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# Eilat virus induces both homologous and heterologous interference

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ABSTRACT

Most alphaviruses are mosquito-borne and exhibit a broad host range, infecting many different vertebrates including birds, rodents, equids, and humans. Occasionally, alphaviruses can spill over into the human population and cause disease characterized by debilitating arthralgia or fatal encephalitis. Recently, a unique alphavirus, Eilat virus (EILV), was described that readily infects mosquito but not vertebrate cell lines. Here, we investigated the ability of EILV to induce superinfection exclusion. Prior infection of C7/10 (*Aedes albopictus*) cells with EILV induced homologous and heterologous interference, reducing the virus titers of heterologous superinfecting viruses (SINV, VEEV, EEEV, WEEV, and CHIKV) by ~10–10,000 fold and delaying replication kinetics by 12–48 h. Similar to *in vitro* infection, prior *in vivo* EILV infection of *Aedes aegypti* mosquitoes delayed dissemination of chikungunya virus for 3 days. This is the first evidence of heterologous interference induced by a mosquito-specific alphavirus *in vitro* and *in vivo*.



## Introduction

The genus *Alphavirus* in the family *Togaviridae* is comprised of small, spherical, enveloped viruses with genomes consisting of a single strand, positive-sense RNA approximately 11–12 kb in length (Griffin, 2007). The genus consists of 31 recognized species classified into ten complexes based on antigenic and/or genetic similarities (Arrigo et al., 2010; Forrester et al., 2012; Nasar et al., 2012; Powers et al., 2001). The two aquatic alphavirus complexes [southern elephant seal virus and salmon pancreas disease virus] are not known to utilize arthropods in their transmission cycles, whereas all the remaining complexes [Barmah Forest, Ndumu, Middelburg, Semliki Forest, Venezuelan (VEE), eastern (EEE), western equine encephalitis (WEE) and Trocara] consist of arboviruses that almost exclusively utilize mosquitoes as insect vectors (Griffin, 2007; La Linn et al., 2001; Villoing et al., 2000; Weaver et al., 2012; Weston et al., 1999). Mosquito-borne alphaviruses can infect mosquito species encompassing at least eight genera (*Aedes*, *Culex*, *Anopheles*, *Culiseta*, *Haemagogus*, *Mansonia*, *Verrallina*, and *Psorophora*) and many vertebrate taxa including equids, birds, amphibians, reptiles, rodents, pigs, as well as human and non-human primates (Griffin, 2007; Karabatsos, 1985; Jupp et al., 1981; Webb et al., 2008). This ability to infect both mosquitoes and vertebrates enables maintenance of alphaviruses in nature in enzootic cycles with occasional spillover into the human population. Old World viruses such as chikungunya (CHIKV), o'nyong-nyong (ONNV), Sindbis (SINV), and Ross River (RRV) are rarely fatal and cause clinical disease characterized by rash and debilitating arthralgia. In contrast, New World viruses such as western (WEEV), eastern (EEEV), and Venezuelan equine encephalitis (VEEV) viruses cause fatal encephalitis (Griffin, 2007). Currently, there are no licensed vaccines or effective antiviral treatments available to prevent or treat human infection by alphaviruses.

Recently, a host-restricted alphavirus, Eilat virus (EILV), was described that is serologically distinct from other alphaviruses but phylogenetically groups within the mosquito-borne clade as a sister to the WEE complex (Nasar et al., 2012). In contrast to all other mosquito-borne alphaviruses, EILV is unable to infect or replicate in vertebrate cells but can readily replicate to high titers ($>10^8$ PFU/mL) in mosquito cells (Nasar et al., 2012). The EILV vertebrate host range restriction is present at the attachment/entry as well as genomic RNA replication levels (Nasar et al., 2015). EILV is the first mosquito-specific alphavirus described, and represents a new complex within the genus (Nasar et al., 2015).

Superinfection exclusion or homologous interference is a phenomenon where prior infection with a virus reduces or prevents a subsequent infection with a closely related virus. This phenomenon was first described with two closely related genotypes of tobacco mosaic virus, where plants previously infected with common-mosaic

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virus could not be subsequently infected with the yellow-mosaic tobacco virus (McKinney, 1926, 1929). This phenomenon has been successfully developed as a tool to prevent or reduce the loss of crops and is termed "cross protection" (Ziebell and Carr, 2010). Following its discovery, superinfection exclusion was also demonstrated with animal and mosquito-only viruses including alphaviruses (Bolling et al., 2012; Bratt and Rubin, 1968a; Claus et al., 2007; Geib et al., 2003; Hobson-Peters et al., 2013; Kenney et al., 2014; Kent et al., 2010; Laskus et al., 2001; Lee et al., 2005; Steck and Rubin, 1966a; Tscherne et al., 2007; Walters et al., 2004; Whitaker-Dowling et al., 1983; Zou et al., 2009). The available *in vitro* data demonstrate that a prior alphavirus infection can reduce replication of a superinfecting homologous or heterologous virus (Adams and Brown, 1985; Eaton, 1979; Karpf et al., 1997; Lennette and Koprowski, 1946; Renz and Brown, 1976; Stollar and Shenk, 1973; Zebovitz and Brown, 1968). These studies raise the intriguing possibility of utilizing alphaviruses as transmission control measures to limit or eliminate human and/or animal infection. However, all of the viruses within the mosquito-borne clade are capable of replication in both vertebrates and insects, and in addition many can cause severe disease in animals and humans (Griffin, 2007). Consequently, the use of these viruses is unsuitable for transmission control measures. In contrast, the host-restricted and therefore vertebrate apathogenic EILV is potentially ideal for this application. Here, we investigated the ability EILV to induce homologous and heterologous superinfection exclusion.

## Results

### *Homologous interference in C7/10 cells*

To assess superinfection with homologous and heterologous viruses *in vitro* in C7/10 cells, an optimal multiplicity of infection (MOI) to establish EILV infection was investigated (Fig. 1). Infection of C7/10 cells at an MOI of 10 plaque-forming units (PFU)/mL with EILV-eRFP established infection in $>90\%$ of cells within 24 h and was subsequently utilized for superinfection experiments (Fig. 2). To investigate exclusion of homologous virus, C7/10 cells were infected with EILV at an MOI of 10, followed by infection with EILV-eRFP at an identical MOI (Fig. 3). Almost all of the mock-infected cells expressed eRFP 24 h post-infection (hpi), whereas little or no expression was observed in EILV-infected cells up to 72 h post-superinfection, indicating exclusion of homologous virus (Fig. 3).

### *Heterologous interference in C7/10 cells*

To investigate heterologous interference, C7/10 cells were infected with EILV at an MOI of 10 to establish initial infection and at 24 hpi cells were superinfected with SINV-eGFP or VEEV-TC83-eGFP at an MOI of 1 or 0.1 (Fig. 1). Heterologous interference was monitored by eGFP expression *via* fluorescent microscopy. Most of the mock-infected cells expressed eGFP 24 hpi following SINV-eGFP or VEEV-TC83-eGFP infection, whereas minimal eGFP expression was detected in EILV-infected cells at either MOI (Fig. 4). At 72 h post-superinfection, increased eGFP expression was observed irrespective of the MOI or virus (Fig. 4).

To further explore heterologous interference, superinfection was also monitored *via* replication kinetics. Following mock or EILV infection, C7/10 cells were superinfected with SINV or VEEV strain TC83 at an MOI of 1 or 0.1 (Fig. 1). EILV-infected cells displayed a statistically significant reduction in superinfecting SINV or VEEV-TC83 titers at early time points [6, 12, and 24 (both viruses) and 48 (SINV only)] at both MOIs, with $p$-values $<0.02$ for all time points (Fig. 5). SINV and VEEV-TC83 titers were reduced by $\sim$100 to 10,000-fold between 6 and 24 hpi (Fig. 5). In addition to the reduction in virus titers, the replication kinetics of superinfecting viruses were delayed by 12–48 h regardless of virus or MOI (Fig. 5). These data corroborated results obtained *via* fluorescent microscopy, and suggested that prior EILV infection was able to induce heterologous interference, reducing superinfecting virus production and delaying replication kinetics.

Next, we investigated the heterologous interference of alphaviruses associated with severe human disease: EEEV strain FL93, VEEV subtype IC strain 3908, WEEV strain McMillan, and CHIKV strain JKT. C7/10 cells were infected with EILV at an MOI of 10 followed by superinfection with heterologous viruses at an MOI of 0.1 (Fig. 1). Similar to results obtained with superinfection of SINV and VEEV-TC83, all heterologous viruses displayed a reduction in virus production and delayed replication kinetics at early time points in EILV-infected cells (Fig. 6). Virus titers of superinfecting viruses were reduced by $\sim$10 to 1,000-fold at 6, 12, 24 and 48 hpi with $p$-values $<0.04$ for all time points (Fig. 6). VEEV and WEEV displayed the greatest reduction of $\sim$1,000-fold at 24 and 48 hpi, respectively (Fig. 6). Replication kinetics of all superinfecting viruses were also delayed by at least 12–48 h (Fig. 6). WEEV was the only virus that replicated to peak virus titers similar to those in mock-infected cells, whereas the peak titers of other three viruses were reduced by $\sim$10 to 52-fold compared to their respective mock-infected counterparts (Fig. 6).

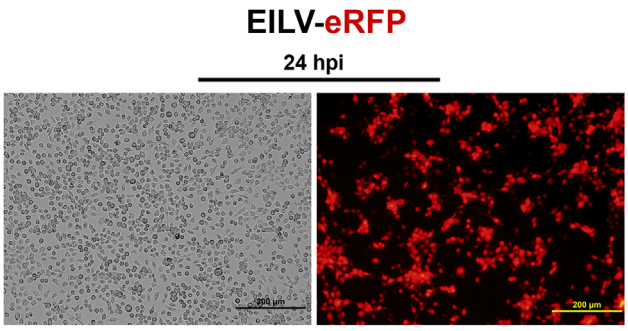



**Fig. 2.** EILV-eRFP infection of C7/10 cells at an MOI of 10 PFU/cell. Phase contrast and fluorescent field micrographs were taken at 24 hpi.

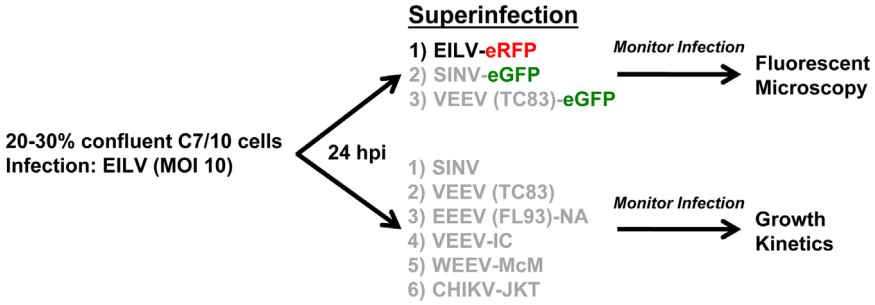



**Fig. 1.** Experimental design to investigate homologous and heterologous interference by Eilat virus *in vitro*.

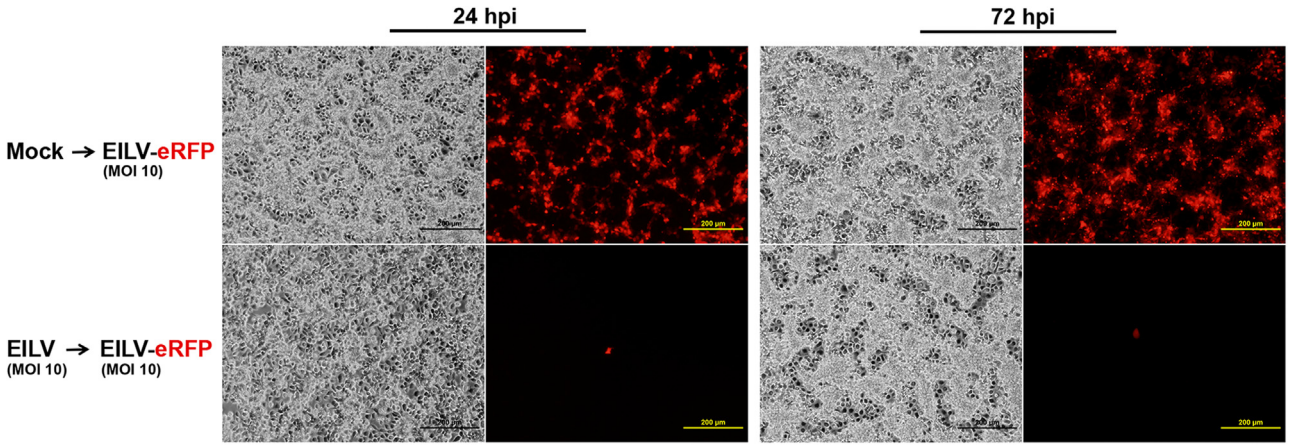



**Fig. 3.** EILV-induced homologous interference in C7/10 mosquito cells determined *via* eRFP expression. Phase contrast and fluorescent photographs were taken at 24 and 72 h post-superinfection.

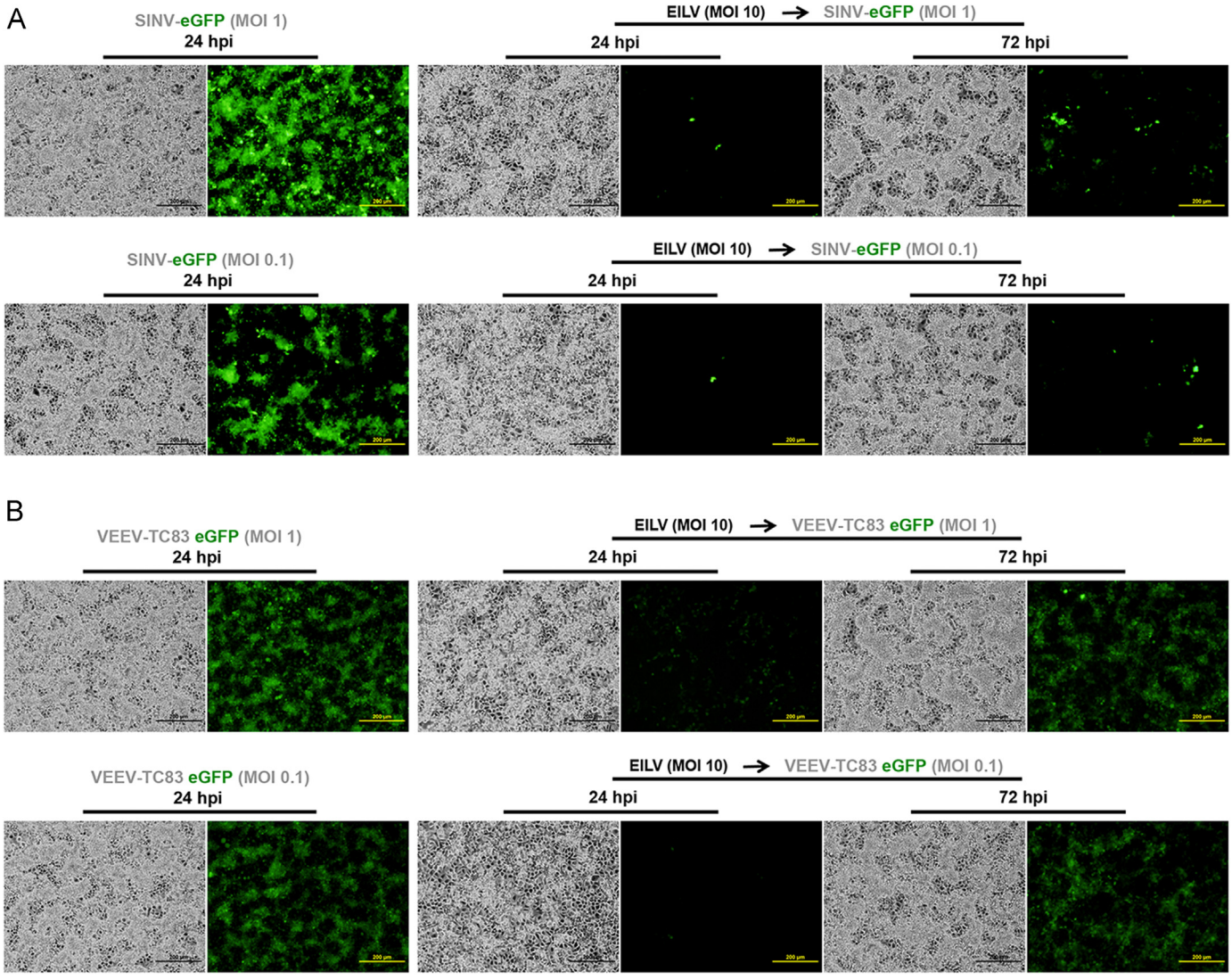



**Fig. 4.** EILV-induced heterologous interference in C7/10 cells, (A) SINV-eGFP and (B) VEEV-TC83-eGFP. Heterologous virus replication was measured *via* fluorescent microscopy. Phase contrast and fluorescent micrographs were taken at 24 and 72 h post-superinfection.

*Heterologous interference in Aedes aegypti mosquitoes*

To investigate heterologous interference in mosquitoes, *A. aegypti* mosquitoes were utilized as they are susceptible to both EILV and CHIKV infection (Karabatsos, 1985; Nasar et al., 2014). Cohorts of *A. aegypti* were injected *via* the intrathoracic (IT) route with ~1 μL of EILV at a titer of $10^7$ PFU/mL to establish initial infection (Figs. 1 and 7A). Five mosquitoes were assayed 7 days

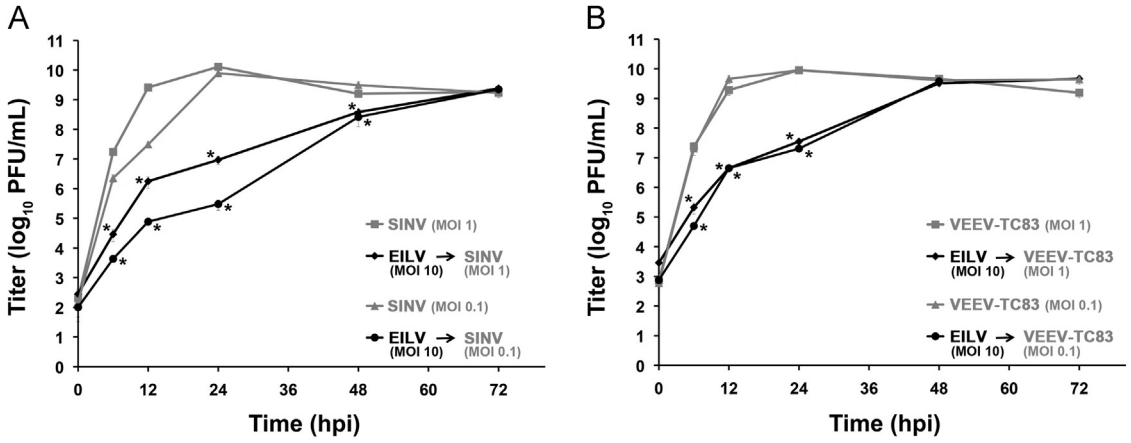



**Fig. 5.** EILV-induced heterologous interference in C7/10 cells, (A) SINV and (B) VEEV-TC83. Heterologous virus titers were determined *via* plaque assay. Each time point represents average of triplicate infections. Bars indicate standard deviations for each time point. *p*-values $< 0.02$ are indicated with *.

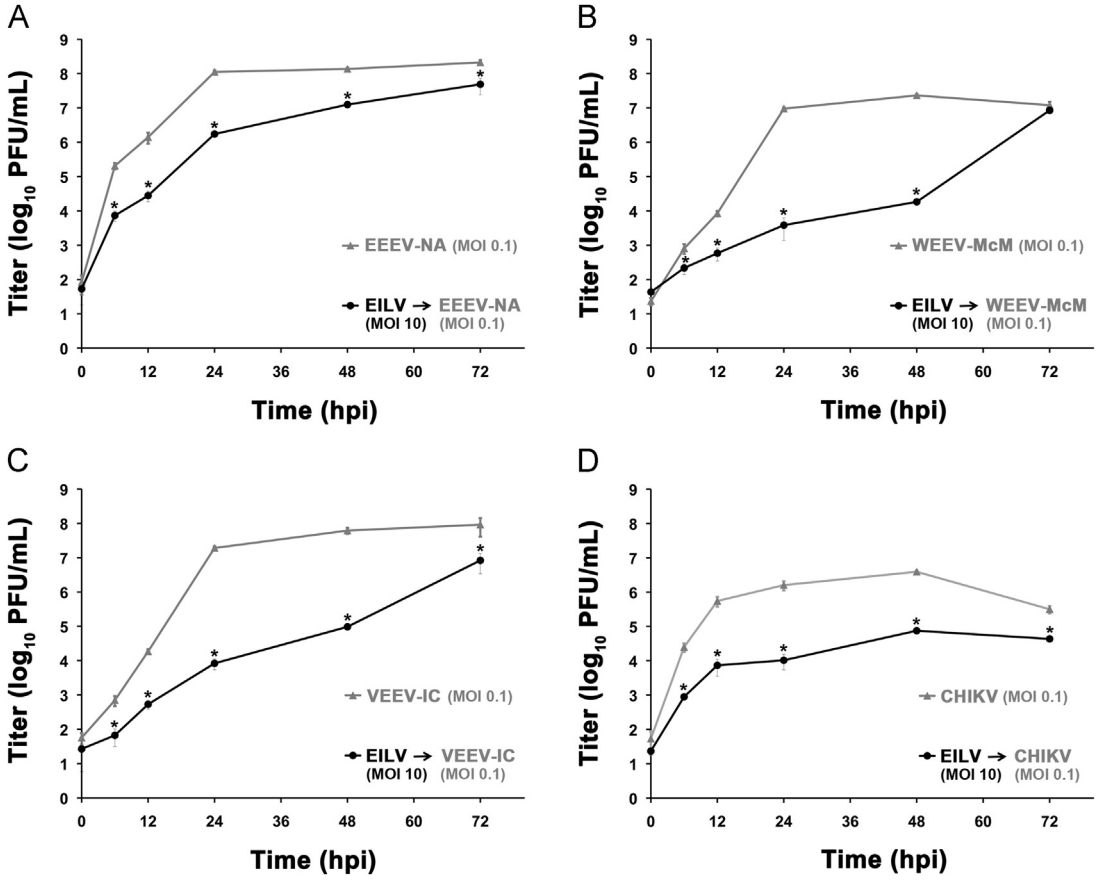


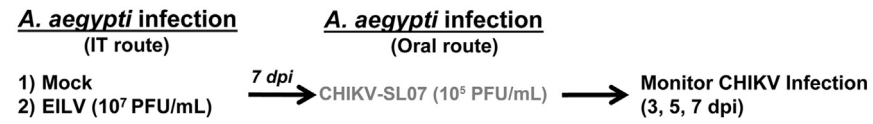

**Fig. 6.** EILV-induced heterologous interference in C7/10 cells, (A) EEEV-NA, (B) VEEV-IC, (C) WEEV-McM, and (D) CHIKV-JKT. Heterologous virus titers were determined *via* plaque assay. Each time point represents average of triplicate infections. Bars indicate standard deviations for each time point. *p*-values $< 0.04$ are indicated with *.

post-injection to confirm EILV infection *via* plaque assays. As expected, all mosquitoes were infected with an average titer of 2.9 $\log_{10}$ PFU/mosquito (Fig. 7B). At 7 days post-EILV injection, mosquitoes were provided bloodmeals containing CHIKV at $10^5$ PFU/mL. CHIKV infection was monitored *via* cytopathic effects (CPE) and plaque assays on Vero cells. Mosquito body infection rates were lower in EILV-infected group than in the mock-infected cohort at 3 and 5 days post-superinfection: 70% *vs.* 95% and 75% *vs.* 85%, respectively (Fig. 7C). However, by days 7 post-superinfection, the body superinfection rate was identical in both groups (75%) (Fig. 7C). EILV infection delayed CHIKV dissemination from the midgut to the legs and wings for 3 days post-superinfection, whereas the dissemination rates were higher or identical at 5 and 7 days post-superinfection, respectively, as compared to the mock-infected group (Fig. 7C). Lastly, CHIKV titers in both bodies and legs/wings were similar at all time points in both EILV- and mock-infected groups (Fig. 7C).

## Disscussion

Homologous and heterologous interference has been investigated previously with alphaviruses in mosquito and vertebrate cell lines. *Aedes albopictus* cells persistently infected with SINV are

A




B

| Group | IT Dose ($log_{10}$ PFU/mL) (+/- SD) | Mosquito Infection# (7 dpi) Percent | Mosquito Infection# (7 dpi) Titer ($log_{10}$ PFU) (+/- SD) | CHIKV Blood Meal Titer ($log_{10}$ PFU/mL) |
|---|---|---|---|---|
| EILV | 7.7 | 100 (5/5) | 2.9 (+/- 0.11) | 5.5 |
| Mock | - | - | - | 5.5 |

C

| Group | 3 dpi Body | 3 dpi Legs/Wings | 5 dpi Body | 5 dpi Legs/Wings | 7 dpi Body | 7 dpi Legs/Wings |
|---|---|---|---|---|---|---|
| Mock → CHIKV | 19/20 (95%) | 7/20 (35%) | 17/20 (85%) | 7/20 (35%) | 15/20 (75%) | 14/20 (70%) |
| Mean Titer ($log_{10}$ PFU) (+/-SD) | 4.0 (+/- 0.92) | 3.2 (+/- 0.82) | 4.6 (+/- 0.40) | 3.2 (+/- 0.86) | 5.1 (+/- 0.10) | 4.4 (+/- 0.90) |
| Titer range ($log_{10}$ PFU) | 1.5 - 5.0 | 2.0 - 4.2 | 4.1 - 5.3 | 1.3 - 3.9 | 5.0 - 5.3 | 3.2 - 4.8 |
| EILV → CHIKV | 14/20 (70%) | **0/22 (0%)** | 14/20 (70%) | 10/20 (50%) | 15/20 (75%) | 13/20 (65%) |
| Mean Titer ($log_{10}$ PFU) (+/-SD) | 4.1 (+/- 0.38) | | 4.5 (+/- 0.73) | 3.3 (+/- 0.87) | 5.4 (+/- 0.30) | 3.9 (+/- 0.70) |
| Titer range ($log_{10}$ PFU) | 3.8 - 5.3 | | 2.6 - 5.2 | 1.3 - 4.5 | 5.1 - 5.8 | 2.5 - 4.3 |

**Fig. 7.** EILV-induced heterologous interference in *A. aegypti* mosquitoes. (A) Outline of experimental design; (B) EILV infection in mosquitoes 7 dpi and CHIKV blood meal titer, and; (C) CHIKV-SL07 superinfection of mosquitoes 3, 5, and 7 dpi. Bold indicates *p*-value=0.003.

resistant to subsequent superinfection with homologous virus (Adams and Brown, 1985; Renz and Brown, 1976; Stollar and Shenk, 1973). Similarly, superinfection of *A. albopictus* cells persistently infected with CHIKV or SINV reduces virus titers of superinfecting heterologous viruses (UNAV, SFV, SINV, CHIKV, AURAV, and RRV) by ~10 to 1,000-fold (Eaton, 1979; Karpf et al., 1997). In addition, studies with chicken embryo cells infected with West Nile, yellow fever or VEEV viruses demonstrated ~25 to 10,000-fold reduction in superinfecting VEEV or EEEV titers (Lennette and Koprowski, 1946; Zebovitz and Brown, 1968).

We investigated whether similar interference can be induced with a mosquito-only alphavirus, EILV. EILV infection almost completely prevented subsequent infection with EILV-eRFP, where only ~2–5 cells expressing eRFP could be observed per $10^5$ cells. In addition, EILV infection reduced superinfecting virus production by ~10 to 10,000-fold and delayed replication kinetics of heterologous superinfecting viruses (SINV, VEEV, EEEV, CHIKV, WEEV) by 12–48 h. These results demonstrate that EILV infection *in vitro* induces homologous and heterologous interference at levels comparable to those shown in previous studies with other alphaviruses.

Limited studies have investigated homologous and heterologous arbovirus interference *in vivo* (Altman, 1963; Beaty et al., 1983, 1985; Borucki et al., 1999; Chamberlain and Sudia, 1957; Davey et al., 1979; Lam and Marshall, 1968a, 1968b; Pesko and Mores, 2009; Rozeboom and Kassira, 1969; Sabin, 1952; Sundin and Beaty, 1988). Elegant studies utilizing temperature sensitive Semliki Forest and La Crosse virus mutants demonstrated homologous interference by either inhibiting or reducing viral production of superinfecting virus by ~10–500-fold (Beaty et al., 1983, 1985; Davey et al., 1979; Rozeboom and Kassira, 1969; Sundin and Beaty, 1988). Investigations into heterologous interference have yielded similar results with reduction in replication, reduction and/or prevention of dissemination, or reduced transmission of superinfecting virus (Altman, 1963; Borucki et al., 1999; Chamberlain and Sudia, 1957; Lam and Marshall, 1968a, 1968b; Pesko and Mores, 2009; Sabin, 1952). However, the investigation of heterologous interference with mosquito-specific Culex flavivirus (CxFV) has yielded contradictory results; *Culex pipiens* naturally infected with CxFV have reduced WNV viral loads and dissemination rates 7 dpi after oral superinfection with WNV (Bolling et al., 2012), whereas *Culex quinquefasciatus* intrathoracically infected with CxFV display no difference in vector competence for WNV than their mock-infected counterparts (Kent et al., 2010). Here, we report the first evidence of heterologous interference with EILV virus in mosquitoes. Prior EILV infection of *A. aegypti* reduced superinfection rates and delayed CHIKV dissemination from the midgut for 3 days post-infection. Further studies are required to investigate this effect with other pathogenic alphaviruses.

The investigation of EILV-mediated heterologous interference *in vitro* with SINV and VEEV clones with and without eGFP yielded some differences at 72 h post-superinfection. The expression of eGFP was limited at 72 h and was more pronounced with SINV-eGFP superinfection, whereas the clones without eGFP cassette were able to achieve similar virus titers as their mock counterparts (Figs. 4 and 5). There are several possible explanations for the incongruent results. First, competition for viral and/or host factors needed for viral replication, transcription, and translation may reduce eGFP expression. The eGFP clones contain an additional subgenomic promoter that likely competes for limited viral and/or host factors involved in the transcription and translation of two subgenomic RNAs. Additionally, the superinfected cells contain two viruses with three subgenomic promoters, which may also compete for limited host factors for transcription and translation; 2) the expression of SINV subgenomic RNAs may require high levels of transcription and translation. Limited data suggest that SINV virion formation is less efficient than that of VEEV, requiring 5–8 fold higher levels of viral RNAs and structural proteins (Volkova et al., 2006). The competition of host and viral factors may prevent and/or delay the levels of transcription and translation required for expression of subgenomic RNAs thus reducing eGFP expression.

EILV-mediated interference was not observed beyond 5 days post-CHIKV superinfection in mosquitoes. There are several possible explanations for this lack of sustained heterologous interference; 1) decreased EILV replication with time leads to reduced interference thus enabling CHIKV replication; 2) EILV and CHIKV may have different cell tropisms *in vivo* and consequently limited coinfection of cells; 3) the intrinsic ability of EILV to induce interference mechanism/s may be lower as a consequence of adaptation to a single (mosquito) host; and 4) CHIKV may be able to overcome EILV-mediated interference mechanism/s. EILV titers at 7 days-post-injection were ~100-fold lower than those reported for CHIKV in *A. aegypti*, suggesting that a decrease in EILV replication may play a role in reduced heterologous interference (Tsetsarkin et al., 2006). However, these hypotheses require further investigation.

Interference mechanisms that prevent or reduce infection of superinfecting viruses have been shown to act on various stages of the virus replication cycle: downregulation of expression of cellular receptors for viruses, reduction/prevention of entry, reduced endocytic vesicle formation, decreased internalization of bound ligands/receptors, competition for coated pits, prevention of disassembly by encapsidation of incoming viral genomes, poor acidification of early endosomes, prevention of fusion with the host membrane, inhibition of nucleocapsid uncoating, inhibition of viral replication, competition for host factors; and induction of RNA silencing by the primary virus, resulting in sequence-specific degradation of superinfecting virus (Bratt and Rubin, 1968a, 1968b; Lee et al., 2005; Lohmann et al., 2003; Lu et al., 1998; Michel et al., 2005; Simon et al., 1990; Steck and Rubin, 1966a, 1966b; Walters et al., 2004). Limited studies have examined interference mechanisms induced by alphaviruses. The mechanisms identified by these studies include reduced binding, inefficient penetration, inhibition of nucleocapsid uncoating, and post-translational inhibition of replication complexes of superinfecting homologous virus (Adams and Brown, 1985; Renz and Brown, 1976; Singh et al., 1997). The latter interference mechanism was suggested to involve cleavage of the superinfecting nsP123 polyprotein, an essential component of the negative strand replicase, by nsP2 of the initially infecting virus, thus reducing negative strand synthesis. Although several studies have produced indirect evidence supporting this hypothesis; however, formal proof is lacking (Ehrengruber and Goldin, 2007; Sawicki et al., 2006). Our *in vitro* results with heterologous superinfecting viruses do not support this hypothesis. The SINV nsP2/3 cleavage site is identical to that of EILV and its nsP1/2 site differs only by one amino acid; consequently there should be greater exclusion of SINV than of WEEV, whose nsP cleavage sites display greater sequence divergence (Fig. 5) (Nasar et al., 2012). Superinfection with SINV and WEEV resulted in almost identical levels of reduction in virus production and delays in replication kinetics (Figs. 5 and 6). In fact, a much greater inhibition effect was measured at 48 h post-superinfection with WEEV (~1000-fold) than with SINV (~10-fold) (Figs. 5 and 6). These data indicate premature cleavage of superinfecting heterologous virus negative strand replicase probably plays little or no role in preventing replication of superinfecting heterologous viruses.

The superinfection interference we observed is likely due to, in part, to competition for host factors and replication sites, as well as RNA interference (RNAi). The latter is an important antiviral defense in mosquitoes in response to alphavirus infection involving multiple RNAi pathways (Adelman et al., 2013; Brackney et al., 2010; Campbell et al., 2008; Cirimotich et al., 2009; Keene et al., 2004; Khoo et al., 2010; Morazzani et al., 2012; Myles et al., 2008; Schnettler et al., 2013; Vodovar et al., 2012). However, the roles of RNAi and other mechanisms in superinfection exclusion require further investigation.

Heterologous interference could theoretically be utilized to control transmission of pathogenic arboviruses. However, vertebrate-pathogenic, mosquito-borne alphaviruses are obviously unsuitable as a potential biological control measure. Host restricted viruses, such as EILV and CxFV, which do not infect vertebrates, could be developed to safely control arbovirus transmission. However, the available data demonstrate that the intrinsic ability of both EILV and CxFV to interfere with pathogenic arboviruses *in vivo* is modest and requires improvement. One promising strategy to increase interference is to genetically engineer EILV and other viruses to deliver siRNA targeting pathogenic viruses (Gaines et al., 1996; Olson et al., 1996; Powers et al., 1996). Infection of C6/36 cells with a SINV clone encoding anti-sense sequences of dengue virus rendered them resistant to infection with dengue-2 (DENV-2) (Gaines et al., 1996). *A. aegypti* mosquitoes infected with the same SINV clone are unable to support DENV-2 replication in salivary glands, and consequently are unable to transmit DENV (Olson et al., 1996). Similarly, SINV clones engineered to target the S segment of La Crosse virus reduced virus replication both *in vitro* and *in vivo* (Powers et al., 1996). Taken together, these data suggest that either the intrinsic EILV properties and/or genetically engineered clones of EILV have potential to be utilized as transmission reducing approaches to control and/or prevent alphavirus disease.

## Materials and methods

### *Viruses and cells*

SINV strain Eg339, EEEV strain FL93-939, VEEV subtype IC strain 3908, WEEV strain McMillan, CHIKV strain JKT, and CHIKV strain SL07 were obtained from World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch. SINV-eGFP strain Tr-339, VEEV-TC83, and VEEV-TC83-eGFP were obtained from internal collections.

Vero cells were obtained from the American Type Culture Collection (Bethesda, MD), and both BHK and C7/10 cells were obtained from internal collections. Cell lines were propagated at 37 °C (Vero and BHK) or 28 °C (C7/10) with 5% $CO_2$ in DMEM containing 10% (V/V) fetal bovine serum (FBS), sodium pyruvate (1 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). C7/10 cell media was additionally supplemented with 1% (V/V) tryptose phosphate broth (Sigma).

### *Superinfection exclusion in C7/10 cells*

To determine an optimal MOI for homologous and heterologous interference experiments, C7/10 cells were counted with a hemocytometer in bright and fluorescent fields to determine the number and percentage of EILV-eRFP infected cells. Similar experiments were performed to estimate number of cells superinfected with homologous virus.

*Ca.* 20–30% confluent C7/10 monolayers seeded overnight were infected with EILV or EILV-eRFP at an MOI of 10 PFU/cell, or mock-infected; 24 h post-EILV or mock infection, monolayers were superinfected with homologous (MOI of 10) or heterologous viruses at an MOI of 1 or 0.1 PFU/cell (Fig. 1). Superinfection exclusion was monitored *via* fluorescent microscopy and replication kinetics. For fluorescent microscopy, infections were performed in 12-well plates and phase-contrast and fluorescent field photographs were taken at 24 and 72 h post-superinfection.

Replication kinetics were performed in triplicate in T-25 $cm^2$ cell culture flasks. Following superinfection with heterologous virus for 1 h at 28 °C, monolayers were rinsed five times with room temperature (RT) DMEM to remove unbound virus, and 5 mL

of growth medium were added to each flask. Aliquots of 0.5 mL were taken immediately afterward as "time 0" samples and replaced with 0.5 mL of fresh medium. Flasks were incubated at 28 °C, and further samples were taken at 6, 12, 24, 48, and 72 hpi. All samples were flash frozen in an ethanol/dry ice bath and stored at −80 °C.

### *Plaque assay*

All virus samples, except EILV, were titrated on 90% confluent BHK (*in vitro* experiments) or Vero (*in vivo* experiment) cell monolayers seeded overnight in six-well plates. Cell monolayers were overlaid with 3 mL of 1× DMEM containing 1% FBS (V/V), penicillin (100 U/mL), streptomycin (100 μg/mL) and 0.2% agarose (V/V) (Lonza Inc., Allendale, NJ). Cells were incubated at 37 °C with 5% $CO_2$ for 2 (BHK) and 3 (Vero) days for plaque development. Eilat virus titrations were performed on ~80% confluent C7/10 cell monolayers seeded overnight in six-well plates. Following infection, cells were overlaid with 3 mL of a 1:1 mixture of 2% tragacanth and 2× MEM containing 5% FBS, 2% tryptose phosphate broth solution (V/V), penicillin (200 U/mL), and streptomycin (200 μg/mL). Cells were incubated at 28 °C with 5% $CO_2$ for 3 days for plaque development. Following development of plaques, the overlays were removed and monolayers were fixed with 10% formaldehyde in PBS for 30 min. Cells were stained with 2% crystal violet in 30% methanol for 5 min at RT; excess stain was removed and plaques were counted.

### *Superinfection exclusion in mosquitoes*

*A. aegypti* eggs from colonies at UTMB were hatched and reared using standard methods (Rosen and Gubler, 1974). Cohorts of 200 adult females collected 5–6 days after emergence from the pupal stage were cold-anesthetized and inoculated *via* the IT route with PBS or with ~1 μL of EILV at $10^7$ PFU/mL. Mosquitoes were given 10% sucrose and held for an extrinsic incubation period of 7 days at 28 °C. Following incubation, mosquitoes were fed an artificial meal consisting of defibrinated sheep blood (Colorado Serum Company, Denver, CO) and CHIKV-SL07 at a final concentration of $10^5$ PFU/mL. Mosquitoes were allowed to feed for 1 h, and following feeding mosquitoes were cold-anesthetized and sorted. Fully engorged mosquitoes at or higher than stage 3 were retained for the study (Pilitt and Jones, 1972). Mosquitoes were provided 10% sucrose and 20 individuals per cohort were sampled 3, 5, 7 days post-CHIKV infection. Mosquitoes were cold-anesthetized, and bodies and legs/wings were removed and placed in 0.5 mL of 1× DMEM containing 20% FBS (V/V), penicillin (200 U/mL), streptomycin (200 μg/mL), and 5 μg/mL amphotericin B. Samples were triturated using a Mixer Mill 300 (Retsch, Newtown, PA), centrifuged at 18,000 × *g* for 5 min and supernatants from each sample were analyzed for CPE on Vero cells. Positive samples from days 3, 5, and 7 post-CHIKV infection were titrated *via* plaque assay on Vero cells. Number of samples tittered: all positive samples (3 day post-CHIKV infection), $N=10$ (5 day post-CHIKV infection), and $N=5$ (7 day post-CHIKV infection).

### *Statistics*

RStudio (Version 0.97, RStudio, Boston, MA) running R (Version 3.0.1, R Development Core Team, Vienna, Austria) software was used for statistical analysis. Significant differences in mean titers during superinfection were determined using two-way ANOVA for all viruses followed by a Tukey Test. Two-tailed Fisher exact test was performed to determine significant differences in dissemination rates.

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