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Aedes aegypti salivary protein "aegyptin" co-inoculation modulates dengue virus infection in the vertebrate host

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ABSTRACT

Dengue virus (DENV) is transmitted in the saliva of the mosquito vector *Aedes aegypti* during blood meal acquisition. This saliva is composed of numerous proteins with the capacity to disrupt hemostasis or modulate the vertebrate immune response. One such protein, termed "aegyptin," is an allergen and inhibitor of clot formation, and has been found in decreased abundance in the saliva of DENV-infected mosquitoes. To examine the influence of aegyptin on DENV infection of the vertebrate, we inoculated IRF-3/7$^{-/-\ -/-}$ mice with DENV serotype 2 strain 1232 with and without co-inoculation of aegyptin. Mice that received aegyptin exhibited decreased DENV titers in inoculation sites and in circulation, as well as increased concentrations of GM-CSF, IFN-γ, IL-5, and IL-6, at 48 h post-inoculation when compared to mice that received inoculation of DENV alone. These and other data suggest that aegyptin impacts DENV perpetuation via elevated induction of the immune response.

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Introduction

Dengue virus (DENV) is maintained in a primarily anthroponotic cycle between humans and the *Aedes aegypti* mosquito (WHO, 2010). Feeding by *A. aegypti* on vertebrate hosts results in the simultaneous introduction of virus and saliva into the skin (Griffiths and Gordon, 1952; Styer et al., 2007; Turell and Spielman, 1992; Turell et al., 1995). This saliva contains many pharmacologically important proteins that modulate host hemostasis and immune responses, which in turn facilitate blood feeding and virus transmission (Schneider and Higgs, 2008; Ribeiro et al., 2007; Calvo et al., 2007). Additionally, *A. aegypti* saliva has been shown to contain allergenic proteins (Peng and Simons, 2007). The vertebrate immune response to DENV infection may be altered as a result of the immunogenic nature of these salivary proteins, thereby altering DENV infection kinetics.

The effects of salivary proteins on mosquito-borne viral infection have been reviewed previously, and include alterations in cytokine production and potentiation of infection in otherwise non-permissive models (Schneider and Higgs, 2008; Schneider et al., 2004; Osorio et al., 1996). More recently, *A. aegypti* saliva has been shown to induce alterations in leukocyte recruitment during West Nile virus infection, and shift the cytokine profile from a Th1 toward a Th2 type immune response during chikungunya virus infection (Schneider et al., 2010; Thangamani et al., 2010). In the context of DENV infection, the addition of *A. aegypti* saliva in vitro resulted in the reduced production of antimicrobial peptides and interferons, thereby increasing viral titers (Surasombatpattana et al., 2012). Allowing *A. aegypti* to feed on IRF-3/7$^{-/-\ -/-}$ mice immediately before intradermal injection of DENV resulted in the down-regulation of multiple innate immune transcripts and increased DENV viremia titers (McCracken et al., 2014). Additionally, *A. aegypti* saliva has increased the prevalence of disease signs and extended the viremic period in DENV-infected, humanized mice (Cox et al., 2012).

As an important step in the characterization of mosquito saliva, researchers have examined the composition of *A. aegypti* salivary glands at the transcriptional level and protein expression level (Ribeiro et al., 2007; Champagne et al., 1995; Valenzuela et al., 2002; Smartt et al., 1995; Thangamani and Wikel 2009; Almeras et al., 2010, 2009; Wasinpiyamongkol et al., 2010). Work has been done to ascribe function to some of these salivary components and investigate their individual effects on vertebrate hemostasis and immune response outside the context of a viral infection (Calvo et al., 2007; Champagne et al., 1995; Ribeiro and Francischetti 2003; Ribeiro 1995; Boppana et al., 2009; Calvo et al., 2006, 2010;

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Champagne and Ribeiro, 1994; Stark and James, 1998; Zeidner et al., 1999). Additionally, *A. aegypti* saliva has been shown to elicit specific IgG and IgE responses in humans (Wasinpiyamongkol et al., 2010; Remoue et al., 2007). Recent studies have described the effect that DENV infection has on protein expression in the salivary glands or expectorated saliva and postulates how these changes could be relevant to virus transmission (Chisenhall et al., 2014a, 2014b). Among the proteins that were found in lower abundance in *A. aegypti* saliva as a result of DENV infection was a member of the GE-rich 30 kDa antigen family, designated in *A. aegypti* as "aegyptins" (Calvo et al., 2007; Ribeiro et al., 2010). The decreased expression of this protein during DENV infection could suggest that it supplies negative pressure on viral perpetuation. This pressure may be inherent to the aegyptin protein family and perhaps impacts transmission or establishment of infection.

The archetypal protein of the 30 kDa antigen family in *A. aegypti* has been shown to perform two distinct roles within the vertebrate. First, as an allergen, aegyptin has been shown to induce positive skin-test reactions and antibody responses in sensitized humans (Peng and Simons, 2004). Second, aegyptin has demonstrated the ability to bind to collagen, inhibiting platelet aggregation and interaction with von Willebrand factor, which could facilitate blood feeding by reducing the formation of blood clots (Calvo et al., 2007, 2010).

Due to the involvement of aegyptin with both the vertebrate immune response and the clotting cascade, we explored the impact of aegyptin within the context of a DENV infection. Recombinant aegyptin was used for in vivo evaluation of the effects of this protein on the murine immune response to DENV and the resulting infection in the inoculation site, draining lymph nodes, and in circulation.

Results

Inoculation site mice

Viral titers

DENV titers were measured at 48 h post-inoculation in the inoculated ears and draining submandibular lymph nodes of mice inoculated with DENV+aegyptin or DENV only. DENV titers in the ears of DENV+aegyptin inoculated mice had a mean of 1.65×10^6 PFU*/mL, while the inoculated ears of mice that received DENV alone had a significantly greater mean titer of 3.38×10^6 PFU*/mL ($p=0.0057$). The lymph nodes of DENV+aegyptin inoculated mice did not differ significantly from mice inoculated with DENV alone ($p=0.0678$) with mean titers of 7.21×10^5 and 1.42×10^6 PFU*/mL, respectively. (Fig. 1)

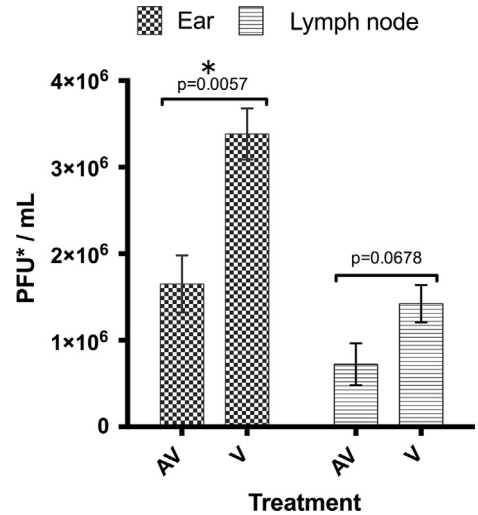

Fig. 1. DENV titers in inoculated ears and draining submandibular lymph nodes at 48 h post-inoculation. DENV titers, expressed as PFU-equivalents/mL (PFU*/mL) were significantly lower in the ears of mice that received co-inoculation of DENV+aegyptin (AV) as compared to the cohort that received DENV only (V, $p=0.0057$), as indicated by an asterisk. DENV titers were not found to be significantly different in the lymph nodes ($p=0.0678$), although they followed a similar trend as those of the ears. Associated bars represent standard error of the means.

Cytokine responses

The cytokines GM-CSF, IFN-γ, IP-10, TNF-α, IL-1a, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 were assessed in the inoculated ears, draining submandibular lymph nodes, and the serum of mice inoculated with DENV+aegyptin, DENV alone, aegyptin alone, and those that received a mock inoculation as outlined above. All differences stated are significant ($p \leq 0.05$). In the ears (Fig. 2A), the concentrations of IFN-γ and IL-2 were found to differ between all treatment groups except DENV+aegyptin compared to DENV alone. IL-10 differed among all comparisons except DENV+aegyptin compared to DENV alone and aegyptin alone compared to mock. IP-10 differed when comparing DENV+aegyptin to aegyptin alone or mock, and mock differed from DENV alone. IL-6 differed only in ears inoculated with aegyptin alone compared to DENV alone. IL-4 and IL-12p70 were below the limit of detection in all treatment groups. All other cytokines displayed no significant differences between treatment groups, and no cytokines were found to differ in the ear between DENV+aegyptin and DENV alone ($p > 0.05$). In the draining submandibular lymph nodes (Fig. 2B), GM-CSF, IFN-γ, and IL-6 were found to differ significantly between DENV+aegyptin compared to DENV alone, aegyptin alone, and mock treatment mice. IL-5 in DENV+aegyptin inoculated mice differed from mice inoculated with DENV alone. TNF-α differed among all comparisons except DENV+aegyptin compared to DENV alone and aegyptin alone compared to mock inoculated mice. IP-10 differed only between aegyptin alone compared to DENV+aegyptin or DENV alone. Again, IL-4 and IL-12p70 were below the limit of detection and all other cytokines displayed no significance between treatment groups ($p > 0.05$). Cytokine concentrations in the serum were not found to be significantly different between any treatment groups ($p > 0.05$, data not shown).

Hematology

The percentages (counts) and concentration (per μL) of circulating leukocytes were determined as described above for all four treatment groups. All stated differences are significant ($p \leq 0.05$) as determined using odds ratios. The eosinophil counts in the DENV+aegyptin inoculated mice were more likely to be elevated when compared to mice inoculated with DENV alone, as well as in mock inoculation compared to DENV alone. The monocyte counts were correspondingly more likely to be lower in these comparisons as well as in mice inoculated with aegyptin alone compared to DENV alone. Neutrophil counts were found more likely to be lower between mice inoculated with aegyptin alone as compared to all other treatments. Lymphocyte counts were correspondingly more likely to be higher in the aegyptin alone group compared to all other treatment groups, as well as in mock inoculated mice compared to those inoculated with DENV alone. All other comparisons of counts, the leukocyte concentrations (data not shown), and total nucleated cell concentrations (data not shown) were not found to differ significantly ($p > 0.05$). The comparison of leukocyte counts from the aegyptin+virus inoculation cohort as compared to the virus only inoculation cohort is displayed in Fig. 3. The odds ratios with associated 95% confidence intervals and p values for this and all other comparisons of counts are in Supplement 1.

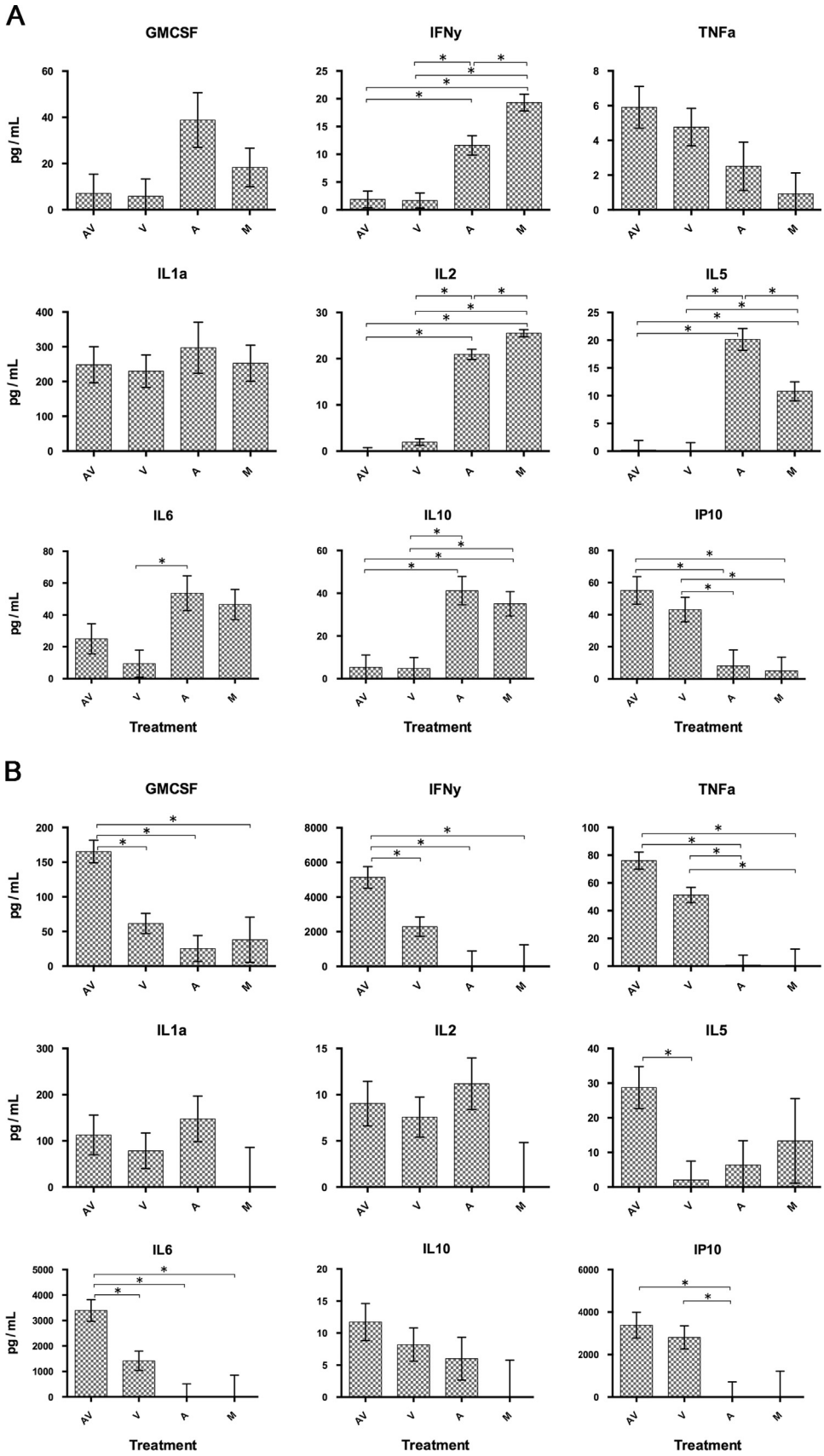

Fig. 2. Cytokine concentrations in the inoculated ears (A) and draining submandibular lymph nodes (B) at 48 h post-inoculation. The *y*-axis displays concentration in pg/mL and the *x*-axis denotes treatment groups (AV = DENV + aegyptin, *V* = DENV only, *A* = aegyptin only, *M* = mock inoculation). Significant comparisons are indicated by an asterisk ($p \leq 0.05$). Associated bars represent standard error of the means.

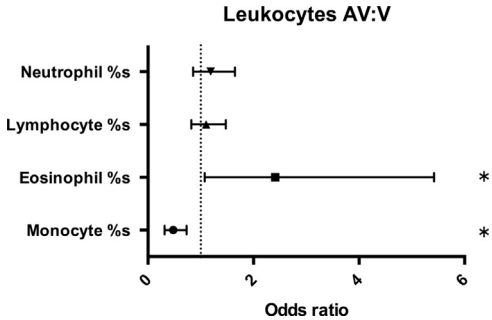

Fig. 3. Comparison of circulating leukocyte percents (counts) at 48 h post-inoculation for the DENV+aegyptin inoculated cohort (AV) compared to the virus only cohort (V). Comparisons of each leukocyte were performed using odds ratios and significance is indicated by an asterisk ($p \leq 0.05$). Ninety-five percent confidence intervals are displayed.

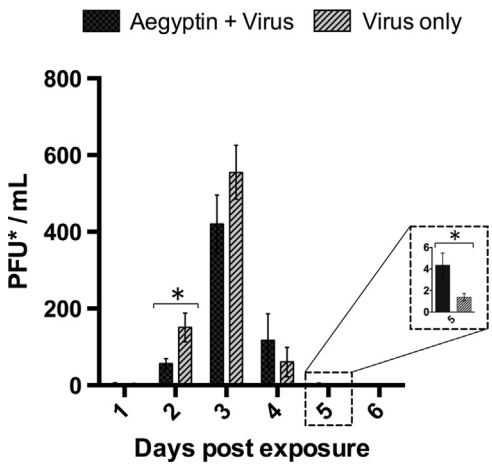

Fig. 4. DENV viremia titers in serum samples on the first six days post-inoculation. DENV titers, expressed as PFU-equivalents/mL (PFU*/mL) were significantly lower in mice inoculated with DENV+aegyptin as compared to those inoculated with virus alone on day 2 post-inoculation. DENV titers were significantly higher in mice inoculated with DENV+aegyptin as compared to those inoculated with virus alone on day 5 post-inoculation. Significance is indicated by an asterisk ($p \leq 0.05$). Associated bars represent standard error of the means.

Serially bled mice

Viremia

DENV viremia titers were measured each day for six days following inoculation in both the DENV+aegyptin and DENV alone treatment groups. These titers were found to differ significantly between the two treatment groups on day two post-inoculation, which corresponds to the difference observed in the inoculation sites at 48 h post-inoculation, and on day five post-inoculation ($p \leq 0.05$) (Fig. 4).

Discussion

The 30 kDa antigen family of proteins contains multiple alleles and splice variants that are compositionally similar, consisting of two domains and possessing similar molecular weights. Prior analysis has revealed that these aegyptins represent two subclades within *Aedes* (designated I and II) (Ribeiro et al., 2010). Analysis of expectorated saliva from DENV2 infected *A. aegypti* using 2D gel electrophoresis and LC-MS/MS has previously identified a subclade I aegyptin (gi|18568322) that was reduced 14.1-fold when compared to saliva from uninfected mosquitoes (Chisenhall et al., 2014b). This aegyptin has also been referred to as "SAAG-4," which has demonstrated the capacity to suppress IFN-γ expression while elevating IL-4 expression by CD4+T cells outside the context of a viral infection (Boppana et al., 2009). The recombinant aegyptin used in the current study in vivo is a member of subclade II (gi|94468546) and is the archetypal aegyptin utilized in the biopharmaceutical characterization of the capacity to inhibit clotting (Calvo et al., 2007, 2010). This aegyptin is also identical in sequence to that of *A. aegypti* salivary allergen "Aed a 3" (gi|205525920), a protein shown to induce allergic responses in both mice and humans (Chen et al., 1998). Additionally, other researchers have found an association between serum reactivity to Aed a 3 and the dengue fever (mild) disease state in clinical patients in Thailand (Machain-Williams et al., 2012). While the two aegyptin groups represent distinct subclades, the acidic (glycine-, aspartic acid-, and glutamic acid-rich) aminoterminal domain and the more complex carboxyterminal domain characteristic of this protein family remain conserved (Ribeiro et al., 2010).

The goal of this study was to determine the impact of the *A. aegypti* salivary protein aegyptin on DENV infection kinetics and the corresponding vertebrate immune response, in light of the allergenic and anti-clotting effects already ascribed to this protein. We designed two in vivo murine experiments examining the two vertebrate facets critical to mosquito-borne viral transmission: establishment of DENV infection within the bite site of the vertebrate host, and systemic infection (circulation) that enables transmission from the vertebrate to naïve mosquitoes. The time point of the first experiment, 48 h post-inoculation, is the day most often observed for the onset of DENV viremia in these mice (Christofferson et al., 2013), and as such potential differences seen in the bite site and draining lymph nodes were assumed to be near their peaks. Mice for the second experiment were examined daily through the end of viremia for differences that have the potential to lead to differential acquisition by the vector, such as differences in the magnitude of viremia titers or day of viremia onset (Nguyet et al., 2013).

The addition of aegyptin to the DENV inoculum resulted in significantly decreased viral titers in the inoculated ears 48 h post-inoculation, which could provide a rationale for the DENV2-induced reduction of aegyptin in the saliva of infected mosquitoes. It may be that this reduction in expectorated aegyptin decreases the likelihood of an allergen-related or otherwise inflammatory immune response at the bite site. As an extreme example, humans bitten by mosquitoes lacking the ability to expectorate saliva failed to manifest wheals (Rossignol and Spielman, 1982). Mosquito saliva also has been shown previously to induce numerous immunological reactions at the site of inoculation including T-cell-mediated hypersensitivities, IgE-independent mast cell degranulation, and immune cell recruitment (Peng and Simons, 2007). Lymph node GM-CSF, IFN-γ, IL-5, and IL-6, as well as the percentage of eosinophils in circulation, were observed to be greater in the DENV+aegyptin mice than in mice inoculated with DENV alone at this time point. GM-CSF was demonstrated previously to promote growth, maturation, and survival of eosinophils, a pro-inflammatory granulocyte typically associated with immune responses to allergens (Metcalf et al., 1986; Vliagoftis et al., 2001). IL-6 is known to be an important mediator of the acute phase response to infection and other antigenic stimuli, and its influences have been reviewed previously (Heinrich et al., 1990). IFN-y and IL-5 promote the activation and survival of phagocytes and eosinophils, respectively, influence B cell isotype switching events, and are involved in allergic immune responses (Cho et al., 2005; Deenick et al., 2005; Horikawa and Takatsu, 2006; Schulten et al., 2013). A reduction or alteration in these immune responses via a reduction in expectorated aegyptin could benefit the establishment of DENV within the vertebrate host. As a consequence of lessened aegyptin in the site of viral establishment, there may be less non-specific destruction of virions or

infected cells by local inflammation, cytokine-mediated cellular activation, and eosinophil-derived degranulation.

Importantly, the addition of aegyptin to the DENV inoculum of the serially bled cohort of mice resulted in an approximately three-fold reduction in viremia titer on day two post-inoculation, which corresponds to the decrease seen in the inoculation site at 48 h post-inoculation. Analogously, a reduction in expectorated aegyptin, as was observed in DENV infected *A. aegypti* mosquitoes, may result in greater viremia titers at this time point, which has been shown to lead to increased acquisition rates by mosquitoes from human clinical patients (Chisenhall et al., 2014b; Nguyet et al., 2013). The increase in DENV+aegyptin viremia titer seen on day 5 post-inoculation (mean=4.36 PFU*/mL) may not result in meaningful differences in transmission when compared to the DENV alone cohort (mean=1.39 PFU*/mL) given the relatively minimal magnitude of these titers. Alternatively, the significance of changes at these two time points may have a more epidemiological importance. Mosquitoes have been shown to successfully acquire DENV up to two days prior to the onset of illness in human cases, at which time these individuals may seek medical attention or otherwise sequester themselves from access by mosquitoes (Nishiura and Halstead, 2007). As such, the minimization of contact between clinically ill individuals and mosquitoes might select for a viral phenotype that induces decreased expression of aegyptin, thereby resulting in greater early (potentially prodromal) viremia rather than late viremia enhancement.

Conclusion

The influence of aegyptin on DENV infections of mice was exemplified by decreased viral titers early in the infection in inoculation sites and in circulation, and by a day with increased viremia titer late in infection. These modulations of DENV infection corresponded to increases in cytokines with known functions in pro-inflammatory and allergen-mediated immune responses, as well as an increased likelihood of eosinophil production. Together, these data support a role for aegyptin in the modification of the host immune response during DENV infection. The role of aegyptin in a naturally introduced DENV infection of humans may be additionally influenced by other, co-expectorated saliva proteins, as well as prior immunity to aegyptin or the same. Future studies should seek to further characterize pro- and anti-viral aspects of mosquito saliva-DENV interactions in the context of human infection and immunity.

Materials and methods

All experiments met the approval and conditions of the LSU Institutional Animal Care and Use Committee (protocol #12-079). LSU IACUC procedures and policies adhere to and comply with the guidelines stated in the NIH Guide for the Care and Use of Laboratory Animals.

Mice

Mice were the generous gift of Dr. M. Diamond (Washington University, St. Louis, MO) with permission from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). These IRF-3/7$^{-/-\ -/-}$ mice are on a C57Bl/6 background and lack functional IRF 3 and 7, and as such have a deficient, but not abrogated, type I IFN response (Daffis et al., 2009).

Virus

Dengue serotype 2 (DENV2), strain 1232 was propagated as described previously, with modification (Christofferson and Mores, 2011). Briefly, we inoculated a T-75 flask of confluent Vero cells with 100 μl of viral stock and incubated for 30 min. Eight mL of Medium 199 with Earle's salts (M199E) with 10% fetal bovine serum and 2% penicillin/streptomycin/amphotericin B was added. The flask was incubated at 37 °C with 5% CO_2 for 5 days and subsequently the supernatant was collected for virus at peak titer. The viral titer of the supernatant was determined using a plaque assay as described previously in Supplemental material, with modification (Alto et al., 2008). The complete medium stated above was used and incubations occurred at 37 °C. This strain was originally isolated from a patient in Indonesia in 1978 (personal communication, R. Tesh). As of this study, it has been passaged four times in Vero cells, and then alternatingly passaged between C6/36 (*Aedes albopictus* cell line) and Vero twice.

Mouse exposure and sample collection

Recombinant aegyptin was generated at a concentration of 9 μM in 25 mM Tris 150 mM NaCl, pH 7.4. It was expressed as described previously in HEK293 cells and has a 6x-His tag (Calvo et al., 2007). Aegyptin was diluted 1/10 in 0.2 μm-filtered 1 × phosphate-buffered saline (PBS) for experimentation.

Inoculation site mice

A total of 17 male and female mice, age 15 weeks, were divided into four treatment groups and inoculated in the pinnae of both ears via 25 μL intradermal injection. These groups were DENV (n=5), DENV+aegyptin (n=5), aegyptin (n=3), and a mock inoculation (n=4). The compositions of the inocula were as follows: DENV – 10 μL DENV (1×10^5 PFU total in cell culture supernatant)+15 μL 1 × PBS; DENV+aegyptin – 10 μL DENV+2 μL aegyptin 1/10+13 μL 1 × PBS; aegyptin only – 10 μL age-matched cell culture supernatant+2 μL aegyptin 1/10+13 μL 1 × PBS; mock – 10 μL age-matched cell culture supernatant+15 μL 1 × PBS. Forty-eight hours later, mice were euthanatized according to our IACUC protocol, and blood was collected via cardiac puncture using ethylenediaminetetraacetic acid (EDTA)-coated syringes and then placed into EDTA-coated BD Microtainer®™ tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ). This blood was used for the creation of blood films for quantification of circulating leukocytes and then centrifuged at 1000 relative centrifugal force (rcf) for 10 min for plasma separation for use in cytokine protein and viral RNA analysis. Inoculated ears and draining submandibular lymph nodes were also collected and processed for cytokine protein and viral RNA analysis. Individual mouse ears and lymph nodes were disrupted and homogenized in 100 μL and 20 μL, respectively, of 1 × PBS using the TissueLyser (QIAGEN, Valencia, CA) for two cycles of 2 min at 25 Hz. For cytokine analysis, 60 μL and 10 μL of the homogenized ear and lymph node solutions were placed in 440 μL and 40 μL, respectively, of radioimmunoprecipitation assay (RIPA) lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) diluted 1/5 in 1 × PBS containing protease inhibitor (cOmplete, Mini, EDTA-free protease inhibitor cocktail tablets, Roche, Indianapolis, IN). For viral RNA detection, 20 μL and 5 μL of the homogenized ear and lymph node solutions were placed into 600 μL and 350 μL, respectively, of QIAGEN's RLT buffer with β-mercaptoethanol and vortexed. RNA was extracted using the RNeasy Tissue Mini Kit (QIAGEN, Valencia, CA).

Serially bled mice

A total of 19 female mice, age 10 ± 2 weeks, were divided into two treatment groups and inoculated in the pinnae of one ear or rear footpad via 25 μL intradermal injection. These groups were DENV ($n=9$) and DENV+aegyptin ($n=10$). The compositions of the inocula were as above, with a DENV titer determined to be 6.7×10^4 PFU total. Mice were bled via submandibular vein puncture immediately prior to inoculation and then daily for the next 6 days (Golde et al., 2005). This blood was collected in microcentrifuge tubes, allowed to clot for thirty minutes at room temperature, and then centrifuged at 3300 rcf for four minutes. Clarified serum was collected and placed into clean microcentrifuge tubes for nucleic acid extraction using the MagMax-96 Total Nucleic Acid isolation kit (Ambion/Life Technologies, Carlsbad, CA) and subsequent quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) measurement of viremia.

Hematology

Differential leukocyte counts were performed in a blinded manner by a board-certified veterinary clinical pathologist, as previously described, in order to examine circulating leukocyte populations during the inoculation site and serially bled mouse studies (Grasperge et al., 2012). Briefly, blood films were made within one hour of collection. Blood films were Wright Giemsa stained and counted in duplicate for differentiation of lymphocytes, monocytes, eosinophils, and basophils. Blood in EDTA was diluted 1:100 in 2% acetic acid and incubated for 10 min at room temperature. Total nuclei were counted in duplicate for each mouse using this blood-acetic acid solution on a hemocytometer in order to derive absolute leukocyte concentrations. Absolute leukocyte concentrations and differential counts were then used to calculate the absolute differential leukocyte concentrations.

Viral detection

Before inoculation, DENV was titered via plaque assay and experimental titers from cell culture supernatant were confirmed by qRT-PCR as previously described (Christofferson and Mores, 2011). DENV RNA was extracted using the MagMax-96 Total Nucleic Acid isolation kit (Ambion/Life Technologies, Carlsbad, CA) or RNeasy Tissue Mini Kit (QIAGEN, Valencia, CA). Serum samples were brought to volume where necessary with BA-1 diluent (M199E, 10% bovine serum albumin, 0.1 g/L L-glutamine, 2.2 g/L sodium bicarbonate, 25 mM HEPES, 2% penicillin/streptomycin/amphotericin B, titrated to 7.4 pH with Tris and HCl) and kits were run per manufacturers' instructions (Chisenhall et al., 2008). Detection of DENV RNA in mice was performed using the Superscript® III Platinum® One-Step qRT-PCR system (Life Technologies, Carlsbad, CA). Serial dilutions of DENV culture supernatant (from above) were quantified via plaque assay, extracted alongside samples, and used to generate the qRT-PCR standard curve for estimation of DENV titer. Thus, viral concentrations are expressed as PFU-equivalents/mL where appropriate, symbolized as PFU*/mL.

Cytokine measurement

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IFN-γ-inducible protein-10 (IP-10), tumor necrosis factor (TNF)-α, interleukin (IL)-1a, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 were measured in the inoculation sites, draining lymph nodes, and serum at 48 h post-inoculation in the inoculation site cohort of mice using the Milliplex® MAP Mouse Cytokine/Chemokine kit (EMD Millipore, Billerica, MA) as per manufacturer's instructions.

Statistical analysis

Tissue viral titers and cytokine levels were statistically analyzed using analysis of variance (ANOVA). Leukocyte values were analyzed using odds ratios. These analyses were performed in SAS 9.13 (Carey, NC). Viremia titers were statistically analyzed using grouped t-test by day in GraphPad Prism version 6.0b for Mac OS X (San Diego, CA). Significance is reported at the $\alpha=0.05$ level.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.019.

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