Analysis of Early Dengue Virus Infection in Mice as Modulated by *Aedes aegypti* Probing

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Dengue virus (DENV), the etiologic agent of dengue fever, is transmitted during probing of human skin by infected-mosquito bite. The expectorated viral inoculum also contains an assortment of mosquito salivary proteins that have been shown to modulate host hemostasis and innate immune responses. To examine the potential role of mosquito probing in DENV establishment within the vertebrate host, we inoculated mice intradermally with DENV serotype 2 strain 1232 at sites where *Aedes aegypti* had or had not probed immediately prior. We assayed these sites 3 h postinoculation with transcript arrays for the Toll-like receptor (TLR), RIG-I-like receptor, and NOD-like receptor signaling pathways of the innate immune system. We then chose TLR7, transcription factor p65 (RelA), gamma interferon (IFN- γ), and IFN- γ -inducible protein 10 (IP-10) from the arrays for further investigation and assayed these transcripts at 10 min, 3 h, and 6 h postinoculation. The transcripts for TLR7, RelA, IFN- γ , and IP-10 were significantly downregulated between 2- and 3-fold in the group subjected to mosquito probing relative to the virus-only inoculation group at 3 h postinoculation. A reduction in these transcripts could indicate reduced DENV recognition and antigen presentation and diminished inhibition of viral replication and spread. Further, mosquito probing resulted in viremia titers significantly higher than those in mice that did not receive probing. *A. aegypti* probing has a significant effect on the innate immune response to DENV infection and generates an early immune environment more permissive to the establishment of infection.

Dengue virus (DENV), the etiologic agent of dengue fever, is an enveloped, positive-sense, single-stranded RNA virus of the genus *Flavivirus*. DENV prevalence has increased in recent decades and now infects an estimated 50 to 100 million humans worldwide annually of approximately 2.5 billion at risk of infection (1). The increased risk of infection can be attributed, in part, to the continued growth and urbanization of human populations and an expanded global distribution of the primary vectors of DENV, the mosquitoes *Aedes aegypti* and *A. albopictus* (2). Feeding by these vectors on vertebrate hosts is initiated by probing that results in physical damage to the epithelium and vasculature, as well as the simultaneous introduction of virus and saliva into host tissues (3–6). This saliva contains many pharmacologically important proteins that modulate host hemostasis and innate immune responses, which in turn facilitate blood feeding and virus transmission (7–9). As exogenous antigens, both the salivary proteins and the virus encounter the vertebrate host immune system and consequently could have an effect on the environment of the bite site during viral establishment.

To investigate the impact of the vector on mosquito-borne viral infection, researchers have analyzed viral titers and/or immunological response markers by using various methods of mosquito involvement, including salivary gland extract (SGE) (10–16), infected mosquitoes (14, 17–23), and spot feeding (11, 12, 14, 24). The immunological effects of mosquito saliva and/or probing in these arbovirus infection experiments were examined at time points of 24 h postexposure or later, with three notable exceptions. Limesand et al. assayed IFN- α/β expression at 4 to 48 h postexposure in response to vesicular stomatitis virus infection *in vitro* following treatment with SGE (10); Thangamani et al. assessed the response to chikungunya virus-infected *A. aegypti* mosquito bites in mice at 3 and 6 h postexposure (22); and Surasombatpattana et al. examined the effect of *A. aegypti* SGE on DENV infection of human keratinocytes *in vitro* at 6 and 24 h postexposure (15).

Three other studies have investigated the effects of *Aedes* saliva on DENV infection of the vertebrate host (17, 25, 26), with observations again gathered at 24 h postexposure or later.

The immunological modulations observed in these DENV studies would likely affect the progression of DENV infection and disease. However, the time points used are distinctly separate from the act of transmission or time of inoculation and therefore may not describe the events that occur *in vivo* during the early hours of DENV infection. The investigation of potential differences at earlier time points will clarify the acute dynamics of DENV transmission at the bite site in the context of mosquito probing and expectorated saliva. Therefore, we examined the early *in vivo* transcriptional changes in murine innate immune recognition and response pathways at sites of DENV inoculation due to contemporaneous *A. aegypti* probing.

Specifically, we assessed potential differences in the murine Toll-like receptor (TLR), NOD-like receptor (NLR), and RIG-I-like receptor (RLR) signaling pathways between skin samples that had or had not been probed by mosquitoes immediately prior to DENV inoculation. TLRs, NLRs, and RLRs are pattern recognition receptors critical to the detection of viral pathogens by the innate immune system. The subsequent signaling pathways initiate a cascade of events that include activation and nuclear translocation of transcription factors, increased transcription of antiviral genes, and

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the production of effector proteins with the ultimate goal of creating an antiviral immune environment. We used commercial quantitative PCR (qPCR)-based transcript arrays of 84 genes each as broad screens for differential gene expression in these pathways at 3 h postinoculation. Following these arrays, we assayed the transcripts of four differentially expressed genes and two housekeeping genes with gene-specific primer sets at 10 min, 3 h, and 6 h postinoculation. The four differentially expressed genes assayed were those that encode TLR7, v-rel reticuloendotheliosis viral oncogene homolog A (avian)/transcription factor p65 (RelA), gamma interferon (IFN- γ), and IFN- γ -inducible protein 10/CXCL10 (IP-10). The two housekeeping genes assayed were those that encode glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L-32. Taken together, the differential regulation of these transcripts, as modulated by mosquito probing, informs on a vertebrate host environment that may benefit the establishment of DENV immediately following transmission.

MATERIALS AND METHODS

All experiments met the approval and conditions of the Louisiana State University (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.

Mosquitoes. The mosquitoes used in this study were field-caught *A. aegypti* mosquitoes from New Orleans, LA, generously provided by the City of New Orleans Mosquito, Termite, and Rodent Control Board and by Dawn Wesson (Tulane University School of Public Health and Tropical Medicine, New Orleans, LA). Mosquitoes were maintained in an environmental chamber at 28°C and 75 to 80% humidity and subjected to a 16:8 light-dark photoperiod regimen until the time of use. Mosquitoes were provided with a water and 10% sucrose solution *ad libitum*, which was removed 24 h prior to experimentation.

Mice. The mice used were a generous gift from M. Diamond (Washington University, St. Louis, MO) with permission from T. Taniguchi (University of Tokyo, Tokyo, Japan). These IRF3/7 $-/-$ mice are on a C57BL/6 background and lack functional IFN regulatory factor 3 (IRF3) and IRF7 and therefore have a deficient, but not abrogated, type I IFN response (27). These mice have been demonstrated previously to be a suitable model for DENV transmission by both mosquito and needle inoculations, with cytokine responses, DENV replication, and resulting viremia evaluated (28).

Virus. DENV serotype 2 strain 1232 was propagated as described previously (29), with modification. Briefly, we inoculated a T-75 flask of confluent Vero cells with 100 μ l of viral stock and incubated it for 30 min. Eight milliliters of M199E medium with 10% fetal bovine serum and 2% penicillin-streptomycin-amphotericin B (Fungizone) (P/S/F) was added. The flask was incubated at 37°C with 5% CO₂ for 5 days, and subsequently the supernatant was collected for virus at peak titer. The supernatant was found to contain 6.7×10^6 PFU/ml with a plaque assay as described previously in the supplemental material of reference 30, with modification. M199E medium containing P/S/F was used, and incubations occurred at 37°C. This strain was originally isolated from a patient in Indonesia in 1978 (R. Tesh, personal communication) and examined previously in mice (28).

Exposure and sample collection. Three *A. aegypti* females were allowed to feed on the pinna of one restricted ear of each mouse for 10 min from the initiation of probing. Mice then immediately received intradermal needle inoculation (10 μ l) of 6.7×10^4 PFU of DENV at the location of mosquito probing and at a similar location on the opposite ear. Thus, both the treatment site—the one ear where mosquitoes had probed—and the control site—the ear where mosquitoes had not probed—were located on the same mouse in order to control for mouse-to-mouse variation. At 10 min ($n = 10$), 3 h ($n = 8$), and 6 h ($n = 10$) postinoculation, mice were CO₂ euthanized and then the pinnae of their ears were removed and

homogenized. Subsequently, RNA was purified and cDNA was then created for each sample. A separate group of mouse ears exposed only to mosquito probing were collected at these same time points (seven, six, and seven ears, respectively). Ten additional unexposed mouse ears were collected for comparison.

Two groups of four additional mice were exposed to DENV as described above with and without mosquito probing. These mice were bled daily for the next 6 days via submandibular vein puncture. Their blood was collected in microcentrifuge tubes, allowed to clot for 30 min at room temperature, and then centrifuged at a relative centrifugal force of 8,000 for 4 min at 4°C. Clarified serum was placed into clean microcentrifuge tubes and stored at -80°C until nucleic acid extraction and subsequent measurement of viremia.

RNA purification and cDNA creation. Individual mouse ears were disrupted and homogenized in 600 μ l of buffer RLT containing β -mercaptoethanol with the TissueLyser (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. RNA was then purified with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) with on-column DNase I treatment (Qiagen, Valencia, CA). Total nucleic acid was extracted from clarified mouse serum with the MagMax-96 total nucleic acid isolation kit (Ambion/Life Technologies, Carlsbad, CA).

A uniform quantity of total RNA per mouse ear, as determined by NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE) A_{260} reading, was used for each pair of ears to create cDNA. cDNA creation was performed with the RT² First Strand kit (SABiosciences, Qiagen, Valencia, CA) for the RT² Profiler PCR arrays or the SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) (Life Technologies, Carlsbad, CA) with oligo(dT) primers for follow-up testing.

Viral titer assessment. RNA was extracted from all ear tissue and serum samples as described above. Titers of DENV were measured by quantitative RT-PCR (qRT-PCR) assay with the One-Step TaqMan qRT-PCR system (Life Technologies, Carlsbad, CA) and DENV concentration standards derived from plaque assays as previously described (29). Viral titers are expressed as PFU equivalents per milliliter.

Transcript assays. All samples were tested by real-time qPCR on the Roche LightCycler 480 in a 384-well format.

The first assays were performed with the TLR (PAMM-018A) and Antiviral (PAMM-122Z) RT² Profiler PCR arrays (SABiosciences, Qiagen, Valencia, CA) as low-replicate screenings of some 3-h postinoculation samples for TLR and antiviral responses in the mouse. These commercial plate arrays assay the expression of 84 different genes along various innate immune pathways, as well as housekeeping genes and quality controls. Samples were prepared according to the manufacturer's instructions.

Follow-up testing of all samples was conducted with individual primer sets (Integrated DNA Technologies, Coralville, IA) for TLR7, IFN- γ , IP-10, RelA, L-32, and GAPDH with EXPRESS SYBR GreenER qPCR Super-Mix Universal (Life Technologies, Carlsbad, CA). The sequences of the primers used are shown in Table 1. Samples were assayed in triplicate with 10- μ l reaction volumes and a cDNA concentration per reaction equivalent to 3.34 ng of starting total RNA (to equilibrate cDNA loads to the RT² Profiler PCR arrays). The run protocol was as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s.

Analysis. The $\Delta\Delta C_p$ method (31, 32) was used in order to calculate fold change and fold regulation differences between the treatment and control groups. The $\Delta\Delta C_p$ method is discussed here briefly. The average crossing point PCR cycle (C_p) for all qPCR technical replicates is calculated first. In our study, this was done on a per-ear basis with the following equation: $C_p^{\text{Avg}} = (C_{p1} + C_{p2} + \dots + C_{pn})/n$. Next, the ΔC_p is calculated, which is the C_p value for the gene of interest minus the C_p value for the designated housekeeping gene, again on a per-ear basis ($\Delta C_p^{\text{Avg}} = C_p^{\text{Avg}}_{\text{GOI}} - C_p^{\text{Avg}}_{\text{HKG}}$). To get the $\Delta\Delta C_p$, the control ΔC_p is subtracted from the treatment ΔC_p . In our study, this was done by subtracting the control ear ΔC_p from the treatment ear ΔC_p on a per-mouse basis ($\Delta\Delta C_p = \Delta C_p^{\text{Avg}}_{\text{TRT}} - \Delta C_p^{\text{Avg}}_{\text{CTRL}}$ per mouse). Fold change values

TABLE 1 Sequences of primers used in this study, source publications, and NCBI reference genes

Gene product	NCBI gene, no.	Primer (5'–3')		Reference
		Forward	Reverse	
TLR7	<i>Tlr7</i> , 170743	GGC ATT CCC ACT AAC ACC AC	TTG GAC CCC AGT AGA ACA GG	51
GAPDH	<i>Gapdh</i> , 14433	TGC ACC ACC AAC TGC TTA GC	TGG ATG CAG GGA TGA TGT TC	51
IP-10	<i>Cxcl10</i> , 15945	CAG TGA GAA TGA GGG CCA TAG G	CTC AAC ACG TGG GCA GGA T	51
IFN- γ	<i>Ifng</i> , 15978	CCA TCG GCT GAC CTA GAG AA	ATG AGG AAG AGC TGC AAA GC	52
RelA	<i>Rela</i> , 19697	CTT CTG GGC CTT ATG TGG AGA T	TCG CAT TTA TAG CGG AAT CG	53 ^a
L-32	<i>Rpl32</i> , 19951	TAA GCG AAA CTG GCG GAA ACC	AGG ATC TGG CCC TTG AAC CT	53 ^a

^a Modified from original sequence publication.

are then equal to 2 raised to the negative $\Delta\Delta C_p$ (Fold change = $2^{(-\Delta\Delta C_p)}$ per mouse).

Since a 1-fold change indicates that the treatment and control group values do not differ, any >1-fold change indicates an increase in the amount of transcript produced. Similarly, a <1-fold change indicates a decrease in the amount of transcript produced. Fold regulation is a manipulation that allows better conceptualization of the data. This adjusts the relative scale of the data such that a 2-fold increase and a 2-fold decrease would have the same absolute value, making zero on the y axis the turning point between the directionality of change, rather than 1 [Fold regulation = fold change for values of ≥ 1 and Fold regulation = $-(1/\text{fold change})$ for values of <1].

Statistical analysis of fold change values was then performed with SAS 9.2 (Cary, NC). Student's *t* test was used with a null hypothesis of the mean of the differences between control and treatment ears equal to 1. Significance was assessed at, and confidence intervals of the differences were calculated based on, an α value of 0.05. Differences in viral titers between treatments at each time point were compared with a one-way analysis of variance (ANOVA) with repeated measures to control for intramouse variation in the two treatment categories. For the serially bled mice, a one-way ANOVA was performed per day postexposure but without repeated measures. Again, significance was assessed at an α value of 0.05.

RESULTS

DENV titers. On the basis of the qRT-PCR assay, the titers of DENV in the ears of mice subjected to mosquito probing were not found to differ significantly ($P > 0.05$) from the DENV titers in the ears of mice that received DENV injection alone at 10 min, 3 h, and 6 h postinoculation (Fig. 1). However, mice subjected to mosquito probing demonstrated significantly greater viremia titers on days 3 and 4 postexposure. In addition, DENV RNA was detectable through day 6

postexposure in the mosquito probing group, whereas DENV RNA was evident in only one mouse on day 5 and undetectable on day 6 postexposure in the virus-only group (Fig. 2).

Preliminary transcript arrays. On the basis of the SABiosciences RT² Profiler PCR arrays, there was differential regulation of many genes for innate immune receptors, including TLR7, *Ifih1*/MDA5, and Nod2; the mitogen-activated protein (MAP) kinases; activators, inhibitors, and adaptors of signaling such as *Ticam1* and *Nfkb1a*; transcription factors such as the subunits of NF- κ B, *Rel* and *RelA*; enzymes such as caspase 1; and cytokines, including IFN- γ and IP-10 (see supplemental material). In the *A. aegypti* spot-feeding treatment group relative to the virus-only control group, the observed differential expression of the transcripts of TLR7 (3.88 $\times$ downregulated), *RelA* (3.4 $\times$ upregulated), IFN- γ (6.13 $\times$ downregulated), and IP-10 (2.86 $\times$ downregulated) was chosen for further study. Out of a total 130 targets, 41 transcripts were at least 2-fold downregulated and 12 were at least 2-fold upregulated (Fig. 3).

During this initial investigation, we observed that the housekeeping genes normally used in these transcript arrays (e.g., the gene for GAPDH) had a more variable response between the two treatments than we thought appropriate for a standardizing method. In some cases, subsequent assays revealed this *Cp* difference to be indicative of a more-than-log-fold difference in GAPDH expression. Thus, we assessed other housekeeping genes, determined that the gene for L-32 was the most stable, and therefore used it as the housekeeping gene for the remainder of the study (Fig. 4).

Subsequent targeting of transcripts. We then used individual primer sets to specifically target the four genes outlined above, as well as our chosen housekeeping gene, that for L-32. Once the $\Delta\Delta C_p$ values were calculated, we observed significant transcript downregulation of all four of our genes of interest for the spot-feeding treatment group relative to the virus-only control group at 3 h postinoculation. The calculated fold regulation values for these transcripts were as follows: TLR7, $-2.33\times$ ($P = 0.008$); *RelA*, $-2.59\times$ ($P = 0.001$); IP-10, $-2.75\times$ ($P = 0.004$); IFN- γ , $-2.17\times$ ($P = 0.036$). These values and the associated 95% confidence intervals are shown in Fig. 5.

When the study time was expanded to 6 h postinoculation, we observed no difference in fold change values relative to 1, indicating a convergence in the expression of the two groups by this time point. We then looked at expression 10 min postcompletion of viral inoculation. As shown in Fig. 5, this cohort exhibited a great deal of variation in the expression levels of the two groups. While there may be a trend toward upregulation at this time point, only IFN- γ shows significant upregulation (3.04 $\times$, $P = 0.034$).

FIG 1 DENV titers in ear samples at 10 min, 3 h, and 6 h postinoculation. DENV titers, expressed as PFU equivalents per milliliter (PFU*/ml), did not differ significantly between samples exposed to mosquito probing and those injected with DENV alone ($P > 0.05$).

FIG 2 DENV viremia titers in serum samples in the first 6 days postexposure. DENV titers, expressed as PFU equivalents per milliliter (PFU*/mL), were significantly higher in mice exposed to mosquito probing plus virus than in those that received only DENV inoculation on days 3 and 4 postexposure, as indicated by asterisks ($P < 0.05$). Error bars represent the standard errors of the means.

Additionally, the relative abundance of expression in all samples (mosquito probing-plus-virus, virus-only, mosquito-only, and unexposed ears) was calculated for each gene of interest relative to the normalization gene (that for L-32), shown in Fig. 6. The expression level of each gene in unexposed ears tended to be lower than in the other three groups, while the expression level in the mosquito-only group in relation to that in the virus-only and mosquito probing-plus-virus groups varied depending on the gene.

DISCUSSION

The preliminary immune pathway transcript arrays revealed that 31.5% of the target transcripts were at least 2-fold downregulated whereas only 9.2% of the transcripts were at least 2-fold upregulated. Importantly, 11 of the downregulated transcripts were receptors and 9 were proinflammatory cytokines and chemokines. The downregulation of these transcripts suggests a propensity for mosquito-induced generalized suppression of the inflammatory response and a reduction in infiltrating leukocytes.

The transcripts for TLR7, RelA/p65, IFN- γ , and IP-10/CXCL10 were examined individually because of the changes in expression observed in our preliminary transcript arrays and the potential for these genes to affect DENV establishment. Plasmacytoid dendritic cells expressed increased levels of the single-stranded RNA (ssRNA) receptor TLR7 in response to DENV exposure and recognize DENV genomic RNA in a replication-independent manner, resulting in type I IFN production that was inversely proportional to the DENV titer (33). Additionally, peripheral blood mononuclear cells from clinical patients in Thailand exhibited downregulation of TLR7 in secondary dengue hemorrhagic fever cases (34). RelA is part of the NF- κ B complex and is responsible for the in-

duction of many inflammatory cytokines, as well as DENV-induced expression of major histocompatibility complex (MHC) class I (35) and early production of IFN- β , leading to resistance to RNA viral replication (36). IFN- γ has manifold effects, including enhanced activation of DENV-infected dendritic cells (37) and, in combination with IFN- β , inhibition of DENV RNA translation (38). The vital importance of IFN- γ is further exemplified by one of the primary mouse models of DENV infection, the AG129 mouse, which lacks a functional IFN- γ response and thereby suffers clinical illness and increased DENV titers (39). IP-10 is an antigen-presenting cell chemokine induced by IFN- γ that was also shown to competitively inhibit DENV attachment to new cells, resulting in a decreased DENV titer (40). Similarly, an approximately 2-fold decrease in the average IP-10 concentration was demonstrated to increase the DENV titer in monocyte-derived dendritic cells (41). As potential internal controls for normalization, we also measured the transcript levels of the genes for GAPDH and L-32. The gene for GAPDH is a traditional housekeeping gene and GAPDH is involved in the glycolysis cycle. The product of the L-32 gene is a component of the 60S rRNA subunit and is a less traditionally used transcription control.

In reference to the choice of the gene for L-32 as our housekeeping gene for the individual transcript assays (as opposed to those used in the preliminary transcript arrays), it is important to note that, in addition to the variation in GAPDH expression we observed between the treatment and control groups, the literature indicates that increased GAPDH expression is often observed in studies of cellular activation and stress-induced apoptosis (42, 43). There is also literature stating that infections with certain viruses, such as Newcastle disease virus, influenza virus, and vaccinia virus, can modulate GAPDH expression (44–46). Therefore,

FIG 3 Preliminary transcript arrays. Differential regulation of genes for transcription factors, innate immune receptors, the MAP kinases, enzymes, cytokines, chemokines, and activators, inhibitors, and adaptors of signaling was detected in mouse ear samples subjected to mosquito probing plus DENV2 inoculation relative to that in mouse ear samples that received DENV2 inoculation alone. A cutoff of 2-fold up- or downregulation was used to assess which genes were differentially expressed (colored bars surpass the gray 2-fold cutoff). The transcripts of RelA ($3.4\times$ upregulated), TLR7 ($3.88\times$ downregulated), IFN- γ ($6.13\times$ downregulated), and IP-10 ($2.86\times$ downregulated) are marked in their respective categories. Out of 130 targets, 41 transcripts (31.5%) were at least 2-fold downregulated and 12 (9.2%) were at least 2-fold upregulated. The colors of the categories correspond to the similarly colored sections in the data in the supplemental material.

we opted to use the more stably expressed gene for L-32 as the gene of reference for data normalization and suggest that this gene is a better choice for future transcript studies involving DENV and mosquito feeding.

This study provides the first transcriptional analysis of the effects of mosquito probing on the *in vivo* immune response to DENV infection at time points prior to 24 h postexposure. In 2010, Thangamani et al. worked to assess the immune response to early chikungunya virus infection by measuring transcript levels at 3 and 6 h postinoculation. Notably, they found that expression of IFN- γ and the double-stranded RNA receptor TLR3 was lower in the infected-mosquito inoculation group than in the needle inoculation group (22), which parallels our IFN- γ and TLR7 results

FIG 4 qPCR amplification curves representing GAPDH and L-32 fluorescence and C_p values for a set of treatment and control samples. Arrows show the approximate cycles where amplification curves cross the threshold. CTRL, control.

from 3 h postinoculation. Although we initially anticipated that RelA would be upregulated on the basis of our preliminary transcript arrays, ultimately, specific investigation of this gene revealed significant downregulation due to probing—perhaps because of the change in the housekeeping gene used to normalize the data.

Interestingly, we observed a general downregulation at 3 h postinoculation, while there was no significant difference between the treatment and control groups at 6 h postinoculation. Similarly, in an *in vitro* experiment by Surasombattattana et al., expression of IFN types I and II was not found to differ during DENV infection between SGE-exposed and unexposed keratinocytes at 6 h postexposure. However, differential expression of other immune effectors was observed (15). It must be considered, then, that the convergence of expression we observed at 6 h postinoculation could be due to two things.

First, the convergence could be due to the waning effects on our targets of salivary components temporally removed from the initial expectoration event. That is, probing and salivary proteins may have a more immediate effect on viral recognition and response components that diminishes with time. Accordingly, Edwards et al. observed that when mice were inoculated with Cache Valley virus in areas of *A. triseriatus* spot feeding with a delay of 1 to 4 h postfeeding, there was an enhancement of infection and serum antibody production. When inoculation was delayed greater than 4 h or when inoculation was performed without delay but at a site distal to the location of spot feeding, no enhancement was observed (24). Similarly, Styer et al. demonstrated that *Culex tarsalis* SGE-induced enhancement of West Nile virus infection in mice was absent when SGE was injected at a distal location (14). These experiments suggest that the influence of salivary components on downstream immunological and infection characteristics is clustered both spatially and temporally at the bite site. In contrast, DENV-2 inoculation delayed 30 to 60 min after *A. aegypti* spot feeding at a distal location resulted in viremia levels similar to those elicited by infected-mosquito delivery in hu-NSG mice (17), warranting further study of the temporospatial nature of the influence of saliva.

Second, our observed convergence could be due simply to the interference of systemic responses reaching the opposing inoculation site, stemming from the location of both the treatment and

FIG 5 Fold change values for each of the four genes of interest at 10 min, 3 h, and 6 h postinoculation in mouse ear samples subjected to mosquito probing plus DENV2 inoculation relative to DENV2 inoculation alone. Asterisks represent the significance of fold change values as different from 1 ($P < 0.05$). Associated bars represent 95% confidence intervals for each gene per time point; thus, bars that do not overlap 1 indicate significance.

control sites on the same mouse. Importantly, significant differences in expression were observed between the mosquito spot-feeding treatment group and the virus-only control group at 10 min and 3 h postinoculation, indicating that the difference in observed expression, while likely conservative, is due to treatment differences and not the result of a systemic response at these time points.

We did not detect a significant difference in DENV titers in the inoculated ears between treatments, as shown in Fig. 1. This lack of difference may be due to residual DENV RNA from the initial viral inoculum clouding the detection of meaningful changes in virion production. Alternatively, DENV replication may not be occurring at detectable levels this early in the course of infection (47, 48). Importantly, we did detect significantly greater levels of virus in the serum of mice subjected to mosquito probing concurrent with DENV inoculations in the days following infection, as shown in Fig. 2. This finding is consistent with a previous DENV infection in this IRF3/7 double-knockout mouse model using this same strain of DENV introduced via infected mosquito (28).

Much of the work examining the effects of mosquito saliva on viral pathogens suggests that there is a shift away from an antiviral T_H1 immune response toward a T_H2 response characterized by increased production of interleukin-4 (IL-4) and IL-10, among other immunological alterations (11, 13, 20, 22). Additionally,

inoculation via mosquito bite and exposure to saliva have been shown to enhance viral infection or potentiate infection in otherwise nonpermissive models (10–12, 14, 15, 17–21, 24). The observed downregulation of TLR7, RelA, IFN- γ , and IP-10 transcripts at 3 h postinoculation appears to reflect a shift away from an antiviral immune response, potentially generating an environment with reduced DENV recognition and antigen presentation and diminished inhibition of viral replication and spread (depicted in Fig. 7). Upon superficial examination, the upregulation of IFN- γ at 10 min postinoculation might seem to contradict this argument. However, mosquito probing causes tissue injury during the search for capillaries, and IFN- γ is known to be upregulated during skin injury repair (49). Additionally, IFN- γ is known to cause increased expression of and antigen presentation by MHC classes I and II (50). Therefore, it is plausible that the tissue damage caused by mosquito probing is the initial stimulus of the production of IFN- γ . This IFN- γ may, in turn, cause an increased presentation of mosquito salivary proteins, some of which are known to have potent allergen potential, thereby instigating a shift away from an antiviral immune response toward a mosquito allergen-based T_H2 immune response. An obvious next step would be to assess these MHC complexes and immune effectors such as IL-4 and IL-10 that could indicate a T_H2 shift, as well as other markers of tissue injury repair and allergen-based immune responses. On the basis of the implications of our findings, the

FIG 6 Relative abundance of expression of each gene of interest normalized to the expression of the gene for L-32 in mouse ears. The groups depicted are unexposed, mosquito probing only, virus inoculation only, and virus inoculation plus mosquito probing at 10 min, 3 h, and 6 h postinoculation. Values represent mean relative abundance, and error bars show standard deviations.

FIG 7 Cellular representation of the signaling pathways assayed and their involvement in DENV infection. DENV enters the cell through receptor-mediated endocytosis, where it (i) fuses with the endosomal membrane to release its ssRNA genome into the cytoplasm or (ii) is recognized by endosomal TLR7, which signals through RelA to induce the production of MHC class I, IFN- β , and inflammatory cytokines that will then induce IFN- γ and IP-10. The DENV double-stranded RNA (dsRNA) replication intermediate can be recognized by RLRs, NLRs, and TLR3 to further induce signaling via RelA. IFN- β and IFN- γ can inhibit DENV translation and replication, while IP-10 can competitively inhibit DENV binding to plasma membrane receptors, reducing the infection of new cells. Mosquito probing immediately prior to DENV inoculation could inhibit many of these interactions via the observed transcript downregulation (represented by X's) and thus allow more successful DENV transcription and translation (represented by dashed arrows).

downregulation of our target transcripts early in the infection process indicates important mechanisms by which mosquito probing and salivary protein expectoration serve to enhance the establishment of DENV infections in the vertebrate host.

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