RESEARCH PAPER

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Expression of dengue virus NS3 protein in *Drosophila* alters its susceptibility to infection

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We developed a *Drosophila* model in which the dengue virus NS3 protein is expressed in a tissue specific and inducible manner. Dengue virus NS3 is a multifunctional protein playing a major role during viral replication. Both protease and helicase domains of NS3 are interacting with human and insect host proteins including innate immune components of the host machinery. We characterized the NS3 transgenic flies showing that NS3 expression did not affect fly development. To further study the links between NS3 and the innate immune response, we challenge the flies with gram-positive and gram-negative bacteria. Interestingly, the *Drosophila* transgenic flies expressing NS3 were more susceptible to bacterial infections than control flies. However ubiquitous or immune-specific NS3 expression affected neither the life span nor the response to a non-infectious stress of the flies. In conclusion, we generated a new *in vivo* system to study the functional impact of DENV NS3 protein on the innate immune response.

Background

The genus *Flavivirus* is a large group of arthropod-borne viruses including major human pathogens, such as West Nile (WNV), yellow fever (YFV), and the 4 serotypes of dengue viruses (DENV types 1-4).[1,2] DENV are mosquitoes-borne pathogens that are transmitted to human by *Aedes* mosquitoes including *Aedes aegypti* and *Aedes albopictus*. In order to develop new therapeutic approaches, a growing scientific community is working on *Aedes* with the rationale that targeting the arthropod vector to interfere with virus transmission should limit its spreading to humans.[3] Indeed, recent studies have demonstrated that infection by endosymbiotic bacterium *Wolbachia* induces resistance to Dengue virus in *Aedes aegypti* and suppresses its transmission.[4,5] In this context, understanding how DENV replication may be controlled in arthropods is of major importance. To achieve this goal, and to better characterize the innate immune response against the virus, several approaches have been followed using either *Aedes* or *Drosophila* models. These approaches include genome wide RNAi interference screen in *Drosophila*,[6] whole-genome microarray in *Aedes aegypti* [7] and evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes.[8] Importantly, the antimicrobial Toll pathway was shown to control Dengue virus infection in *Aedes aegypti* [9-11] but was not required for *Wolbachia* mediated dengue virus interference in *Drosophila*.[12]

The evolutionary conservation of innate mechanisms of host defense has made *Drosophila* a useful model to study the genetic control of innate immune response. Indeed, the 2 NF-κB signaling cascades that regulate *Drosophila* antimicrobial defense responses share striking similarities with the vertebrate innate immune responses. Activation of the Toll pathway in particular triggers the expression of a set of antimicrobial peptide genes including *Drosomycin* (*Drs*) in a Cactus dependent manner.[13] Cactus is the *Drosophila* ortholog of the mammalian kinase IκBα. In steady state condition; Cactus inhibits the nuclear translocation of Dif, Dorsal-related Immunity Factor (ortholog of p65). After activation, Cactus releases Dif from the cytoplasm allowing its translocation to the nucleus.[14] The Toll cascade is highly similar to the mammalian TLR (Toll-like receptor) receptor pathway.[15]

In order to identify interactions between cellular and DENV proteins, we have constructed a Flavivirus-host interaction map using a genome-wide high-throughput yeast 2-hybrid screen (Y2H).[16] The global analysis of the cellular targets highlighted the enrichment of host proteins involved in RNA binding, transcription regulation, vesicular transport or innate immune response regulation. Using both Y2H and co-immunoprecipitation approaches, we previously found that NS3, a non-structural protein of DENV targets IκBα, TRAF4 and AZI2, 3 cellular proteins of the Toll-like receptor (TLR) pathway.[16] NS3 is a multifunctional protein that contains a N-terminal protease domain and a C-terminal domain that displays nucleotide 5′-triphosphatase (NTPase), RTPase, and helicase activities. In association with NS5 and NS2B, NS3 plays a crucial role in DENV RNA replication and viral protein maturation.[17] As our screen

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unraveled the interactions of NS3 helicase domain with innate immune response components, we aimed to assess the functional consequences of these interactions *in vivo*.

We thus used the UAS–Gal4 system to express the dengue NS3 either as full-length protein (NS3 Fl) or as NS3 helicase (NS3 Hel) functional domain in *Drosophila melanogaster*. Following a challenge with the pathogenic bacterial strains *Staphylococcus aureus* and *Pseudomonas aeruginosa*, we observed a significant difference in flies survival rate in NS3 expressing flies compared to control flies. In contrast, we found that ubiquitous or immune-specific NS3 or NS3 Hel expression was well tolerated by the flies and did affect neither their life span nor their response to a non-infectious stress.

Methods

Transgenic Drosophila

We cloned DENV2 NS3 helicase domain and DENV2 full-length NS3 protein cDNA into the GAL4-responsive pUAST expression vector,[18] and transgenic strains were generated by embryo injection by Bestgene Company (Chino Hills, CA, USA). Both NS3 helicase and full-length NS3 cDNA carried a 3XFlag Tag in 5′.[16] Standard crosses with flies carrying appropriate balancers were performed to establish stable heterozygous or homozygous transgenic lines. The following Gal4 driver lines were obtained from Bloomington stocks: the ubiquitious drivers Act-Gal4,[19] the engrailed domain-specific driver en-Gal4,[20] the immune specific driver (fat body and hemocytes) collagen type IV-specific enhancer-promoter Cg-Gal4.[21] Flies expressing the Gal4 factor were used as controls: Act-Gal4>+, en-Gal4>+, Cg-Gal4>+. The wild type chromosome from the control heterozygotes was derived from the w^{1118} strain.

Western blot

Third instar larvae were crushed in lysis buffer (20mM Tris-HCl pH 7,4; 100 mM NaCl; 1% NP-40; 2 mM DTT) supplemented with a protease inhibitor cocktail (Sigma). Larvae lysates were incubated on ice for 30 min, and then centrifuged at 14,000 g for 20 min. Total protein concentrations were then determined using the Bradford protein assay (Biorad). 15 μg of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels with MOPS running buffer (SDS-PAGE) and transferred to nitrocellulose membrane. 3XFlag- tagged proteins were detected with a mouse monoclonal peroxidase-conjugated anti-FLAG M2 antibody (Sigma) and revealed with ECL detection reagent (GE Healthcare).

Wing imaginal discs staining

Wing imaginal discs were dissected and stained as previously described.[22] Briefly, wandering third instar larvae were dissected using inside-out technique directly in PBS. Tissues were fixed in 4% formaldehyde in PBS 20 min at room temperature with mild agitation. Imaginal discs were then washed 3 times in PBS-triton, incubated for 20 min at room temperature in blocking solution (PBS 0.1% triton, 5% BSA, 2.5% NGS) and incubated overnight with anti-flag M2 antibody coupled with cya3 (Sigma) diluted in blocking solution (dilution 1/600). Tissues were then washed 3 times in PBS triton 0.1% and incubated with the phalloidin-FITC 10 μg/mL (Sigma) for 30 min at room temperature. Finally and after 3 additional washing imaginal discs were mounted in a 50% glycerol-PBS solution.

Picture acquisition

All microscopic pictures have been taken using an upright confocal microscope (Axioplan2 LSM510 Zeiss) and a Plan Neofluar 40x/1.3 Oil Ph3 objective. All images were processed with Adobe Photoshop or ImageJ softwares.

Life span

Act-Gal4 and Cg-Gal4 X UAS-NS3 Fl or UAS-NS3 Hel crosses were performed, newly F1 eclosed female flies were collected, allowed mating for 48 h after emerging then sorted and aged at 25°C on standard medium. Act-Gal4 and Cg-Gal4 X w^{1118} crosses were performed to generate control flies. One hundred flies per genotype were aged in individual vials containing no more than 20 flies each and assayed for longevity. Food was changed every 2 or 3 d, and the number of dead flies was recorded. Statistical analysis was done using the nonparametric log rank (Mantel-Cox) test.

Cold stress and recovery

All tests were performed using synchronized 3- to 5-day-old virgin flies as previously described.[23] Briefly, groups of 20 flies were placed in glass vials (without food), which were immersed in a 10% glycol solution cooled to 0°C to induce chill coma. Flies were sampled after 0.5, 3, 6 and 9h of cold stress. For each cold stress duration, 40 males of each genotype (w^{1118}; Cg-Gal4/UAS-NS3 Hel, w^{1118}; Cg-GAL4/UAS-NS3 Fl and w^{1118}; Cg-Gal4/UAS-LacZ) were allowed to recover at 25°C, and the recovery time was recorded. Flies were considered to have recovered when they stood up.

Drosophila infection

P. aeruginosa (PAO1), E. coli 1106 and *E. aerogenes* were used as Gram-negative bacterial strains. *M. luteus* and *S. aureus* were used as Gram-positive strains. All strains were grown as previously described.[24] For septic infection, 30 to 50 adult male flies of 3- to 5-day-old were pricked in the upper part of the thorax with a thin needle previously dipped into an overnight concentrated bacterial culture. Results are expressed as percentage of surviving infected flies at different time points following infection. Statistical analysis was done using the nonparametric log rank (Mantel-Cox) test.

Results

Characterization of *Drosophila* lines expressing the Dengue virus NS3 protein

We generated transgenic flies carrying either a UAS-NS3 full length or a UAS-NS3 helicase domain expressing transgene. The

two corresponding constructs are bearing a 3Xflag tag. Two independent transgenic lines were generated for each construct using the PhiC31 recombination strategy, which allows similar level of expression between transgenes inserted on the same insertion site. To characterize the transgenic lines, we first crossed the UAS-NS3 flies to flies carrying the well-defined engrailed-Gal4 (en-Gal4) driver. As expected we observed the specific expression of NS3 in the engrailed domain in the posterior part of the wing imaginal disc for all transgenic lines (**Fig. 1**). We then performed a western blot analysis and could detect the NS3 tagged proteins at the expected size, 58 kDa and 80 kDa for the NS3 helicase and the NS3 respectively (**Fig. 1F**). Our data indicate that NS3 full length or NS3 helicase expression in *Drosophila* wing tissue do not affect wing development.

Expression of dengue virus NS3 protein in *Drosophila* alters its susceptibility to bacterial infection

In order to examine if NS3 expression would be sufficient to affect flies susceptibility to bacterial infection, flies expressing NS3 full length or NS3 helicase domain under the control of the immune Cg-Gal4 driver were infected with several bacterial species by septic injury. Age and sex match control flies were infected

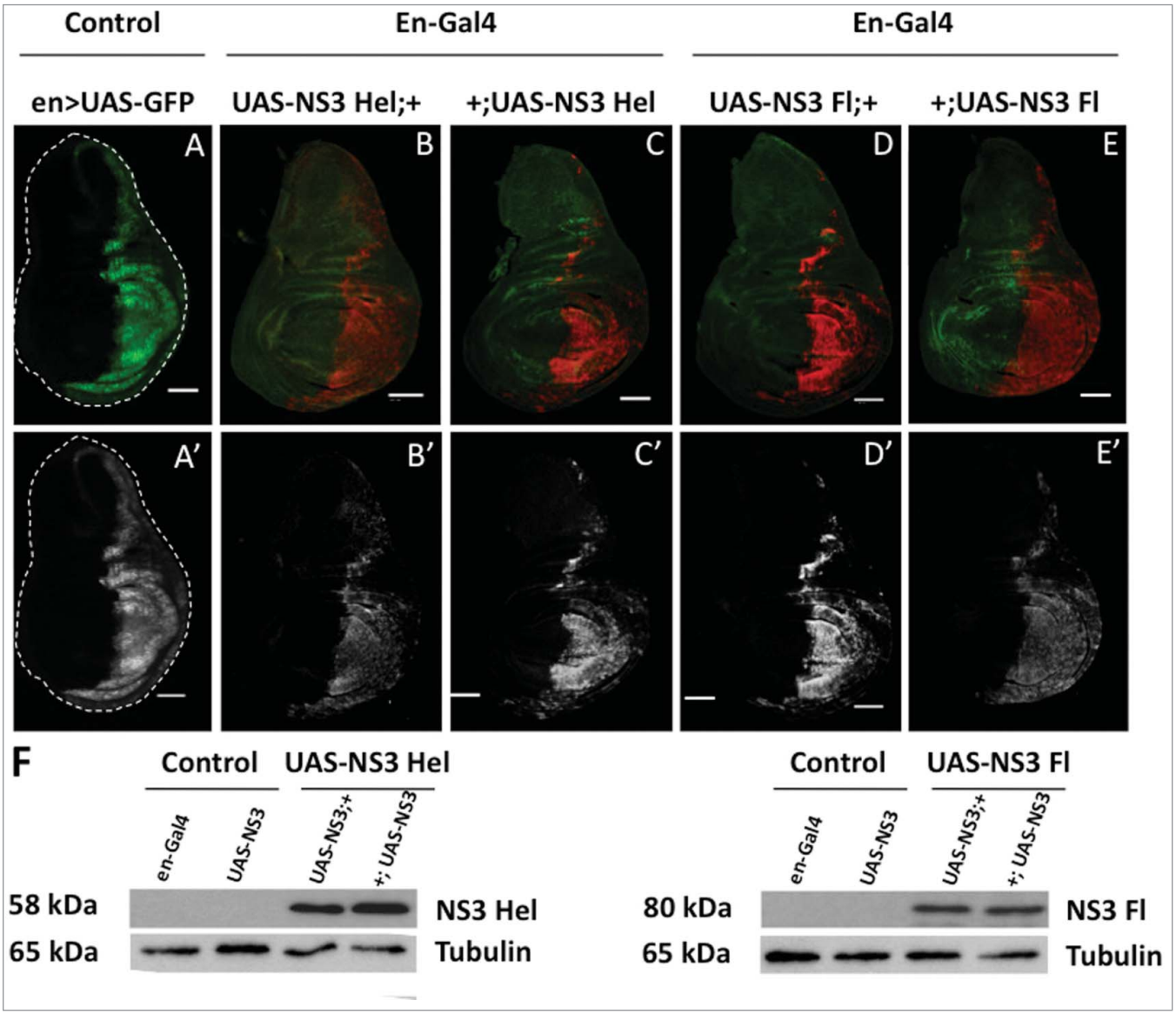

Figure 1. Expression of NS3 helicase in developing wing imaginal discs. Males carrying UAS-NS3 helicase or UAS-NS3 full-length expressing transgenes were mated with engrailed-GAL4 (en-GAL4) females. The different lines represent UAS-NS3 Hel and UAS-NS3 Fl expressing transgenes inserted on chromosome II (UAS-NS3;+) and III (+; UAS-NS3) respectively. (**A**) en>UAS-GFP line was used as a control for the engrailed expression pattern. (**B–E**) Late third instar wing imaginal disks were dissected and stained with phalloidin (in green) and with an antibody against the 3x flag tagged viral proteins (red). Bottom panel (**A′–E′**) represents NS3 protein specific staining. Scale bars are 50 μm. (**F**) Third instar larvae protein extracts were analyzed by western-blot. 3x flag tagged viral proteins were detected at 58 kDa and 80 kDa for NS3-Helicase and NS3 full-length respectively. Tubulin detection was used as loading control.

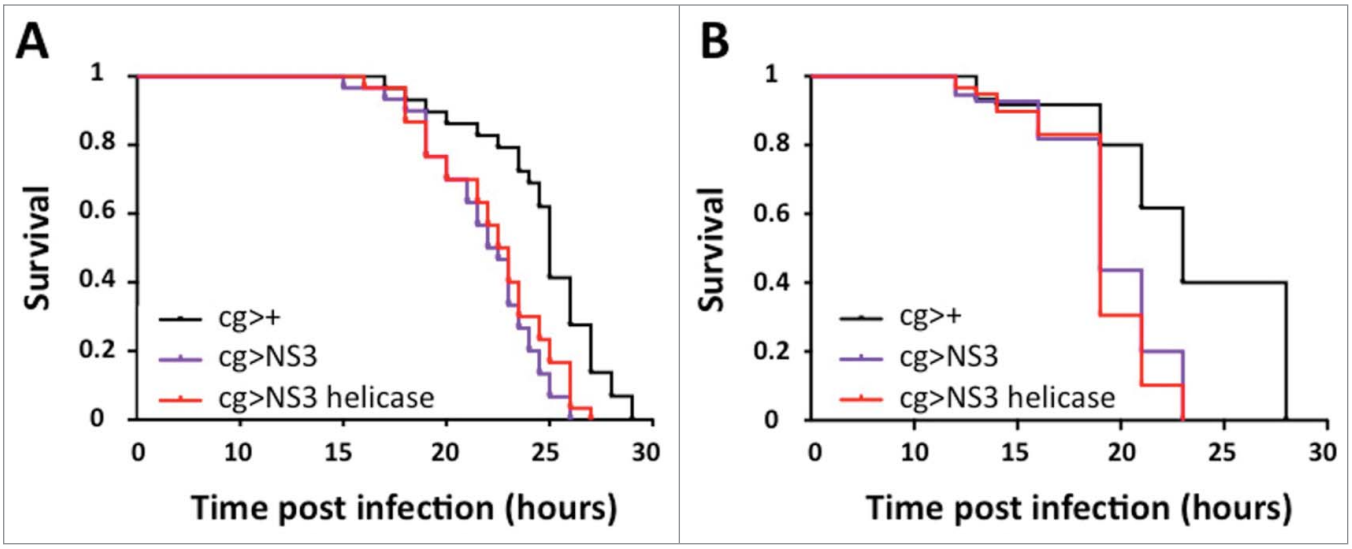

Figure 2. Flies expressing Dengue virus NS3 are sensitive to *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection. Sixty males flies expressing NS3 Fl or NS3 Hel under the control of Cg-Gal4 driver (Cg > NS3 Fl and Cg > NS3 Hel) and control flies (Cg > +) were infected with Gram-positive bacteria, *Staphylococcus aureus* or Gram-negative bacteria *Pseudomonas aeruginosa* by septic injury. Survival rates of infected *Drosophila* were followed at 25°C for indicated times post-infection [(**A**) *Staphylococcus aureus* and (**B**) *Pseudomonas aeruginosa*)]. One representative experiment out of 3 biological independent experiments is presented. Non parametric Log-rank Mantel-Cox test: Cg > + vs. cg > UAS-NS3 Hel < 0,0009 and Cg > + vs. Cg > UAS-NS3 Fl < 0,0001 (**A**) and Cg > + vs. cg > UAS-NS3 Hel < 0,0001 and Cg > + vs. Cg > UAS-NS3 Fl < 0,0001 (**B**).

in the same conditions. *Escherichia coli, Pseudomonas aeruginosa* and *Enterobacter aerogenes* were used as Gram-negative bacterial strains; *Staphylococcus aureus* and *Micrococcus luteus* were used as Gram-positive strains. Both wild-type and NS3 expressing flies were insensitive to non-pathogenic bacteria *E. coli, E. aerogenes* and *M. luteus*. Indeed, we observed normal fly viability 2 weeks post-infection in both flies groups (**Fig. S1**). In contrast, we found that flies expressing either the full length or the helicase domain of NS3 were more sensitive than control flies to infection with the highly pathogenic bacteria *S. aureus* and *P. aeruginosa* (**Fig. 2A and B**).

NS3 expressing flies display normal phenotype, life span and resistance to thermal stress

We then checked the phenotype of NS3 flies carrying either the immune specific Cg-Gal4 driver or the ubiquitous Actin-

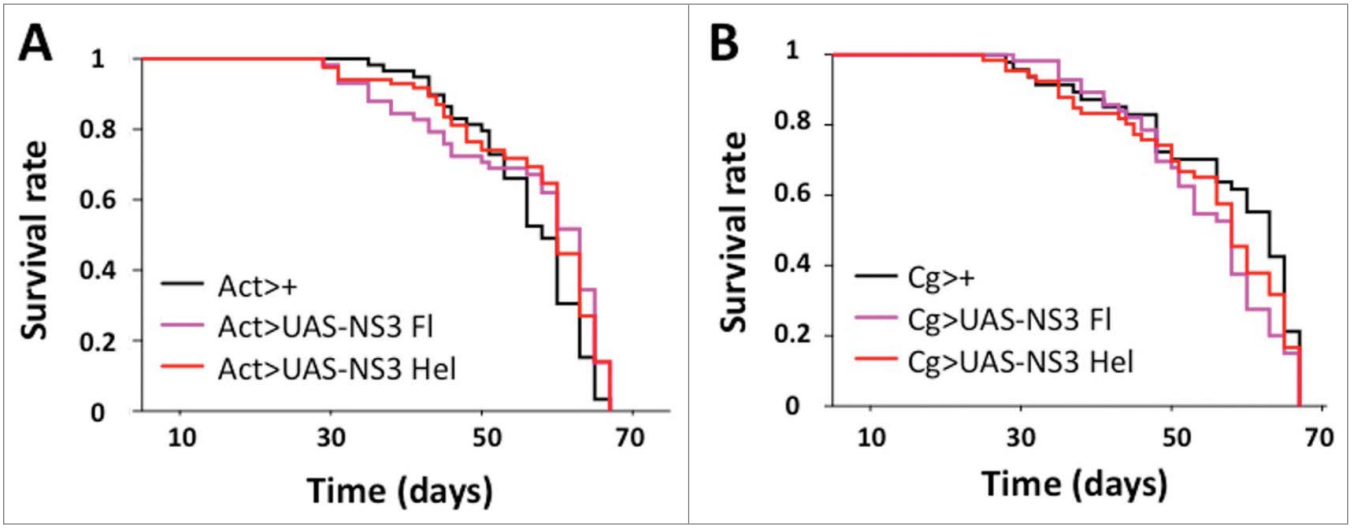

Figure 3. NS3 transgenic flies display normal life span. (**A and B**) UAS-NS3 and w^{1118} (control) flies were mated to flies carrying either the immune specific Cg-Gal4 (**A**) or the ubiquitous Act-Gal4 (**B**) driver. One hundred F1 females per genotype were aged and assayed for longevity. Statistical analysis did not reveal any significant difference between NS3 expressing and control flies.

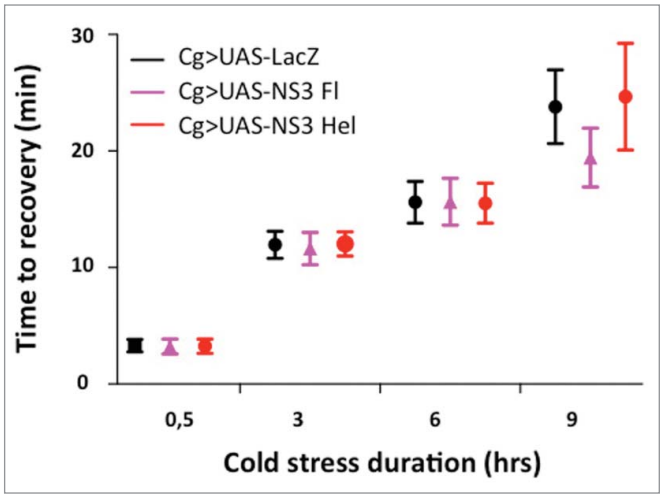

Figure 4. NS3 transgenic flies display normal resistance to cold stress. For cold stress experiment, 40 male flies per genotype were kept at 0°C for 30, min 3 h, 6 h or 9 h. The time before waking was then assessed for each fly group, Cg-Gal4 > UAS-LacZ and the NS3 expressing *Drosophila*, Cg-Gal4 > UAS-NS3 Hel and Cg-Gal4 > UAS-NS3 Fl. For cold stress results, one representative experiment out of 3 biological independent experiments is presented.

Gal4 driver. In both cases, embryonic and larval developments gave rise to NS3 adult flies that displayed a normal phenotype and express NS3 (data not shown). And the viral protein expression either as full length or as helicase functional domain did not affect the *Drosophila* life span (**Fig. 3**).

We finally examined the response capacity of NS3 flies to a non-infectious thermal stress. Transgenic or control flies were kept at 0°C for 30 min, 3 h, 6 h or 9 h, and we then measured the time before waking for each genotype. We did not observe any significant difference between the control flies (w^{1118};Cg-Gal4/UAS-LacZ) and the NS3 expressing *Drosophila* (w^{1118};Cg-Gal4/UAS-NS3 Hel or w^{1118};Cg-Gal4/UAS-NS3 FL) (**Fig. 4**).

Discussion

In the present work, we developed a *Drosophila* model in which the dengue virus NS3 protein is expressed in a tissue specific and inducible manner. We observed an increased susceptibility of transgenic flies to bacterial infection. But ubiquitous or immune-specific NS3 expression affected neither the life span nor the response to a non-infectious stress of the flies.

In order to test the sensitivity of the NS3 transgenic flies compare to wild type flies, we infected them with *S. Aureus* and *P. Aeruginosa* pathogenic bacteria and with non-pathogenic *E. coli, M. luteus* and *E. aerogenes* bacteria. These latest are known to induce lethality in Imd immuno-compromised mutant *Drosophila* but not in control flies.[25] We could not see any increased mortality in the NS3 flies as compare to wild type when infected with non-pathogenic bacteria. However, in a context of infection with pathogenic bacteria, we observed an increased susceptibility of the transgenic flies. This suggests that NS3 expression is not sufficient to induce a robust immuno-suppression in the flies but that NS3 is able to modulate the antibacterial immune response and viability in infected flies.

In a previous study, we have shown that human proteins of the innate immune system interact with NS3 DENV protein.[16] Like other innate immune components, molecules of the TLR pathway are highly conserved among evolution; indeed CACTUS is the ortholog of IκBα, and TRAF4 of TRAFd1 in *Drosophila*. We were not able to find homologous genes for AZI2. As we demonstrated that NS3 binds to IκBα and TRAFd1 in human, it would be important to test if these interactions are conserved in *Drosophila* and in *Aedes* as well. In this regard several studies suggested that DENV infection modulates Toll pathway in *Aedes* supporting the putative interaction between the virus and its arthropod vector innate immune system. Indeed Cactus, the ortholog of IκBα plays a very important role in the control of viral load in *Aedes*. In this organism, *cactus* gene expression is modulated during viral infection, and its down regulation by RNAi induces a diminution of viral load.[11] In a recent study, DENV infection of primary dendritic cells induced a blockage of TLR induced NF-κB activation and a subsequent down regulation of cytokine production.[26] We could then hypothesize that NS3 interaction with IκBα/Cactus might be part of a viral strategy to evade Toll dependent immune response, like it has been recently demonstrated for polydnavirus, which are carried by parasitoid wasps.[27] Even if this phenomenon would occur during DENV infection a robust innate immune response including activation of the Toll pathway, the JAK-STAT pathway, and the RNAi-mediated antiviral defenses is induced in *Aedes*.[28] Whether these defense mechanisms might be then less efficient to constrain a novel infection is not known. Our previous results indicated that other immune proteins, such as TRAF4 and AZI2 interact with the NS3 helicase domain of DENV in human cells.[16] In *Drosophila*, TRAF4 is the ortholog of TRAFd1 which has been shown to regulate Dif/NF-κB activity when associated to Pelle [29] although the regulation of the transcription of antimicrobial peptide genes *Diptericin* and *Drosomycin* was assigned to dTRAF2 rather than dTRAF1.[30] Thus, a putative interaction between NS3 and dTRAF2 or dTRAF1 should be further explored to understand NS3 activity on the transcription of Toll-dependent target genes such as anti-microbial peptide.

Additional experiments in the context of a viral infection would be of great value to further decipher the importance of putative NS3-Cactus interaction.

Conclusions

Overall, we generated a *Drosophila* model in which the dengue virus NS3 protein is expressed *in vivo*. This model will be a valuable tool to assess mechanisms related to the interactions between NS3 and evolutionary conserved host proteins of the innate immune system.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

Authors' Contributions

MQ, MLD and ND carried out the experiments. ND designed the experiments and drafted the manuscript. BM gave his advices to conceive the project, and to write the manuscript. All authors read and approved the final manuscript.

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