinase transmembrane receptor	XP_001654515.1	404	KALKE
Lachesin	XP_001656517.1	315	KVGH – – LNL
Integrin alpha-ps	XP_001663561.1	416	KVGDLNM
Acetylcholine receptor protein alpha 1	XP_001648284.1	90	VKQKW
Cadherin	XP_001661464.1	2,131	KQKW
Leukocyte receptor cluster (lrc) member	XP_001658437.1	398	VKNKW
Importin alpha 1a, putative	XP_001650334.1	99	MKGTLGL
Low-density lipoprotein receptor	XP_001661035.1	397	KGTLG
Dicer 2	AEX31636.1	755	MKFFVTLGL
Putative salivary C type lectin	ABF18475.1	2	MKGTL
Monocarboxylate transporter	XP_001651679.1	602	GTKTN
Gustatory receptor	XP_001655087.1	376	TKQLTNLS
Kinesin-like protein KIF17	XP_001656089.1	259	TKINLS
Kinesin	XP_001653597.1	1,674	SKLMN
Ubiquitin specific protease	XP_001662280.1	613	KLMNQ
Olfactory receptor, putative	XP_001651429.1	299	KLMN
Transferrin	XP_001663123.1	463	LMNH
ATP-binding cassette transporter	XP_001651693.1	661	DLRLSRQ
Restin	XP_001663875.1	201	ARLSR
Myosin heavy chain	XP_001651388.1	1,232	DARLS
Leucine-rich transmembrane protein	XP_001656813.1	982	DMRLSR
Prenylated rab acceptor	XP_001654904.1	44	RLTRQ
Cdk1	XP_001653367.1	245	TQNN

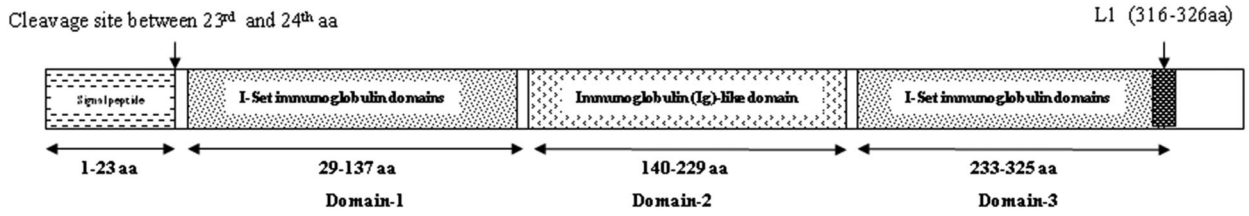

FIGURE 1 Domain mapping of lachesin. Representation of complete lachesin protein template of 334 amino acids, indicating N terminal signal peptide (1–23 aa) and three domains 1–3. Domain 1 (I-set immunoglobulin domain; 29–137 aa) and domain 3 (I-set immunoglobulin domain; 233–325 aa) were located at N- and C-terminal respectively whereas domain 2 (immunoglobulin [Ig]-like domain; 140–229 aa) was found in between domain 1 and domain 3. Finally, EDIII interacting region (316–326 aa) of lachesin protein was indicated as L1

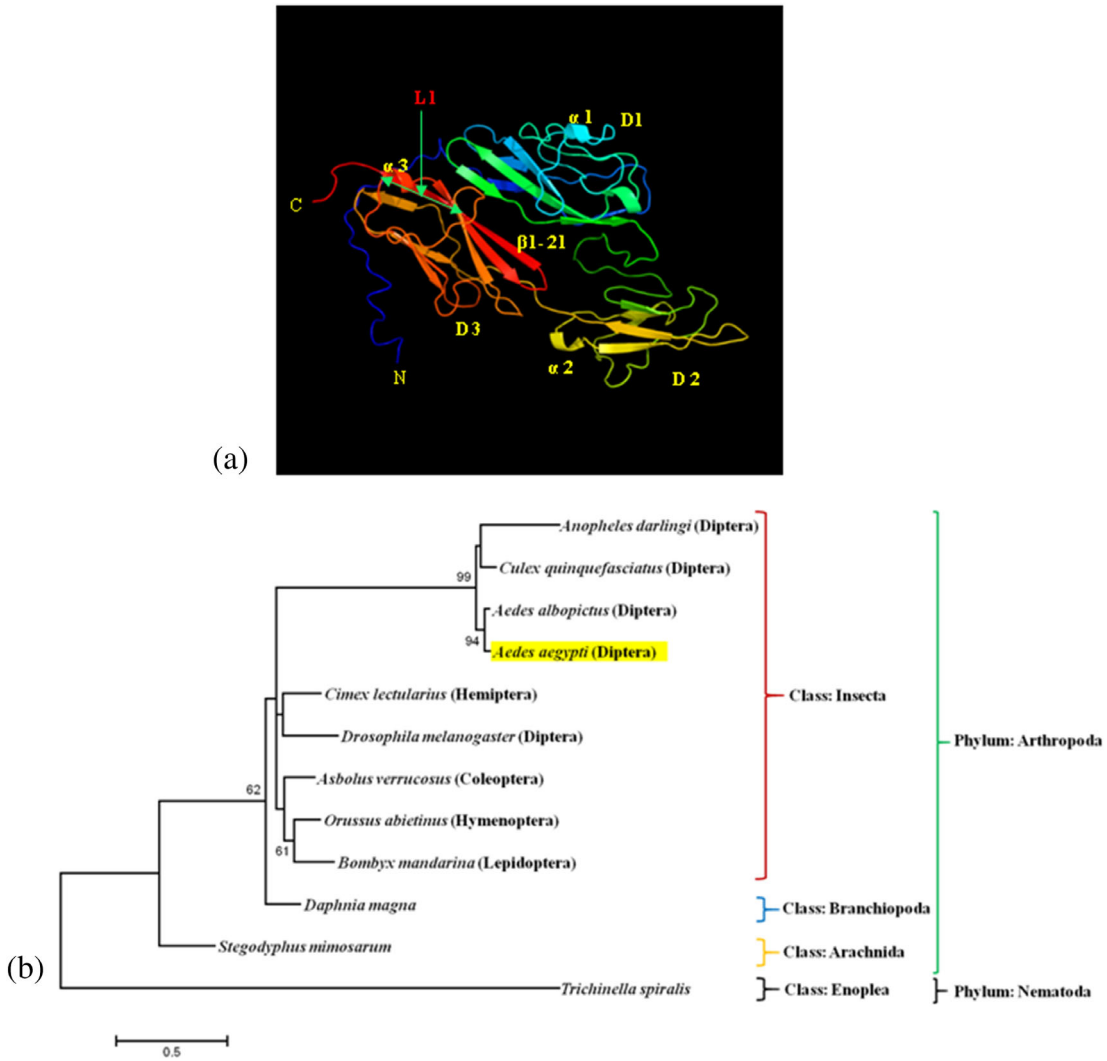

FIGURE 2 (a) The 3D structure of lachesin protein of *Aedes aegypti*. The β-strands, α-helices and three immunoglobulin domains (D1, D2 and D3) are shown. In addition to these structures, EDIII of DENV-2 interacting region of lachesin also indicated as L1. (b) Phylogenetic tree of lachesin protein of *Ae. aegypti* and organisms, were constructed using MEGA 7 with maximum likelihood method. Numbers on each internal branch in the phylogenetic tree node represent the boot strap value from 1,000 replicates. Lachesin sequences include *Anopheles darlingi* (ETN58523.1), *Culex quinquefasciatus* (XP_001862096.1), *Aedes albopictus* (XP_019528163.2), *Aedes aegypti* (XP_001656517.1), *Cimex lectularius* (XP_014249298.1), *Drosophila melanogaster* (NP_523713.2), *Asbolus verrucosus* (RZC40221.1), *Orussus abietinus* (XP_012288477.1), *Bombyx mandarina* (XP_028031731.1), *Daphnia magna* (KZS19971.1), *Stegodyphus mimosarum* (KFM71118.1) and *Trichinella spiralis* (KRY34442.1). Amino acid sequence identities (accession number) of organisms are given inside the bracket

to any significant malignant pathological consequence and alteration in mosquito behaviours or life span (Xiao et al., 2015). Earlier, it was suggested that DENV infection to chemosensory apparatus may lead to change in behavioural aspects like upregulation in chemosensory and locomotor ability (Lima-Camara et al., 2011; Platt et al., 1997). A subsequent study, found the upregulation of two odorant binding proteins, that is, OBP10 and OBP12 in salivary glands upon DENV infection, which are the key players in probing behaviour, further suggesting DENV could facilitate its transmission by modulation mosquito probing behaviour (Sim, Ramirez, & Dimopoulos, 2012).

Study of the nervous system and how it works, is one of the most challenging areas in biology. Virus infections to the neuron have provided insight of virus pathogenesis to basic biology. As a result of evolution, nervous system has evolved rather complicated barriers which block the virus entry but facilitate the access to nutrients and contact with the outside world. Nevertheless, due to any reason if number of barriers decreases, nervous system infection is possible. Once virus infects the neuron, it can be devastating and not easily manageable even with good antiviral drugs. The mosquitoes, as a principle insect vector of many flaviviruses have developed the mechanism, which allow the infection to neurons for optimal virus transmission without causing the serious damage to brain. Characterisation of such sophisticated interaction between viruses and their vector may provide novel strategies for arboviral disease control in the future.

4 | EXPERIMENTAL PROCEDURES

4.1 | Mosquito rearing and collection

The *Ae. aegypti* mosquitoes were obtained from a cyclic colony being maintained in an insectary at National Institute of Malaria Research, New Delhi, which were collected from Dabri, New Delhi (28.6097°N 77.0895°E), in September 2016. Mosquito populations were maintained at 27 ± 1°C and 80% relative humidity according to standard rearing procedure. *Ae. aegypti* samples were further stored in RNA*later* (Ambion) and kept at −70°C till further use. DENV (ATCC VR-1584) was used for interaction and viral replication studies. The experiment to study the role played by lachesin protein in DENV-2 infection in *Ae. aegypti* was performed at ICGEB ACL facility.

4.2 | Source of EDIII of DENV-2

The EDIII of DENV-2 was purchased commercially (ProSpec). According to the details provided by manufacturing company, a ~13 kDa His-tagged EDIII was expressed and purified in *E. coli* BL-21 bacterial expression system. The protein was made available at a concentration of 1.78 mg/ml in 1× phosphate-buffered saline (PBS [pH 7.4]). It was dispensed in small aliquots to minimise freeze thaw

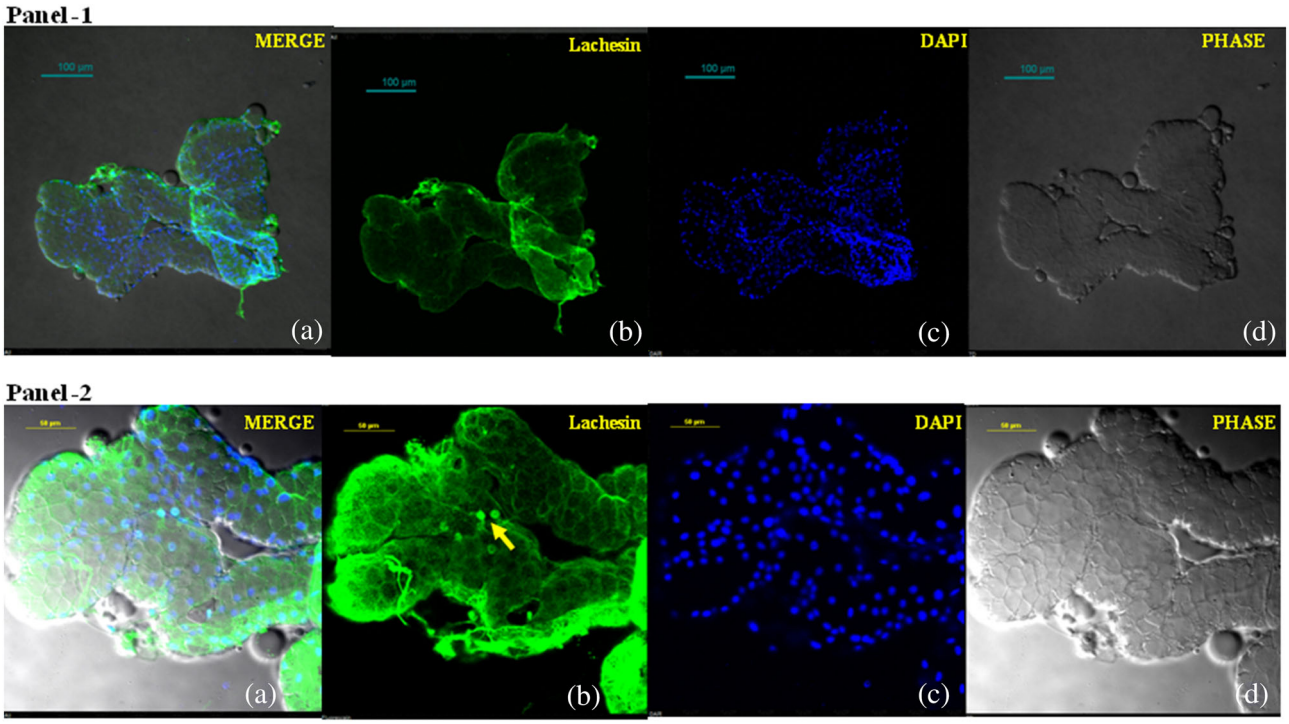

FIGURE 3 Confocal analysis of lachesin protein in salivary glands of *Aedes aegypti*. Panel 1: Lachesin protein expression in dissected salivary gland of *Ae. aegypti* at 10× magnification. (a) Merge image from b, c and d. (b) Lachesin expression was detected with primary anti-lachesin antibody and secondary FITC conjugated anti-mouse antibody. (c) DAPI (blue) staining for nucleus detection. (d) Bright field image. Panel 2: Salivary glands were further magnified at ×40 and analysed for lachesin expression. (a) Merge image from b, c and d. (b) Lachesin expression was detected with primary anti-lachesin antibody and secondary FITC conjugated anti-mouse antibody. (c) DAPI (blue) staining for nucleus detection. (d) Bright field image

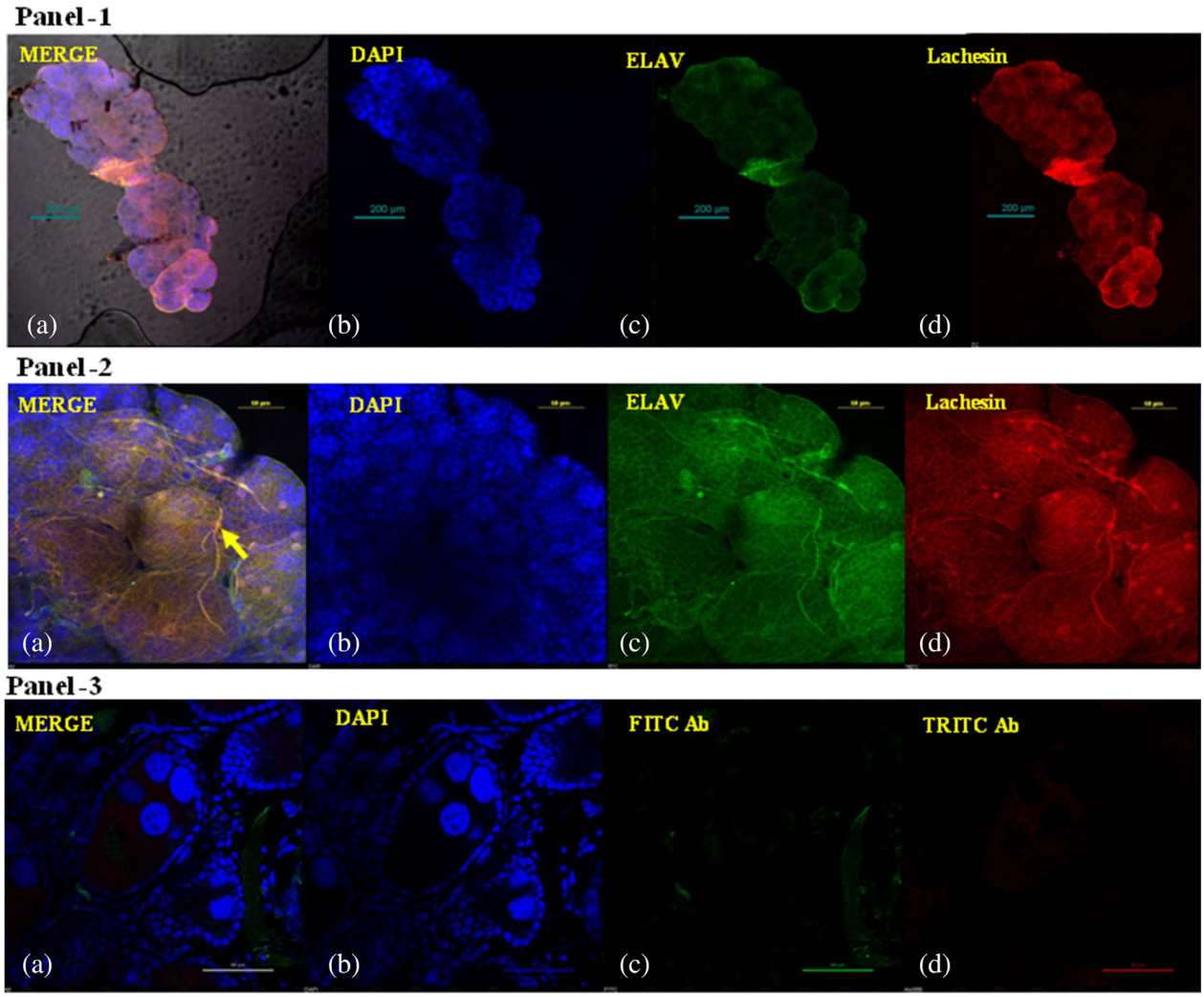

FIGURE 4 Confocal analysis of lachesin protein in ovary of *Ae. aegypti*. Panel 1: Lachesin protein expression in dissected ovary of *Ae. aegypti* at ×10 magnification. (a) Merge image from bright phase, b, c and d. (b) DAPI (blue) staining for nucleus localisation. (c) Neurons were localised using primary anti-ELAV antibody (rat) and secondary FITC-labelled anti-rat antibody. (d) Lachesin expression was detected with primary anti-lachesin antibody and secondary TRITC conjugated anti-mouse antibody. Panel 2: Ovary was further magnified at ×40 and analysed for lachesin expression. (a) Merge image from bright phase, b, c and d. (b) DAPI (blue) staining for nucleus localisation. (c) Neurons were localised using primary anti-ELAV antibody (rat) and secondary FITC-labelled anti-rat antibody. (d) Lachesin expression was detected with primary anti-lachesin antibody and secondary TRITC conjugated anti-mouse antibody. Panel 3: As a control experiment, dissected ovary was incubated with secondary antibodies only. (a) Merge image from bright phase, b, c and d. (b) DAPI (blue) staining for nucleus localisation. (c) Ovary was incubated with FITC-labelled anti-rat secondary antibody whereas no primary antibody was used. (d) Ovary was incubated with TRITC-labelled anti-mouse secondary antibody whereas no primary antibody was used

cycles and stored at −70°C till further use. To purify EDIII, the gene encoding EDIII was synthesised commercially and cloned into pMAL-c2X expression vector (New England Biolab, MA). *E. coli* BL-21 expression strain was transformed with prepared construct. Further EDIII expression was achieved by adding IPTG to final concentration of 0.01 mM at 16°C for 16 hr. The pellet of induced cells was re-suspended in native buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1× protease inhibitor [GE Healthcare]) and lysed by sonication. Lysate was centrifuged at a high speed of 18,514*g* for 20 min at 4°C. Soluble fraction of lysate was incubated with pre-equilibrated Dextrin beads (GE Healthcare) in native buffer for 2 hr at 4°C on Nutator. After incubation, beads were washed twice with washing buffer (50 mM Tris-Cl [pH 7.5], 300 mM NaCl, 1 mM EDTA, 10% glycerol, 1× protease inhibitor, 1% Triton X-100 [vol/vol]). Bound protein fractions were eluted with elution buffer (10 mM Maltose [Sigma], 50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1× protease inhibitor). Eluted fractions were checked on SDS-PAGE and dialysed against native buffer. The dialysed protein samples were stored at −70°C until further use.

4.3 | Phage display library screening

The Ph.D.-C7C phage display library (NEB, MA) was used for screening against EDIII. Total 100 μg/ml of His-tagged EDIII protein (ProSpec) was used as direct target coating on ELISA plate for each

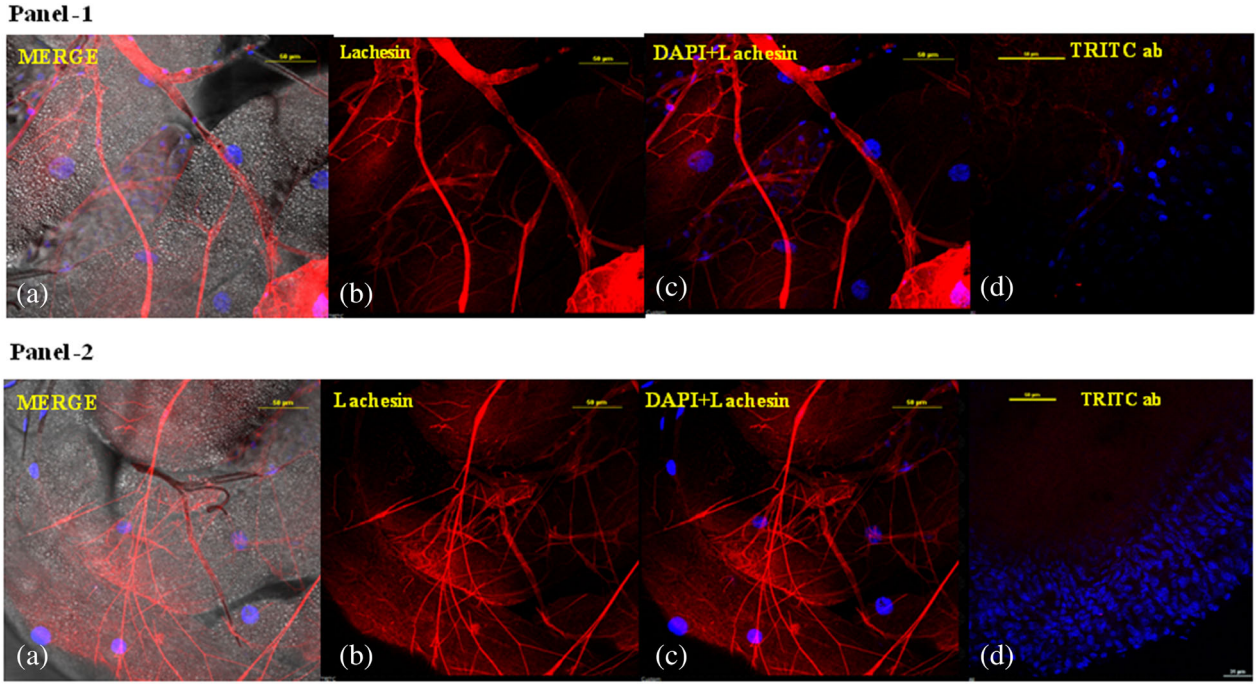

FIGURE 5 Confocal analysis of lachesin protein in gut of *Ae. aegypti*. Two random locations of gut in Panel 1 and Panel 2 were analysed for lachesin protein expression. Panel 1: Lachesin protein expression in dissected gut of *Ae. aegypti* at ×40 magnification. (a) Merge image from bright phase, b and c. (b) Lachesin expression (red) was detected with primary anti-lachesin antibody and secondary TRITC conjugated anti-mouse antibody. (c) Merge image from DAPI (blue) staining and lachesin localisation. (d) As a control, gut was also incubated with TRITC conjugated anti-mouse antibody, whereas no primary antibody was used. Panel 2: Lachesin protein expression from another region in dissected gut of *Ae. aegypti* at ×40 magnification. (a) Merge image from bright phase, b and c. (b) Lachesin expression (red) was detected with primary anti-lachesin antibody and secondary TRITC conjugated anti-mouse antibody. (c) Merge image from DAPI (blue) staining and lachesin localisation. (d) As a control, gut was also incubated with TRITC conjugated anti-mouse antibody, whereas no primary antibody was used

panning and the entire procedure for library screening was performed as per manufacturer's instructions (NEB). In short, commercially available phage library was incubated with EDIII protein coated on polystyrene microtiter plate. After incubation, unbound phages were washed whereas bound phages were eluted by lowering the pH. The eluted phages were then amplified through additional binding/amplification cycle (panning) to enrich the pool of binding phages. After three rounds of panning, individual plaques were isolated and sequenced commercially (Macrogen, South Korea). To rule out the possibility of non-specific binding, during each panning, wells were washed six times with high stringent wash buffer (TBS) containing 0.5% Tween 20 and 150 mM NaCl. The sequences of phages were translated into peptides using BioEdit software and characterised through BLASTp programme against *Ae. aegypti* proteins database available in NCBI server. *Ae. aegypti* proteins showing similarities to peptides were selected for future work. The genes of respective proteins were amplified, cloned and expressed using bacterial expression system.

4.4 | Bioinformatics

The sequences of *lachesin* genes in insects were obtained from the NCBI database. The unrooted phylogenetic tree was built with the maximum likelihood method using MEGA software. The bootstrap consensus tree was inferred from 1,000 replicates. The functional modules of lachesin were predicted using the InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search) and Pfam (http://pfam.sanger.ac.uk/) applications.

4.5 | Cloning, expression and purification lachesin protein

The putative receptor peptide sequence corresponding to lachesin was identified from NCBI-BLASTp against *Ae. aegypti*. The complete and truncated *lachesin* gene corresponding to interactive peptide domain were prepared by designing gene specific primers (for complete gene, Fwd1 [5′-CGGGATCCCATATGGGGCTTTTACGGCTGAACC-3′] and Rev [5′-GCGTCGACTCAATAGGGAACTGGGATAACCG-3′]; and for truncated gene, Fwd2 [5′-ATGAGGATCCCGACCGAAGGTAGCACAAGCTAC-3′] and Rev).

Total RNA was isolated from 10 mosquitoes and was used for cDNA preparation by using RevertAid First Strand cDNA synthesis kit as per manual's instruction (Thermo Fisher Scientific). Prepared cDNA template was further used for PCR amplification of *lachesin* gene. The amplified PCR product of the desired size were eluted and cloned into TA cloning vector pGEM T Easy (Promega). The positive clones were sequenced and *lachesin* gene was sub-cloned into pET-32a expression

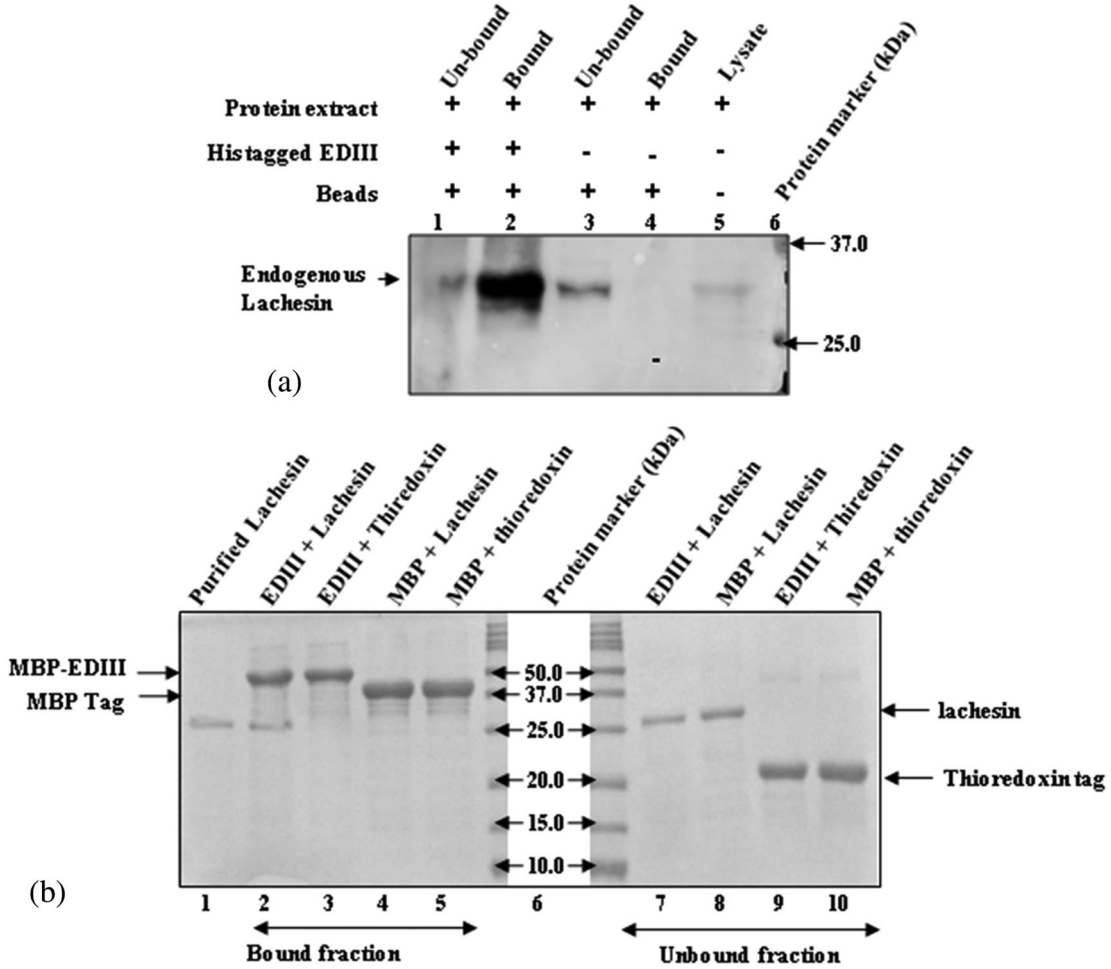

FIGURE 6 In vitro interaction between lachesin and DENV. (a) Interaction of endogenous lachesin protein with His-tagged EDIII. Lanes 1 and 2 are unbound and bound fractions respectively, when His-tagged EDIII bound with Ni-NTA beads were incubated with *Ae. aegypti* protein extract. A prominent band of endogenous lachesin protein was observed in bound fraction, suggesting endogenous lachesin protein interacts with His-tagged EDIII protein. Lanes 3 and 4 are unbound and bound fraction respectively, when Ni-NTA beads were incubated with *Ae. aegypti* protein extract only. No band was observed in bound fractions, suggesting endogenous lachesin protein does not interact with Ni-NTA unspecifically. Lane 5: *Ae. aegypti* protein extract lysate. Lane 6: Protein marker. (b) in vitro interaction of thioredoxin-tagged truncated lachesin with MBP-tagged EDIII. Lane 1: Purified thioredoxin-tagged truncated lachesin protein. Lanes 2–5 are of bound fractions whereas Lanes 7–10 are of unbound fractions. Lanes 2 and 7: thioredoxin-tagged truncated lachesin was incubated with MBP-tagged EDIII. In bound fraction, a 56 kDa band of MBP-tagged EDIII was observed along with the ~29 kDa band of thioredoxin-tagged truncated lachesin whereas in unbound fraction only small amount of unbound thioredoxin-tagged truncated lachesin protein was observed, suggesting the positive interaction of thioredoxin-tagged truncated lachesin with MBP-tagged EDIII. Lanes 3 and 9: EDIII was incubated with theoridoxin tag. In bound fraction only single 56 kDa band of EDIII was observed whereas in unbound fraction a single band of thioredoxin tag was observed, suggesting that thioredoxin tag was not involved in thioredoxin-tagged truncated lachesin interaction with EDIII. Lanes 4 and 8: MBP tag was incubated with thioredoxin-tagged truncated lachesin. In bound fraction a single 45 kDa band of MBP tag was observed whereas in unbound fraction thioredoxin-tagged truncated lachesin protein was found, suggesting that MBP tag was not involved in thioredoxin-tagged truncated lachesin interaction with MBP-tagged EDIII. Lanes 5 and 10: MBP tag was incubated with thioredoxin tag. In bound fraction a single 45 kDa band of MBP tag was observed whereas in its unbound fraction thioredoxin tag (~20 kDa) was found suggesting that MBP tag and thioredoxin tags are not involved in thioredoxin-tagged truncated lachesin interaction with MBP-tagged EDIII

vector followed by *E. coli* BL21 expression strain transformation with these constructs. Transformed cell were induced by adding IPTG to final concentration of 0.01 mM at 16°C for 16 hr. The pellet of induced cells was re-suspended in buffer A (20 mM sodium phosphate [pH 7.5], 150 mM NaCl, 1 mM 2-mercaptoethanol, 0.5% N-tetradecyl-N-N-dimethyl-3-ammonio-1-propane-sulfonate [SB 3-14] [G Bioscience] [wt/vol], 1× protease inhibitor [GE Healthcare]) and lysed by sonication. Lysate was centrifuged at high speed 18,514*g* for 20 min at 4°C. The soluble fraction of lysate was incubated with pre-equilibrated Ni-NTA beads in buffer A without SB3-14, for 2 hr at 4°C. After binding, the beads were washed with washing buffer (20 mM sodium phosphate [pH 7.5], 150 mM NaCl, 1× protease inhibitor, 40 mM imidazole, 1% Triton X-100 [vol/vol]) twice. Bound protein fractions were eluted with elution buffer (20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 250 mM imidazole, 1× protease inhibitor). Eluted fractions were checked on SDS-PAGE and dialysed against buffer B

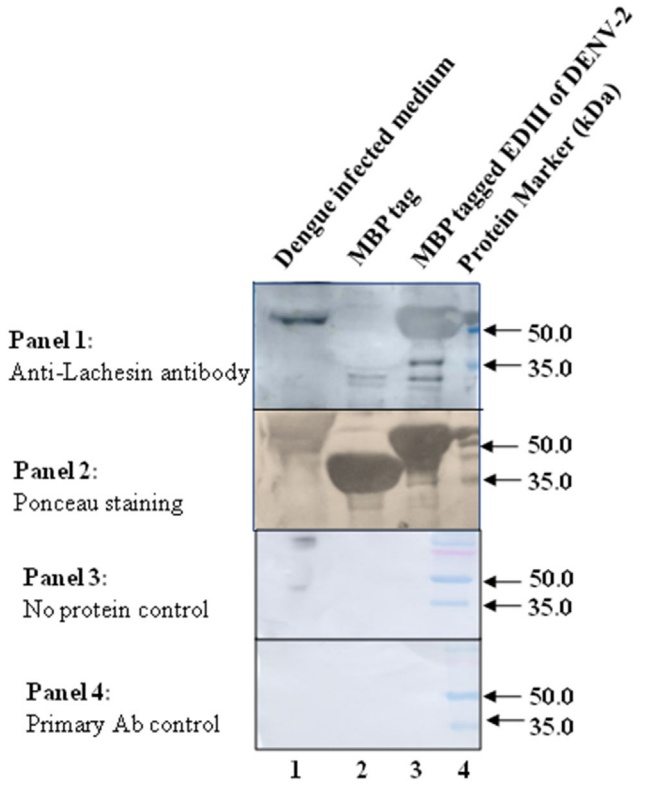

FIGURE 7 Virus overlay protein binding assay (VOPBA). DENV-2 particles, MBP tag and MBP-tagged EDIII of DENV-2 (indicted in Lanes 1, 2 and 3 respectively) were resolved using SDS-PAGE and blotted on nitrocellulose membrane. Blotted nitrocellulose membrane was incubated with truncated lachesin protein. Lachesin protein was detected using primary anti-lachesin antibodies and secondary anti-mouse HRP-labelled antibodies. Panel 1: Lane 1: A band of 55 kDa was observed, suggesting that lachesin protein directly interacts to envelope protein (E) of DENV-2. Lane 2: No band of lachesin protein was observed, suggesting that MBP tag alone does not interact with lachesin protein. Lane 3: A band of ~55 kDa was observed, suggesting the positive interaction between lachesin and EDIII proteins. Panel 2: showing the amount of various proteins loaded into the each well. Proteins bands were visualised using ponceau staining. Panel 3: As a control, the blots were not incubated with purified truncated lachesin protein and were detected using primary anti-lachesin antibody followed by secondary HRP-labelled anti-mouse antibody. Signals were not observed suggesting the specificity of the result observed in Panel 1. Panel 4: As a control, the blot was overlaid with purified truncated lachesin protein but the primary anti-lachesin antibody was not used, rather the blot was incubated with secondary HRP-labelled anti-mouse antibody for detection using ECL detection system. Signals were not observed suggesting the specificity of the result observed in Panel 1

(50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1× protease inhibitor, and 20% glycerol). The dialysed protein samples were stored at −70°C until further use.

4.6 | Anti-lachesin antibody generation

Six-to-eight weeks old BALB/c mice were used for antibody generation. Mice were sub-cutaneously injected with 100 μg of purified

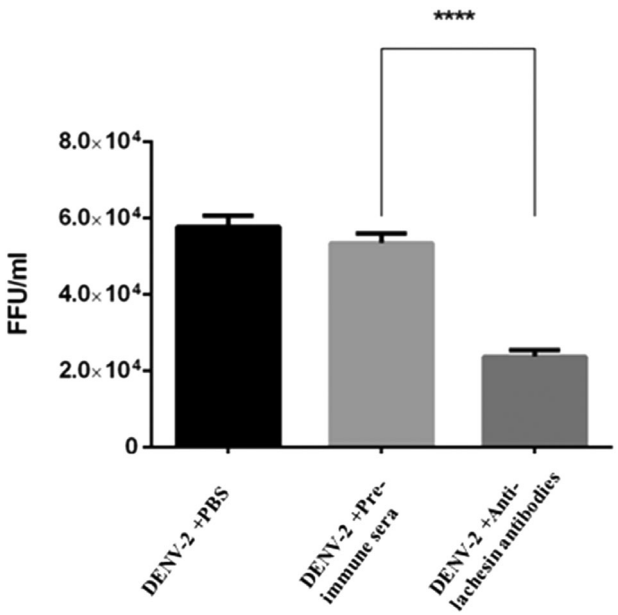

FIGURE 8 DENV focus forming assay. To assess the role of lachesin protein in DENV-2 infection in *Ae. aegypti*, DENV-2 particles were microinjected into mosquitoes in three combinations (DENV + PBS, DENV + Pre-immune serum and DENV + anti-lachesin antibodies). After 6 days of microinjections, DENV-2 titre was quantified using focus forming assay. Mosquitoes injected with DENV-2 and anti-lachesin antibodies, showed nearly 50% reduction as compared to control groups (DENV-2 + PBS or DENV-2 + Pre-immune serum). Statistical significance was checked using one-way ANOVA and unpaired *t* test (****$p < .0001$)

recombinant lachesin protein as antigens. Two mice were used for antibody production. Purified recombinant protein was emulsified with Freund's complete adjuvant (Sigma-Aldrich) for the first immunisation and Freund's incomplete adjuvant (Sigma) for the subsequent booster dosages. Antisera was prepared and stored at −20°C. Anti-lachesin antibodies IgG fraction was purified using "Protein A Sepharose Fast Flow" (GE Healthcare).

4.7 | Endogenous lachesin detection in mosquito protein lysate

A total of 15 mosquitoes (mixed population) were homogenised in 200 μl of homogenisation buffer (10 mM Na_2HPO_4 [pH 7.5], 1 mM EDTA, 500 mM NaCl and protease inhibitor cocktail [Sigma]). After complete homogenisation, mosquito extract was centrifuged at 1,485*g* for 5 min. Supernatant was collected in a separate tube. Protein detection was performed by harvesting one-fifth of the supernatant along with SDS-PAGE loading dye. Samples were resolved in 12% SDS-PAGE gel and transferred onto nitrocellulose membrane. Western blotting was performed with anti-lachesin at 1:2,000 dilution as primary and anti-mouse-HRP at 1:3,000 dilution as secondary antibody. Bands were visualised using enhanced chemiluminescence (ECL) system (Millipore) and documented through GE Healthcare Gel-Doc system.

4.8 | Confocal microscopy experiments

To perform confocal microscopy experiments, mosquito organs (guts, ovary and salivary glands) were dissected under stereomicroscope in PBS. Dissected organs were fixed in 4% paraformaldehyde for 30 min and then, permeablised by incubation with PBST (PBS with 0.05% Triton X-100) for 1 hr. Finally, the organs were incubated with primary (1/200, anti-lachesin produced in mice) and secondary antibodies (1/400, Tetramethylrhodamine [TRITC]-labelled anti-mouse, Vectashield) for 2 hr each following blocking with 3% BSA (bovine serum albumin). For co-localisation, neuronal antibodies, Drosophila anti-embryonic lethal abnormal vision (anti-ELAV) antibody produced in rat (1/200) were incubated along with lachesin primary antibodies (1/200) for 2 hr. FITC-labelled anti-rat secondary antibodies (Sigma) were used to detect neuronal antibodies at 1/400 dilution for 2 hr. Organs were washed thrice with PBS after each incubations and finally mounted on slide using fluoroshield with DAPI (Sigma). In the case of control experiment, dissected organs were incubated with secondary antibodies only.

4.9 | Protein–protein interaction experiments

The interaction experiments were conducted in two ways, firstly using endogenous lachesin present in *Ae. aegypti* protein extract and secondly through bacterially expressed and purified truncated lachesin protein for testing direct protein–protein interaction. In first approach, purified EDIII bound with Ni-NTA agarose beads was incubated with whole mosquito protein extract prepared in homogenisation buffer (10 mM Na_2HPO_4 [pH 7.5], 1 mM EDTA, 500 mM NaCl and protease inhibitor cocktail [Sigma]) on shaker at 4°C for 2 hr. After incubation, the beads were centrifuged at 1,485*g* at 4°C. Unbound fractions were separated and beads were washed twice with washing buffer containing 0.01% Triton X-100 in binding buffer. Beads were mixed with 40 μl sample buffer and boiled at 100°C for 10 min. After incubation, 20 μl of each reaction was resolved in 12.5% SDS-PAGE and blotted to nitrocellulose membrane. Blot was developed using anti-lachesin and anti-mouse-HRP-labelled antibodies. Blots were developed by ECL using Clarity western ECL substrate (Biorad), and signals were recorded on gel documentation system (Amersham Imager 600, GE Healthcare). In second approach, purified truncated lachesin protein (239–335 aa) was incubated with MBP-tagged EDIII bound to dextrin beads in binding buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$ and 0.01% NP-40) for 2 hr at 25°C. After incubation, beads were washed with washing buffer (binding buffer containing 400 mM of NaCl and 0.05% NP-40). Proteins bound to beads were eluted by directly boiling the beads in 40 μl SDS-sample buffer for 10 min followed by short centrifugation. A total 20 μl of sample containing eluted protein was resolved on SDS-PAGE and detected using coomassie brilliant blue (CBB) staining.

4.10 | Immunoblotting and virus overlay protein binding assay

To check the presence of DENV-2 particles in samples, infected C6/36 supernatants were preheated with sample buffer (2% [wt/vol] SDS, 50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol) for 10 min at 95°C. Prepared samples were resolved using SDS-PAGE and further blotted on nitrocellulose membrane. After blocking in 3% (wt/vol)

TABLE 2 IgSF receptors used by diverse groups of viruses, to gain entry to the host cell

Virus	Receptor name	Abbreviation	Reference
Adenovirus C	Coxsackievirus–adenovirus receptor	CAR	Carson (2001)
Mouse hepatitis virus	Carcinoembryonic antigen	CEACAM-1/CD66a	Dveksler et al. (1991)
Feline calicivirus	Junction adhesion molecule 1	JAM-1	Makino et al. (2006)
Coxsackie A virus type 21	Intercellular adhesion molecule 1	ICAM-1	Shafren, Dorahy, Greive, Burns, and Barry (1997)
Coxsackie B virus	Coxsackievirus–adenovirus receptor	CAR	Carson (2001)
Encephalomyocarditis virus	Vascular cell-adhesion molecule 1	VCAM-1	Huber (1994)
Major receptor group rhinovirus	Intercellular adhesion molecule 1	ICAM-1	Staunton et al. (1989)
Poliovirus	Poliovirus receptor	PVR/CD155	Mendelsohn, Wimmer, and Racaniello (1989)
Reovirus	Junctional adhesion molecule A	JAM-A	Barton et al. (2001)
Dengue virus (DENV)	Natural killer cell p44-related protein	NKp44	Hershkovitz et al. (2009)
DENV	T-cell immunoglobulin and mucin domain 1	TIM-1	Meertens et al. (2012)
Mouse hepatitis virus	Carcinoembryonic antigen-related cell-adhesion molecule 1	CEACAM-1/CD66a	Dveksler et al. (1991)
Herpes simplex virus	Poliovirus receptor related protein 1	HveC	Geraghty et al. (1998); Warner et al. (1998)

BSA in PBS, membranes were incubated with the primary antibody anti-envelope mouse antibody (1:2,000) (Santa Cruze Biotechnology) PBS for 2 hr at room temperature. After several washes in PBS, 0.05% (wt/vol) Tween 20 (PBST), the secondary HRP-labelled anti-mouse antibody was applied 1:4,000 in PBST for 1 hr at room temperature. Blots were developed using ECL detection system. To perform virus overlay protein binding assay (VOPBA), DENV particle, MBP-tagged EDIII and MBP were resolved using SDS-PAGE and further blotted on nitrocellulose membrane. After blocking with 3% BSA, membrane was probed with recombinant purified truncated lachesin protein for 4 hr at 4°C. Following incubation, membrane was washed three times with 0.05% Tween 20 containing PBS. To detect the bound lachesin protein to membrane, primary antibody anti-lachesin and HRP-labelled anti-mouse antibody was used. Further ECL detection system was used to develop the bands. For VOPBA control experiments, two controls were performed; in the first control the blots were not incubated with purified truncated lachesin and were detected using primary anti-lachesin antibody followed by secondary HRP-labelled anti-mouse antibody. In the second control, the blot was overlaid with purified truncated lachesin protein but the primary anti-lachesin antibody was not used, rather the blot was incubated with secondary HRP-labelled anti-mouse antibody for detection using ECL detection system.

4.11 | Blocking of lachesin protein and DENV-2 infection in *Ae. aegypti*

To block lachesin protein and to check its effect on DENV replication, 26 μg/μl of anti-lachesin antibody and 1.38×10^7 focus forming unit (FFU) per millilitre of DENV-2 (ATCC VR-1584) were used. The antibody and the virus were mixed in 1:1 ratio and 69 ηl of this mixture was injected into the thorax of *Ae. aegypti* mosquitoes. Similarly 1/10 and 1/100 dilution of the antibody was also prepared and injected. In the control experiments, pre-immune sera and PBS were injected along with DENV-2. After 6 days, mosquitoes were analysed for virus titre using focus forming assay (FFA). To conduct FFA, five mosquitoes were taken from each group and homogenised in 1 ml of Dulbecco's Modified Eagle Media (DMEM) separately. After centrifugation, supernatant was collected and filtered using 0.22 μm syringe filters. These samples were used to perform FFA for DENV-2 quantification. Briefly, Vero cells were seeded in 96 well plates, followed by inoculation of the homogenised mosquito samples at different dilutions (starting at 1:50). Viral adsorption was allowed to proceed for 2 hr at 37°C. An overlay containing 5% fetal bovine serum and 1% carboxymethyl cellulose in DMEM was added at the end of adsorption. The infected monolayer was incubated at 37°C. After 72 hr of infection, the overlay medium was removed from the wells, and cells were washed with cold PBS. The cells were fixed for 30 min in acetone:methanol (1:1). After washing with PBS, the cells were blocked with 5% skim milk/PBS for 30 min. Infected cells were detected with a monoclonal anti-dengue antibody. After washing with PBS, antibody-labelled cells were detected with a secondary antibody conjugated to Horseradish peroxidase (HRP). The labelling was visualised with 3,3′,5,5′-tetramethylbenzidine. The FFUs were counted, and the viral titres were determined by times dilution factor. The FFUs were counted using the formula: FFU/ml = No. of foci/dilution × volume. All the experiments were performed a minimum of three times. One-way ANOVA and unpaired *t* test were performed using GraphPad Prism for statistical significance.

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CONFLICT OF INTEREST

The authors have no competing financial interests.

AUTHOR CONTRIBUTIONS

Raman Rajagopal, Vipin Singh Rana and Sonam Popli conceived and designed the experiments. Vipin Singh Rana, Sonam Popli, Gunjan Kumar Saurav, Karuna Yadav, Ankit Kumar and Narendra Kumar performed the experiments. Vipin Singh Rana, Sonam Popli, Gunjan Kumar Saurav, Karuna Yadav, Ankit Kumar, Sujatha Sunil, Om Prakash Singh, Krishnamurthy Natarajan and Raman Rajagopal analysed the data. Vipin Singh Rana, Sonam Popli, Gunjan Kumar Saurav, Karuna Yadav, Sujatha Sunil, Om Prakash Singh, Krishnamurthy Natarajan and Raman Rajagopal wrote the paper. All authors read, discussed, edited and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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