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Aedes aegypti ML and Niemann-Pick type C family members are agonists of dengue virus infection

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ABSTRACT

Upon exposure to dengue virus, the *Aedes aegypti* mosquito vector mounts an anti-viral immune defense by activating the Toll, JAK/STAT, and RNAi pathways, thereby limiting infection. While these pathways and several other factors have been identified as dengue virus antagonists, our knowledge of factors that facilitate dengue virus infection is limited. Previous dengue virus infection-responsive transcriptome analyses have revealed an increased mRNA abundance of members of the myeloid differentiation 2-related lipid recognition protein (ML) and the Niemann Pick-type C1 (NPC1) families upon dengue virus infection. These genes encode lipid-binding proteins that have been shown to play a role in host–pathogen interactions in other organisms. RNAi-mediated gene silencing of a ML and a NPC1 gene family member in both laboratory strain and field-derived *Ae. aegypti* mosquitoes resulted in significantly elevated resistance to dengue virus in mosquito midguts, suggesting that these genes play roles as dengue virus agonists. In addition to their possible roles in virus cell entry and replication, gene expression analyses suggested that ML and NPC1 family members also facilitate viral infection by modulating the mosquito's immune competence. Our study suggests that the dengue virus influences the expression of these genes to facilitate its infection of the mosquito host.

1. Introduction

Dengue fever and dengue hemorrhagic fever are the most important arthropod-borne viral diseases, with an estimated 390 million infections occurring annually across more than 100 countries in the tropics and subtropics (Bhatt et al., 2013). A dramatic increase in dengue incidence in recent decades has resulted from an increased global prevalence of its primary vector, *Aedes aegypti*, along with its secondary vector, *Aedes albopictus* (Guzman et al., 2010; Whitehead et al., 2007). Dengue can be caused by any of four antigentically distinct serotypes (DENV serotype 1–4), and there are currently no anti-DENV drugs or vaccines available. Thus, disease control relies mainly on mosquito-targeted intervention programs. However, the conventional mosquito elimination programs depend on the use of insecticides and environmental management, which raise ecological, environmental, and effectiveness concerns (Ault, 1994; Dong, 2007; Gubler, 1998; Rivero et al., 2010). For this reason, the development of novel vector and disease control strategies is essential, and a molecular understanding of the mosquito's immune responses against these viruses is needed.

DENV is transmitted from infected humans to other individuals through mosquito bites. After mosquitoes feed on infectious blood, the virus infects the mosquito midgut epithelium and propagates to establish the infection (Black et al., 2002). Virus levels in the midgut generally peak at 7–10 days, with the virus then disseminating to other parts of the body through the trachea. The virus finally infects the salivary glands, from which it can be transmitted to another host through a mosquito blood meal, which typically occurs about 10 days after the original infectious blood meal (10 dpbm) (Salazar et al., 2007).

The publication of the *Ae. aegypti* genome in 2007 (Nene et al., 2007) has opened new avenues for the study of the mosquito's response to DENV infection. Through genome-wide transcriptomic analyses, in conjunction with RNAi-mediated gene silencing, we have identified the Toll and JAK–STAT pathways as key DENV antagonists that act by controlling virus restriction factors (Souza-Neto et al., 2009; Xi et al., 2008). DENV infection-responsive transcriptome analyses have revealed that the transcript abundance of five members of two lipid-binding protein gene families, the myeloid differentiation 2-related lipid recognition protein (ML) and Niemann Pick-type C1 (NPC1) families, is increased in response to DENV infection. Since DENV is an enveloped virus and its outer shell is lipid-based, these lipid-binding proteins are likely to play a role(s) in mosquito–virus interactions.

The ML domain is a lipid recognition protein domain found in several proteins with lipid-binding properties (Inohara and Nunez, 2002). Members of this family have diverse functions associated

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with lipid recognition, including pathogen recognition, lipid trafficking and metabolism, and pheromone perception (Chang et al., 2006; Gruber et al., 2004; Horácková et al., 2010; Starostina et al., 2009). A role for the ML domain in immune recognition has been described for the vertebrate MD2 protein and its insect homologs. MD2 is a secreted glycoprotein that mediates the activation of the vertebrate Toll-like receptor 4 (TLR4) upon exposure to bacterial lipopolysaccharide (LPS) (Shimazu et al., 1999). *Drosophila* MD2 homologs have been shown to mediate the activation of the immune deficiency (IMD) immune signaling pathway upon exposure to lipopolysaccharide (LPS) (Shi et al., 2012). The *Anopheles gambiae* homolog of ML, AgMDL1, is involved in the mosquito's immune defense against *Plasmodium falciparum* infection (Dong et al., 2006). Niemann-Pick disease type C1 (NPC1) is another class of lipid-binding proteins that is responsible for cholesterol transport and homeostasis; these proteins function together with the NPC2 proteins in the late endosomal/lysosomal system (Chang et al., 2006; Garver and Heidenreich, 2002). NPC1 has been shown to be required for the Ebola virus to escape from the vesicular compartment (Carette et al., 2011; Côté et al., 2011), but the immune function of the NPC1 family in the mosquito has yet not been investigated.

Although these lipid-binding protein families have been shown to be involved in virus–host interaction and immune responses in various systems, little is known about their function in the *Ae. aegypti*–DENV interaction. Here, we investigated the role of *Ae. aegypti* ML and NPC1 gene family members in modulating DENV infection in the mosquito by conducting RNAi-mediated gene silencing and gene expression studies. Our results suggested roles for an ML and a NPC1 protein as agonists of DENV in the mosquito. Furthermore, our data suggest that the virus might influence the expression of these genes to facilitate its infection, emphasizing the importance of lipid-binding proteins in viral infection of insects.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: M006H300). Commercial anonymous human blood was used for DENV infection experiments. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

2.2. Bioinformatics analyses and genes selection

The gene sequences and gene annotations for the insect ML and NPC gene families were obtained from the ImmunoDB (http://cegg.unige.ch/Insecta/immunodb) and Vectorbase (http://aaegypti.vectorbase.org/) databases (Lawson et al., 2009; Waterhouse et al., 2007). To compare sequence similarity, a multiple sequence alignment (MSA) was generated using T-coffee software (http://www.tcoffee.org/) (Di Tommaso et al., 2011). The MSA was then used to generate a phylogenetic tree using MEGA 5.05 software (Tamura et al., 2011). ML and NPC genes that potentially play a role in DENV infection were suggested from the transcriptional changes of these genes in previous microarray-based transcriptome studies (Sim et al., 2012; Xi et al., 2008) Dimopoulos group, unpublished data).

2.3. Mosquito strains and mosquito maintenance

The mosquitoes used for most of the experiments were of the *Ae. aegypti* Rockefeller/UGAL strain. The other mosquito strain used to confirm that the results were common among different mosquito strains was a recently colonized *Ae. aegypti* population obtained from the Caribbean island of Saint Kitts (sixth to seventh generation) (Sim et al., 2013). The mosquitoes were maintained on a 10% sugar solution at 27 °C and 95% humidity with a 12-h light/dark cycle, following the protocol described previously (Xi et al., 2008).

2.4. Cell culture

The *Ae. albopictus* C6/36 cell line was used to propagate DENV. It was maintained in complete minimum essential medium (MEM) supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 1% MEM non-essential amino acids, 10 unit/mL penicillin, and 10 μg/mL streptomycin at 32 °C and 5% CO_2.

The baby hamster kidney (BHK) cell line was used for plaque assays. It was maintained in complete Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 10 unit/mL penicillin, 10 μg/mL streptomycin, and 5 μg/mL plasmocin at 37 °C and 5% CO_2.

2.5. Genes silencing by RNA interference

The role of the ML and NPC1 genes in DENV infection in *Ae. aegypti* was assessed using RNA interference-mediated gene silencing as described previously (Garver and Dimopoulos, 2007). In brief, dsRNAs were constructed using in vitro transcription with the HiScribe™ T7 In Vitro Transcription Kit (New England Biolabs). Approximately 200 ng of dsRNA was injected into the thorax of cold-anesthetized 3–4 days old female mosquitoes using a nano-injector. The dsRNA-injected mosquitoes were kept in the insectary under the conditions mentioned above. Gene silencing efficiency, evaluated using real-time PCR, was determined by comparison to the GFP dsRNA-injected group at 3 days after dsRNA injection.

2.6. DENV propagation and viral infection in the mosquito

The DENV strain used in these experiments was DENV serotype 2 (New Guinea C strain, DENV-2). The virus was propagated in the C6/36 cell line according to a protocol previously described (Das et al., 2007). In brief, DENV stock was added to a 75-cm^2 flask of C6/36 cells at 80% confluence to yield a multiplicity of infection of 1. The virus-infected cells were harvested 6 days after infection. The virus was extracted from the cells by freezing and thawing for two cycles in dry CO_2 and a 37 °C water bath, centrifuged at 800*g* for 10 min, and mixed 1:1 with commercial human blood. The infectious blood meal ($\sim 10^6$–10^7 colony-forming units [cfu]/ml) was maintained at 37 °C for 30 min prior to membrane feeding to 5–7 days old or 3-day post-dsRNA-injected mosquitoes. Blood-fed mosquitoes were separated on ice and maintained under the conditions mentioned above.

2.7. DENV titration by plaque assay

Virus titers in the midguts were determined at 7 dpbm according to an established protocol (Das et al., 2007; Xi et al., 2008). Mosquito midguts were dissected in sterile 1×PBS and stored in complete DMEM medium at −80 °C until used. Midgut samples were homogenized using a homogenizer (Bullet Blender, Next Advance) with 0.5-mm glass beads. The virus-containing