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Aedes aegypti antiviral adaptive response against DENV-2

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ABSTRACT

Priming is the conceptual term defining memory phenomenon in innate immune response. Numerous examples of enhanced secondary immune response have been described in diverse taxa of invertebrates; which naturally lacks memory response. In mosquitoes, a previous non-lethal challenge with some specific pathogens modify their immune response against the same microorganism; developing an improved antimicrobial reaction. In this work, we explore the ability of *Aedes aegypti* to mount a higher antiviral response upon a second oral DENV challenge. When previously challenged with inactive virus, we observed that the posterior infection showed a diminished number of DENV infectious particles in midguts and carcasses. In challenged tissues, we detected higher *de novo* midgut DNA synthesis than control group, as determined by DNA incorporation of 5-bromo-2-deoxyuridine. We demonstrated that inactive DENV particle are capable to induce DNA synthesis levels comparable to infective DENV. We considered the *Drosophila melanogaster hindsight* and *Delta-Notch* mosquitoes orthologues as potential *de novo* DNA synthesis pathway components (as observed in fly oocyte development and midgut tissue renewal). We showed that *Aedes aegypti hindsight* transcript relative expression levels were higher than control during DENV infection and inactive DENV particle alimentation. Also, *Aedes aegypti* second challenge with active DENV induced higher *hindsight*, *Delta* and *Notch* transcriptions in the primed mosquitoes (compared with the primary infection levels). Considering that the mosquito *de novo* DNA synthesis is concomitant to viral particle reduction, this finding opens a new perspective on the mechanisms underlying the vector antiviral immune response and the effector molecules involved.

1. Introduction

Immune memory has been extensively studied in mammals, and this process is, in evolutionary terms, a recent adaptation to recurrent biotic stresses. This mechanism provides the ability to neutralize secondary encounters with potential pathogens during the life-time of the animal. This phenomenon is attributed to adaptive mechanisms such as antibody isotype switch and T- and B-memory cells generation (Gasper et al., 2014; MacLeod et al., 2010; Shivarov et al., 2009; Weng et al., 2012).

In the last few years, evidences of memory-like response generated by the innate immune systems have been accumulating. The mechanism has been termed *priming* for invertebrates and innate immune memory for vertebrate NK cells and macrophages (Kurtz, 2004; Milutinović et al., 2016; Pulendran and Ahmed, 2006; Saeed et al., 2014; Sun et al., 2014, 2011).

In the case of the invertebrates, *priming* has been investigated in different taxonomic groups. The existence of this acquired-immunity in mosquitos is a crucial issue to limit the spread of infectious diseases transmitted by arthropods. These diseases affect millions of people in developing countries.

Recent evidences demonstrate that, upon a second encounter with extracellular pathogens (bacteria, parasites or fungi), the innate immune system responds more effectively. In the case of virus infection, examples of this differential immune response are scarcer. For instance, *Penaus monodon* shrimps White Spot Syndrome Virus resistance and *Plodia interpunctella* moth *Pi Granulosis virus* resistance upon a second encounter have been reported, but these viral invertebrate immune *priming* examples are related to dsDNA viruses resistance (Rungrassamee et al., 2013; Syed Musthaq and Kwang, 2011; Tidbury et al., 2011). RNA viruses transgenerational *priming* has been observed in *C. elegans* infected with Orsay virus; though *Drosophila melanogaster* did show no

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evidence of *priming* with two lethal doses of infective *Drosophila C virus* (Longdon et al., 2013; Rechavi et al., 2011; Sterken et al., 2014). In *C. elegans,* transgenerational resistance mechanism is mediated small interfering RNAs expression silencing the viral genome replication through piwi RNAs and chromatin remodeling factors expression (Rechavi et al., 2011).

In our workgroup, we previously observed that *Anopheles* mosquitoes primed against *Plasmodium* had reduced midgut oocyst infection when compared to naïve mosquitoes (Contreras-Garduño et al., 2015). Also, we observed that primed *Aedes aegypti* mosquitoes were resilient to otherwise lethal doses of pathogen bacteria (Vargas et al., 2016). Dengue virus infection triggers the activation of *Aedes aegypti* Toll, IMD and Jak/STAT transcriptional cascade as well as the mosquito RNA interference pathways (Dostert et al., 2005; Franz et al., 2006; Sánchez-Vargas et al., 2009; Sim and Dimopoulos, 2010; Souza-Neto et al., 2009; Xi et al., 2008). Upregulation of these pathways decrease the viral replication. Interestingly, several genes related to cell cycle control are repressed during DENV infection (Schonhofer et al., 2016; Xi et al., 2008; Zou et al., 2011), hence opening the possibility of a correlation between *Aedes aegypti* DNA synthesis and immune response.

In this work we investigate if *Aedes aegypti* immune response can be primed by Dengue virus, thereby limiting the viral replication. We investigate the participation of DNA synthesis in a memory mechanism of the insect immune system (Contreras-Garduño et al., 2015; Hernández-Martínez et al., 2013).

The *Drosophila melanogaster Delta-Notch* cascade signaling is involved in the fly midgut epithelial renewal and intestinal stem cell differentiation to enterocytes subtypes (Baechler et al., 2016; Edgar et al., 2014; Sun and Deng, 2007). This DNA synthesis signaling cascade could provide a mechanistic explanation for the DNA synthesis *de novo* in *Aedes aegypti* midguts tissue in response to biotic challenge.

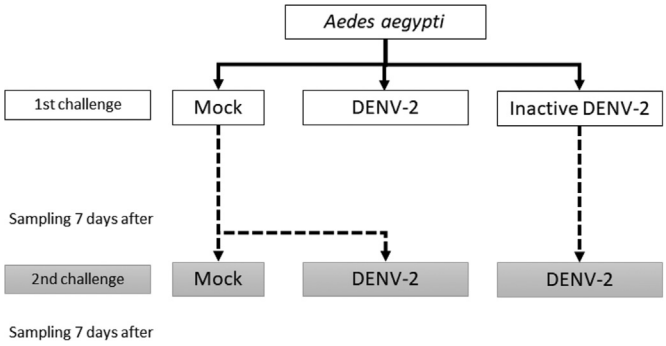
This signaling cascade initiates with the induction of *Delta* ligand expression; which binds to *Notch* receptor. The binding induces Notch intracytoplasmic domain cleavage and translocation to the nucleus. Notch activates the transcription of the genes required for DNA synthesis. *Hindsight* acts as a cytoplasmic repressor for mitotic cyclins, switching from mitotic cycles to endocycles. This phenomenon allows a rapid gene transcription required for the synthesis of larger amounts of protein (Baechler et al., 2016; Edgar et al., 2014; Kasner et al., 2014; Shen and Sun, 2017; Zielke et al., 2013). Therefore, analysis of the transcriptional levels of these three molecules during *priming*/endoreplication events corroborates their involvement in the *priming* mechanism of insects.

2. Materials and methods

2.1. Mosquitoes oral virus challenges

Aedes aegypti Rockefeller strain were reared in the insectary of the INSP (*Instituto Nacional de Salud Pública*) as described elsewhere (Ramos-Castañeda et al., 2008). Adult females were orally challenged with 1×10^8 Foci Forming Units per mililiter of DENV-2 New Guinea C virus propagated in C6/36 cells, mixed 1:1 with rabbit erythrocytes resuspended in MEM media (Thermo Scientific). This inoculum is sufficient to infect 100% of the mosquitoes (Carrington and Simmons, 2014). Mock infections inoculum consists in uninfected C6/36 lysate plus erythrocytes resuspended in MEM media. Viral inactivation was realized mixing the virus with 0.02 mg/mL of Psoralen (Sigma) followed by a 1-h UV-light exposition at 4 °C (Aubry et al., 2016).

2.2. Experimental design



Five days post-emergence, groups of 100 female *Aedes aegypti* mosquitoes were mock-fed (uninfected C6/36 cell lysate in rabbit erythrocytes), DENV-fed (titrated DENV-2 infected C6/36 cell lysate in rabbit erythrocytes) and inactive DENV-fed (titrated DENV-2 infected C6/36 cell lysate treated with psoralen-UV in rabbit erythrocytes). Seven days post-first challenge, midgut and carcasses were dissected for further analysis. A second challenge was realized with remaining individuals. The mock-infected mosquitoes were divided in two groups: the first one was mock fed, and the second was DENV-fed. The third group (previously fed with inactive DENV-2) was challenged with infectious DENV-2. Seven days post-second challenge (fourteen days after first challenge), midguts and carcasses tissues were dissected individually for posterior analysis in infectivity titer assay. Ten midguts pools per group were placed in Trizol reagent for viral load through PCR.

2.3. Viral titration and NS1 relative determination

For NS1 antigen detection, freshly dissected midguts were cultured in MEM media mixed with antibiotics for 24 h. Supernatants were tested in a direct ELISA assay for NS1 viral antigen relative determination (Platelia kit, Bio-Rad). Midguts and carcasses were dissected 7 days post infection in both challenges, and frozen at −70 °C in MEM media, tissue viral titers were determined through Focus Forming Units assay. We used an immunocytochemistry protocol using LLC-MK_2 cells (ATCC CCL-7) infected with Log_{10} serial dilutions of macerated samples. Infection foci were detected using anti Dengue M Protein mouse antibody (AB_1240702), secondary goat anti-mouse-IgG-horseradish peroxidase (AB_955395) and DAB substrate to reveal immunoassay. Foci were counted and virus titer was calculated using Reed-Muench formula described elsewhere (Baer and Kehn-Hall, 2014).

Total viral genome copies were determined through real time PCR amplification. Serial Log_{10} dilutions of a Dengue insert containing plasmid were used to establish a standard curve for known number of dengue genome copies (1×10^7 Dengue genome copies per microliter, a kind gift donated by Dr. Rosa Ma. Del Angel, CINVESTAV, IPN). Total viral load per midguts pools were extrapolated from the samples Ct values.

2.4. DNA synthesis de novo in mosquitoes

During *Aedes aegypti* infection kinetics, mosquitoes were fed *ad libitum* with a cotton soaked in sterile 10% sucrose solution with 100 μg/mL of BrdU. DNA was extracted with Phenol-Chloroform-Isoamyl Alcohol mixture as per manufacturer's instructions. DNA was quantified in a Nanodrop spectrophotometer at 230/260 nm