Negeviruses reduce replication of alphaviruses during co-infection

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Abstract

Negeviruses are a group of insect-specific virus (ISV) that have been found in many arthropods. Their presence in important vector species led us to examine their interactions with arboviruses during co-infections. Wild-type negeviruses reduced the replication of several alphaviruses during co-infections in mosquito cells. Negev virus (NEGV) isolates were also used to express GFP and anti-chikungunya virus (CHIKV) antibody fragments during co-infections with CHIKV. NEGV expressing anti-CHIKV antibody fragments was able to further reduce replication of CHIKV during co-infections, while reductions of CHIKV with NEGV expressing GFP were similar to titers with wild-type NEGV alone. These results are the first to show that negeviruses induce superinfection exclusion of arboviruses and to demonstrate a novel approach to deliver anti-viral antibody fragments with paratransgenic ISVs. The ability to inhibit arbovirus replication and express exogenous proteins in mosquito cells make negeviruses a promising platform for control of arthropod-borne pathogens.

Importance

Negeviruses are a group of insect-specific viruses (ISVs), viruses known to only infect insects. They have been discovered over a wide geographical and species range. Their ability to infect mosquito species that transmit dangerous arboviruses makes negeviruses a candidate for a pathogen control platform. Co-infections of mosquito cells with a negevirus and an alphavirus demonstrated that negeviruses can inhibit the replication of alphaviruses. Additionally, modifying Negev virus

(NEGV) to express a fragment of an anti-CHIKV antibody further reduced the replication of CHIKV in co-infected cells. This is the first evidence to demonstrate that negeviruses can inhibit the replication of important arboviruses in mosquito cells. The ability of a modified NEGV to drive the expression of antiviral proteins also highlights a method for negeviruses to target specific pathogens and limit the incidence of vector borne diseases.

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48 **Introduction**

49 Many insect-specific viruses (ISVs) have been discovered in wild-caught and
50 laboratory colonies of mosquitoes and in mosquito cell cultures (1). ISVs are only
51 known to replicate in arthropods or insect cell lines. While posing no threat to
52 human or animal health, ISVs may affect the transmission of more dangerous
53 vector-borne pathogens. Highly insect-pathogenic ISVs have been suggested for use
54 as biological control agents to reduce populations of vector competent mosquitoes
55 (2-4). Several recent studies have demonstrated that ISVs may play a more direct
56 role by inhibiting the replication of arboviruses within the insect host. The majority
57 of these experiments have attempted to define a relationship based on
58 superinfection exclusion, a phenomenon in which an established virus infection
59 interferes with a secondary infection by a closely related virus. For example, insect-
60 specific flaviviruses, such as cell fusing agent virus (CFAV), Nhumirim virus (NHUV)
61 and Palm Creek virus (PCV) have demonstrated an ability to reduce viral loads of
62 vertebrate pathogenic flaviviruses, like West Nile virus (WNV), Zika virus (ZIKV),
63 dengue virus (DENV), Japanese (JEV) and St. Louis encephalitis (SLEV) viruses (5-
64 10). Similarly, the insect-specific alphavirus Eilat virus (EILV) was shown to reduce
65 or slow replication of the pathogenic alphaviruses chikungunya virus (CHIKV),
66 Sindbis virus (SINV), eastern (EEEV), western (WEEV) and Venezuelan equine
67 encephalitis (VEEV) viruses in cell culture or in mosquitoes (11). Less information is
68 available about the effect of unrelated viruses during superinfection. Cell cultures
69 chronically infected with Aedes albopictus densovirus (AalDNV) limit replication of
70 DENV (12), cell cultures with established CFAV and Phasi Charoen-like virus (PCLV)

infections reduced ZIKV and DENV replication (13), and co-infections with Yichang virus, a mesonivirus, reduced DENV replication in cells and dissemination in mosquitoes (14). The mechanism for these reduced titers has not been elucidated, but the relationships appear to be virus specific and even host specific (5, 15, 16).

The genus *Negevirus* is a recently discovered, unclassified group of ISVs (17). Members of this genus have been isolated from several species of hematophagous mosquitoes and sandflies, and negev-like viruses have also been found in other non-vector insects (18-25). Phylogenetic studies have placed this group of viruses most closely to members of the genus *Cilevirus*, plant pathogens that are transmitted by mites (17, 24). These viruses have a single-stranded, positive-sense RNA genome of ~9-10 kb, and contain three open reading frames (ORFs) (17). The ORFs encode for the replication machinery (ORF1), a putative glycoprotein (ORF2), and a putative membrane protein (ORF3). Electron microscopy has shown the structural proteins to be arranged in a hot air balloon morphology, a round particle with a single protrusion that is likely the glycoprotein structure (26-28). Little is known about the infectivity, transmission dynamics, and species range of negeviruses. However, they are commonly found in field collected mosquitoes (29, 30).

The association of negeviruses with important vector species over a wide geographical range raises the question of possible interactions or interference of negeviruses with vertebrate pathogenic viruses. Few studies exist that demonstrate the ability of unrelated viruses to induce superinfection exclusion, but evidence for this phenomenon with negeviruses could provide a platform to control vector-borne viral diseases in many arthropod vector species. In this study, three negevirus

isolates from the Americas were assessed for superinfection exclusion in cell cultures with VEEV, CHIKV Mayaro virus (MAYV), o'nyong'nyong virus (ONNV), and Semliki Forest virus (SFV). The use of a Negev virus (NEGV) infectious clone also allowed manipulation of the virus genome to provide a greater ability to exclude superinfection with CHIKV.

Results

Wild-type negevirus growth curves

All wild-type negeviruses reached titers greater than 10$\log_{10}$ pfu/mL within 48 hours when infected at a MOI of 1 (Figure 1A). NEGV and PIUV-Lutzomyia neared peak titer by 12 hours post infection (hpi), while PIUV-Culex neared peak titer at 24 hpi. Infections of NEGV with MOIs of 1 and 5 produced similar growth curves (Figure 1B).

Superinfection exclusion of alphaviruses with wild-type negeviruses

To determine the effect of negeviruses on the replication of alphaviruses in cell culture, negevirus isolates were co-infected with VEEV-TC83 or CHIKV isolates. NEGV was able to significantly reduce replication of VEEV-TC83, with reductions of 5.5-7.0 $\log_{10}$ pfu/mL of VEEV at 48 hours (Figure 2A). There were no significant differences in VEEV-TC83 titers when co-infected with NEGV inoculated at MOIs of 1 or 5. There were also no significant differences in VEEV-TC83 titer when NEGV inoculation preceded VEEV-TC83 inoculation by 0-, 2- or 6 hours. Co-infection with PIUV-Culex or PIUV-Lutzomyia also significantly reduced replication of VEEV-TC83

117 across all time points (Figure 2B-D). A similar reduction of VEEV-TC83 was
118 observed during all negevirus co-infections, as VEEV-TC83 was reduced 4.6-7.2
119 log_{10} pfu/mL at 48 hours.

120 Co-infections with CHIKV and NEGV also resulted in significantly lower titers
121 of CHIKV at all time points, but only reducing the titer of CHIKV by 0.65-0.93 log_{10}
122 pfu/mL after 48 hours (Figure 3A). Varying the MOI of NEGV and timing of CHIKV
123 inoculation only produced differing titers of CHIKV at the 12-hour timepoint.
124 However, titers of CHIKV during co-infection with different negeviruses varied
125 greatly (Figure 3B, C), with the largest variance of CHIKV titers, reductions of 0.7
126 log_{10}, 2.4 log_{10} and 5.3 log_{10} pfu/mL, observed when inoculated 6 hours post-
127 inoculation with NEGV, -PIUV-Culex and -PIUV-Lutzomyia, respectively (Figure 3D).

128 NEGV and PIUV-Lutzomyia were also able to reduce replication of several
129 other alphavirus isolates by varying amounts when infected simultaneously. Titers
130 of VEEV-IC were reduced by 2.8-3.0 log_{10} pfu/mL at 48 hours post co-infection
131 (Figure 4A). Three isolates of MAYV, Guyane, BeAn343102, and BeAr505411, were
132 reduced by 1.9-3.2 log_{10} pfu/mL at 48 hours post co-infection (Figure 4B). Outputs
133 between MAYV co-infections were similar with significant differences at 48 hours
134 only seen between MAYV-Guyane co-infected with NEGV and PIUV-Lutzomyia,
135 respectively. ONNV titers were reduced by 2.4-3.2 log_{10} pfu/mL, and SFV was
136 reduced by 1.2-1.8 log_{10} pfu/mL after 48 hours (Figure 4C, D).
137
138 *Replication of modified NEGV isolates*

139 The sequence for GFP was successfully cloned as both a fusion- and cleaved
140 protein at several sites along the NEGV infectious clone (Table 1, Figure 5A).
141 Following electroporation, viable virus was rescued from isolates with GFP inserted
142 as a fusion- and cleaved protein on the C-terminal of ORF3, and as a cleaved protein
143 on the C-terminal of ORF1.
144 Further experiments used isolates with GFP added to the ORF3 (NEGV GFP-
145 fusion and NEGV GFP) and also with GFP swapped with scFv CHK265, a partial
146 sequence for an anti-CHIKV antibody (Figure 5A). Mutated isolates were rescued
147 and had titers ranging from 9.6-10.4 $\log_{10}$ pfu/mL, with similar growth curves to the
148 wild-type NEGV (Figure 5B). Cells infected with NEGV GFP-fusion demonstrated
149 brilliant, punctate fluorescence (Figure 5C), while cells infected with NEGV GFP
150 (cleaved) demonstrated dull, diffuse fluorescence (Figure 5D). The number of
151 fluorescent plaques and overall titer of NEGV isolates expressing GFP remained
152 stable for 5 passages in C7/10 cells (Figure 5E, F).
153
154 *Superinfection exclusion of alphaviruses with modified NEGV*
155 NEGV isolates expressing GFP or scFv-CHK265 were used to infect cells for
156 co-infection with VEEV-TC83 or CHIKV. The results for co-infections with VEEV-
157 TC83 were similar to the reduction in titer seen with wild-type viruses. At the 48-
158 hour timepoint, VEEV-TC83 was reduced by 4.5-5.5 $\log_{10}$ pfu/mL when co-infected
159 with NEGV isolates (Figure 6A), 4.6-5.8 $\log_{10}$ pfu/mL when infected 2 hours post
160 NEGV isolates (Figure 6B), and 5.6-6.9 $\log_{10}$ pfu/mL when infected 6 hours post
161 NEGV isolates (Figure 6C). When infected simultaneously with CHIKV, titers were

reduced by 0.7-1.1 $\log_{10}$ pfu/mL during co-infections of NEGV expressing GFP, and by 2.9-3.8 $\log_{10}$ pfu/mL during co-infections of NEGV expressing scFv-CHK265 at the 48-hour timepoint (Figure 7A). When inoculated 2 hours post NEGV infection, the titer of CHIKV after 48 hours was reduced 0.7-0.9 $\log_{10}$ pfu/mL with NEGV expressing GFP and 3.7-4.5 $\log_{10}$ pfu/mL with NEGV expressing scFv-CHK265 (Figure 7B). Delaying CHIKV infection 6 hours post NEGV resulted in reductions of 1.2-1.9 $\log_{10}$ pfu/mL and 5.2-5.7 $\log_{10}$ pfu/mL after 48 hours of co-infection with NEGV expressing GFP and scFv-CHK265, respectively (Figure 7C).

Discussion

The microbiome of arthropod vectors is known to influence host-pathogen interactions (31-33). The precise mechanisms of pathogen inhibition are unknown, but there is increasing evidence that interference from ISVs is one mechanism (5-7, 10). Interactions between related viruses has led to the theory of superinfection exclusion, in which an established infection interferes with or inhibits a secondary infection by a closely related virus. For example, a CFAV mosquito isolate reduced the replication of DENV and ZIKV during co-infections in mosquitoes and mosquito cells (5).

To investigate if superinfection exclusion occurred with other virus combinations, pathogenic alphaviruses and negeviruses were used in co-infection experiments. Titers of multiple VEEV isolates and MAYV isolates were consistently reduced during co-infection experiments with negeviruses. Reductions varied during CHIKV-negevirus co-infections. These results provide further evidence that

185 superinfection exclusion of alphaviruses is pathogen specific, but differ from a
186 previous report demonstrating no reduction in titer of VEEV TC-83 and a significant
187 reduction of wild-type VEEV-IC (strain 3908) after 48 hours when co-infected with
188 EILV, an alphavirus ISV (11). However, the potential for superinfection exclusion of
189 pathogens is different for each ISV, despite their relatedness. These differences have
190 been demonstrated among several insect-specific flaviviruses, Nhumirim virus
191 (NHUV) and Palm Creek virus (PCV) were capable of superinfection exclusion; CFAV
192 gave varying results; and Culex flavivirus (CxFV) did not reduce titers of pathogenic
193 arboviruses (5-10, 15, 16, 34-37). In our experiments with negeviruses, PIUV
194 isolates were more capable than NEGV at inhibiting important arboviruses.
195 While ISVs show promising results to block arbovirus replication in
196 mosquito vectors, their unknown mechanism of action may limit their use against a
197 wide range of pathogens, but paratransgenic ISVs could be used to provide antiviral
198 molecules that specifically interfere with pathogen transmission (38). To this end,
199 we used an infectious clone of NEGV to deliver a fragment of an antibody known to
200 neutralize CHIKV (39). A scFv consists of the variable regions of the heavy and light
201 chains of an antibody, joined by a soluble linker. These antibody fragments can
202 possess the neutralizing qualities of their full-size versions in only ~27kDa. Co-
203 infections with scFv-expressing NEGV isolates greatly reduced titers of CHIKV,
204 whereas co-infections with control NEGV isolates expressing GFP or wild-type NEGV
205 only modestly reduced CHIKV titers. The use of parastransgenic NEGV expressing
206 scFvs demonstrates a novel approach to disrupt pathogen infection in mosquitoes.
207 This method adapts two existing techniques for pathogen control: *Wolbachia*

infected mosquitoes and the CRISPR-Cas-aided integration of scFv sequences into the mosquito genome. *Wolbachia* is a ubiquitous species of bacteria found in many insects that has been shown to block replication of some viral pathogens in cell cultures and mosquitoes. The use of *Wolbachia*-infected vectors has been widely adapted to curb mosquito-borne viral diseases, propelled by its natural ability to colonize mosquitoes (40). Negeviruses also possess this attribute, having been discovered in numerous mosquito species on 6 continents, along with sandflies and other diverse insect species (20-23, 25). Insertion of gene-editing scFv sequences into mosquito genomes has also been used to prevent *Plasmodium* and DENV infection (41, 42). By using CRISPR-Cas9 to insert a scFv targeting *Plasmodium*, infection was blocked in *Anopheles* mosquitoes, and gene drive ensured the production of the scFv in the offspring. In this study, we used scFv expression strategy by cloning an anti-CHIKV scFv into the NEGV genome. Using NEGV as a vehicle for paratransgensis is advantageous, because an isolate can infect multiple host species, and it is suspected to be vertically transmitted in mosquitoes and in theory could become established in multiple generations of the infected host species (17, 22, 43).

Modifications to certain parts of the NEGV genome were tolerated as both cleaved and fusion proteins. Expression of extraneous proteins in viruses is common with 2A sequences to produce separate proteins or under a separate subgenomic promoter (44, 45). However, extraneous proteins expressed as a fusion with a structural virus protein is uncommon. ORF3 is ~25kDa and is suspected to be the membrane protein, the dominant structural protein; and ORF2 is ~40kDa and is

the putative glycoprotein predicted to form a bud projecting from one end of the virion (26). The viability of the NEGV isolates with GFP- or scFv-fusion at ORF3 isolates was surprising because these inserts double the size of the membrane protein, which must interact with itself and ultimately support the projection of the glycoprotein. The modifications to ORF2 resulting in non-viable virus are not surprising as the glycoprotein is suggested to be important for cell attachment and entry (26). GFP preceded by a 2A sequence was also successfully cloned onto the C-terminal of ORF1. As 2A allows for separation of the two proteins, this insertion only added 17 residues to a ~268kDa protein. However, ORF1 will likely be expressed at lower levels compared to ORF3. By using NEGV to express anti-CHIKV scFvs, the cleaved and fused inserts may provide distinct advantages. Cleaved scFvs are free to be transported around the cell, accessing many different locations where they may encounter CHIKV proteins. In contrast, fused proteins are bound to the membrane protein of NEGV and are limited to compartments of the cell where NEGV proteins are expressed, and virions are assembled. In theory, increasing the concentration of the scFvs in specific areas of the cell, should inhibit CHIKV virion assembly and egression. By using both cleaved and fused NEGV isolates, the scFv sequence can also be easily replaced to target a new pathogen, adding to the versatility of this technique.

The current experiments demonstrate the ability of some negeviruses, both wild-type and paratransgenic isolates, to inhibit the replication in mosquito cells with co-infected arboviruses. The next question is whether genetically altered negeviruses will survive and replicate in live mosquitoes; and if so, will they be

254 vertically transmitted or transovarially transmitted in the insects? This will be our
255 next area of investigation. If successful, then the use of paratransgenic negeviruses
256 could be another novel method to alter the vector competence of mosquitoes for
257 selected arboviruses.
258
259 **Materials and Methods**
260 *Cell culture and viruses*
261 *Aedes albopictus* (C7/10) cells (46) were obtained from the World Reference
262 Center for Emerging Viruses and Arboviruses (WRCEVA). African green monkey
263 kidney (Vero E6) cells were obtained from the American Type Culture Collection
264 (ATCC). C7/10 cells were maintained in Dulbecco's minimal essential medium
265 (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% minimal essential
266 medium non-essential amino acids, 1% tryptose phosphate broth and 0.05 mg/mL
267 gentamycin in a 30°C incubator with 5% CO_2. Vero cells were maintained in DMEM
268 supplemented with 10% FBS and 0.05 mg/mL gentamycin in a 37°C incubator with
269 5% CO_2.
270 NEGV was rescued in C7/10 cells from an infectious clone as previously
271 described and without further passage (47). The sequence was derived from NEGV
272 strain M30957 isolated from a pool of *Culex coronator* mosquitoes collected in
273 Harris County, Texas, USA in 2008 (17). Piura virus (PIUV) strain EVG 7-47 (PIUV-
274 Culex) isolated from a pool of *Culex nigripalpus* mosquitoes from Everglades
275 National Park, Florida, USA in 2013 (22). PIUV EVG 7-47 was passaged four times in
276 C6/36 cells and obtained from the WRCEVA. PIUV strain CO R 10 (PIUV-Lutzomyia)

was isolated from a pool of *Lutzomyia evansi* sandflies caught in Ovejas, Sucre, Colombia in 2013 (22). The isolate PIUV CO R 10 was passaged twice in C6/36 cells and also obtained from the WRCEVA. CHIKV isolate 181/25 (48) was rescued in Vero cells from an infectious clone as previously described (49). Rescued CHIKV 181/25 was subsequently passaged once in C7/10 cells and once in Vero cells. Venezuelan equine encephalitis virus (VEEV) vaccine strain, TC-83 (50), was rescued in baby hamster kidney (BHK) cells from an infectious clone without further passage. VEEV isolate P676 (VEEV-IC), and SFV isolate A774/C2/A were attained from Public Health England and passaged once in Vero cells. ONNV isolate UgMP30, and Mayaro virus (MAYV) isolates Guyane, BeAn344102, and BeAr505411 were attained from BEI Resources and passaged once in Vero cells.

Cloning NEGV for exogenous gene expression

The NEGV infectious clone was used as the backbone to express exogenous genes. Green fluorescent protein (GFP; 717bp) was inserted along several sites of the NEGV genome and the single chain variable fragment (scFv) of anti-CHIKV neutralizing antibody CHK265 (771bp including linkers) (39) was inserted on the C-terminal of ORF3 as either a fusion protein, or with a 2A sequence (EGRGSLLTCGDVEENPGP) (Figure 1A). The cloned scFv CHK265 sequence contained a N-terminal linker (LAAQPAMA) for articulation from the viral ORF3 protein, and a domain linker ($(G_4S)_4$) between the variable heavy (V_H) and variable light (V_L) domains (Integrative DNA Technologies) (Figure 1B). Cloning was performed using In-Fusion HD Cloning Kit (Takara Bio) as per the manufacturer's

protocol. Correct insertion was confirmed by sequencing. Infectious clones of NEGV containing exogenous genes were rescued in C7/10 cells as previously described and without further passage (47). Passaging of NEGV isolates expressing GFP was performed by inoculating C7/10 cells with a MOI of 1 and collecting media supernatant at 48 hours post infection.

Virus growth curves

Negevirus and alphavirus growth curves were done in C7/10 cells maintained at 30°C and 5% CO_2. Negeviruses were inoculated at a multiplicity of infection (MOI) of 1 or 5. Alphaviruses were inoculated at a MOI of 0.1. Virus was added to the cells which were incubated at 30°C for one hour. Inoculum was removed, cells were washed with PBS, and fresh media was added to the wells. Cells were incubated in a 30°C incubator with 5% CO_2. Samples were collected in triplicate at 2-, 6-, 12-, 24- and 48-hours post infection (hpi). Samples were clarified by centrifugation at 1962 *x g* for 5 minutes. Supernatant was removed and stored at -80°C until used for plaque assays. Negevirus titers were determined by plaque assay in C7/10 cells as previously described (47). Alphavirus titers were determined by standard plaque assay in Vero cells.

Negevirus-alphavirus co-infections

C7/10 cells were inoculated with negevirus isolates at a MOI of 1 or 5 to establish infection in a high proportion of cells. The cells were also inoculated with an alphavirus at a MOI of 0.1 at 0, 2, or 6 hours post negevirus infection. Media was

removed after 1 hour of simultaneous incubation with negevirus and alphavirus inocula. Cells were then washed with PBS, and fresh medium was added to the wells. Cells were held in a 30°C incubator with 5% CO_2. Samples were collected in triplicate at 12-, 24- and 48-hours, or 24- and 48-hours post alphavirus infection. Samples were clarified by centrifugation at 1962 *x g* for 5 minutes. Supernatant was removed and stored at -80°C until used for plaque assays. Alphavirus titers were determined by standard plaque assay in Vero cells.

Statistical analysis

Differences in virus growth curves were determined by two-way ANOVA followed by Tukey's test. Comparison of NEGV growth curves with different MOIs was determined by multiple t tests followed by Holm-Sidak method. All statistical tests were performed using GraphPad Prism 6.0.

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Author Contributions

EIP designed the experiments. EIP, TK, MAC-G and HG completed the experiments. EIP undertook analysis. EIP, TK, RBT, GLH and NLF wrote and edited the manuscript and all authors agreed to the final version. RBT, GLH and NLF provided supervision. EIP and NLF acquired the funding.

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Figure legends

Figure 1. Growth curve for wild-type negeviruses. A) The titer of each virus, Negev virus (NEGV), Piura virus-Culex (PIUV-Culex) and Piura virus-Lutzomyia (PIUV-Lutzomyia) at different time points following infection at MOI of 1 in C7/10 cells. B) Growth curve of NEGV with MOI of 1 and 5 in C7/10 cells. All points represent mean of n=3, ± SD. Letters indicate significant differences ($p < 0.0001$).

Figure 2. Growth curves of VEEV-TC83 in C7/10 cells during co-infections with wild-type negeviruses. A) Growth curves of VEEV-TC83 when inoculated on cells at 0-, 2- and 6 hours post NEGV infections. NEGV was inoculated at MOI 1 or 5. B) Growth

curves of VEEV-TC83 when inoculated on cells at 0 hours post negevirus infection, C) 2 hours post negevirus infection, and D) 6 hours post negevirus infection. Negeviruses were inoculated at MOI of 1 (B-D). VEEV-TC83 was inoculated at a MOI of 0.1 in all conditions. All points represent mean of n=3, ± SD. Letters indicate significant differences ($p < 0.0001$).

Figure 3. Growth curves of CHIKV in C7/10 cells during co-infections with wild-type negeviruses. A) Growth curves of CHIKV when inoculated on cells at 0-, 2- and 6 hours post NEGV infections. NEGV was inoculated at MOI 1 or 5. B) Growth curves of CHIKV when inoculated on cells at 0 hours post negevirus infection, C) 2 hours post negevirus infection, and D) 6 hours post negevirus infection. Negeviruses were inoculated at MOI of 1 (B-D). CHIKV was inoculated at a MOI of 0.1 in all conditions. All points represent mean of n=3, ± SD. Letters indicate significant differences ($p < 0.0001$).

Figure 4. Growth curves of VEEV-IC, MAYV isolates, ONNV, and SFV in C7/10 cells during co-infections with wild-type negeviruses. A) Growth curves of VEEV-IC when inoculated on cells at 0 hours post negevirus infections. B) Growth curves of MAYV-Guyane, MAYV-BeAn343102, and MAYV-BeAr505411 when inoculated on cells at 0 hours post negevirus infection. C) Growth curves of ONNV when inoculated on cells at 0 hours post negevirus infection. D) Growth curves of SFV when inoculated on cells at 0 hours post negevirus infection. Negeviruses were inoculated at MOI of 1 in all conditions. Alphaviruses were inoculated at a MOI of 0.1 in all conditions. All

580 points represent mean of n=3, ± SD. Letters indicate significant differences ($p <$
581 0.0001).
582
583 Figure 5. Rescued paratransgenic NEGV infectious clones. A) Schematic of NEGV
584 genomes for wild-type, and GFP-expressing viruses. NEGV GFP-fusion added the
585 GFP sequence onto ORF3 and NEGV GFP separated the ORF3 and GFP with a 2A
586 sequence to produce the proteins separately. GFP was replaced by scFv-CHK265 for
587 NEGV scFv-CHK265-fusion and NEGV scFv-CHK265. B) Growth curves of NEGV
588 wild-type and NEGV mutants expressing GFP or scFv-CHK265. All points represent
589 mean of n=3, ± SD. Letters indicate significant differences ($p < 0.0001$). C)
590 Fluorescent microscopy of C7/10 cells infected with NEGV GFP-fusion. Cells
591 demonstrate brilliant, punctate fluorescence. D) Fluorescent microscopy of C7/10
592 cells infected with NEGV GFP. Cells demonstrate dull, diffuse fluorescence. E) The
593 ratio of plaques to fluorescent plaques over 5 passages in C7/10 cells with NEGV
594 GFP-fusion and NEGV GFP. All points represent mean of n=3, ± SD. F) Titer of NEGV
595 GFP-fusion and NEGV GFP during 5 passages in C7/10 cells. All points represent
596 mean of n=3, ± SD. All NEGV isolates were inoculated at MOI of 1.
597
598 Figure 6. Growth curves of VEEV-TC83 during co-infections with paratransgenic
599 NEGV. Growth curves of VEEV-TC83 when inoculated on cells at A) 0 hours post
600 NEGV infection, B) 2 hours post NEGV infection, and C) 6 hours post NEGV infection.
601 All NEGV isolates were inoculated at MOI of 1. VEEV-TC83 was inoculated at a MOI

of 0.1 in all conditions. All points represent mean of n=3, ± SD. Letters indicate significant differences ($p < 0.0001$).

Figure 7. Growth curves of CHIKV during co-infections with paratransgenic NEGV. Growth curves of CHIKV when inoculated on cells at A) 0 hours post NEGV infection, B) 2 hours post NEGV infection, and C) 6 hours post NEGV infection. All NEGV isolates were inoculated at MOI of 1. CHIKV was inoculated at a MOI of 0.1 in all conditions. All points represent mean of n=3, ± SD. Letters indicate significant differences ($p < 0.0001$).

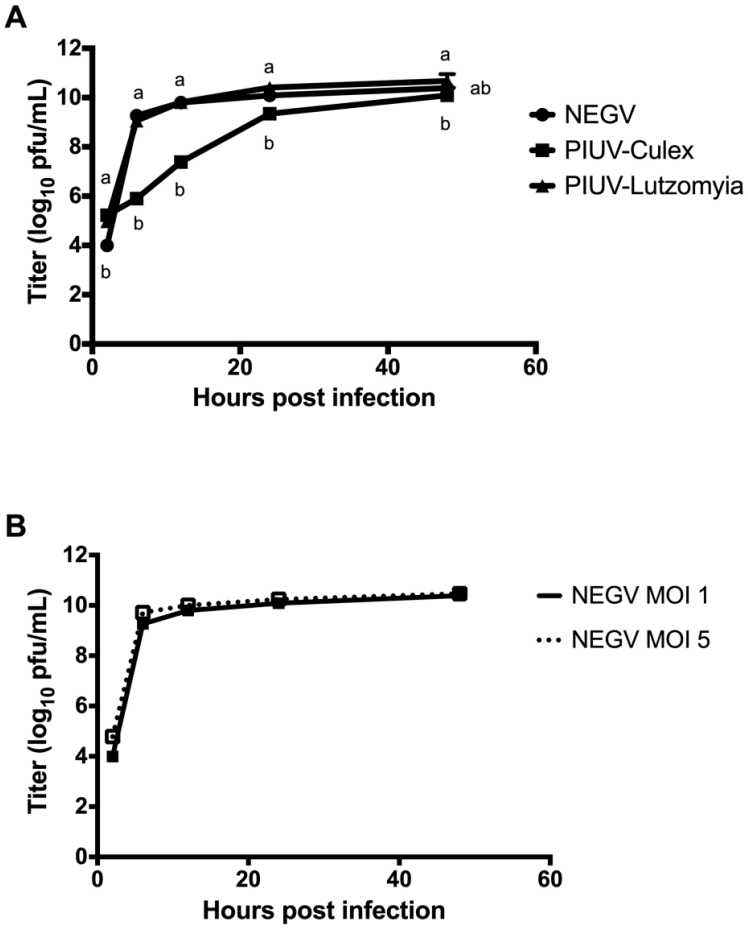
611 **Tables**

612 Table 1. Rescue and passage of NEGV infectious clones with GFP inserted at different
613 sites of the genome. Transcribed RNA was electroporated for P0 stock and
614 supernatant was collected to generate P1 stock.

Insertion site	GFP expression P0	GFP expression P1	CPE
ORF1-2A-GFP	+	+	+
GFP-ORF2	+	-	-
ORF2-GFP	+	-	-
ORF2-2A-GFP	+	-	-
GFP-ORF3	+	-	-
ORF3-GFP	+	+	+
ORF3-2A-GFP	+	+	+

615

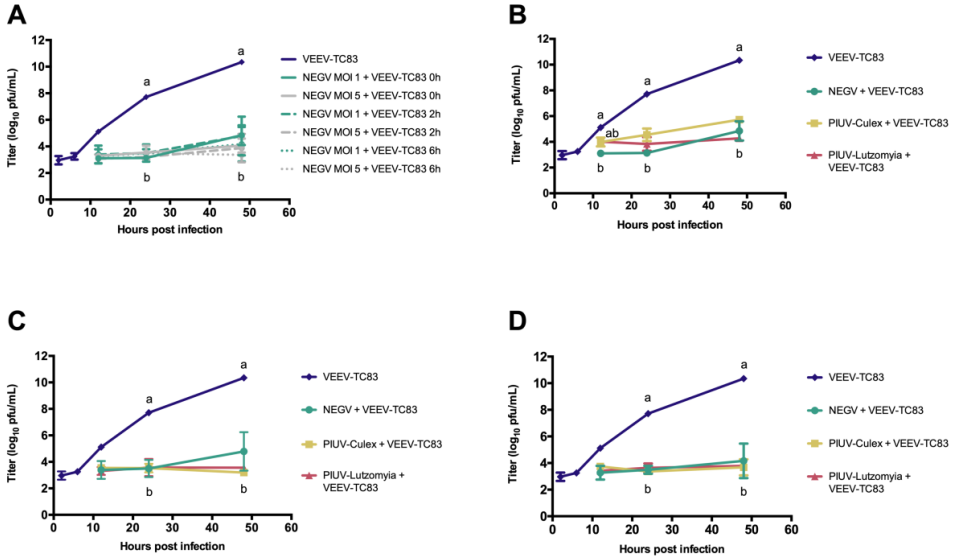
616 **Figures**



617

618 Figure 1.

619



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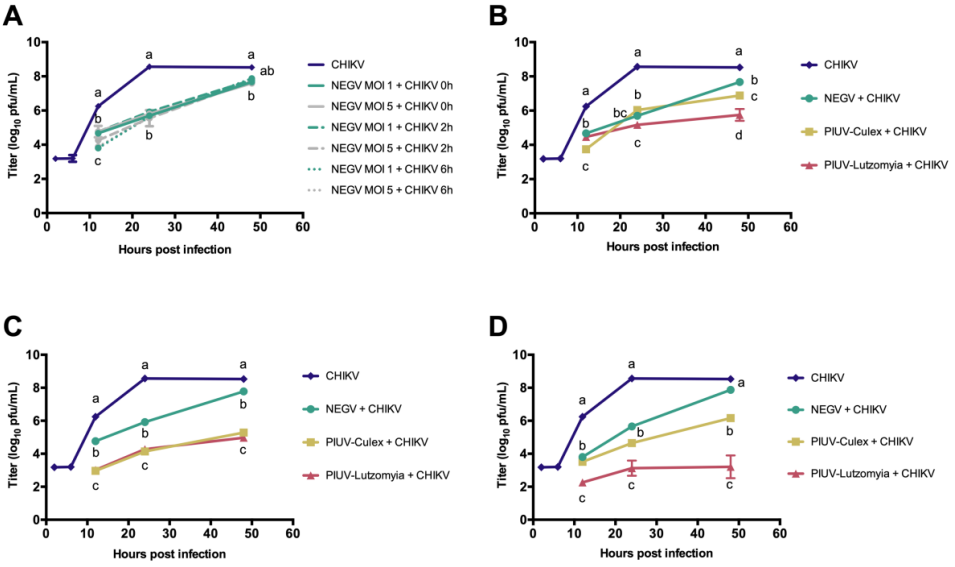
621 Figure 2.

622



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624 Figure 3.

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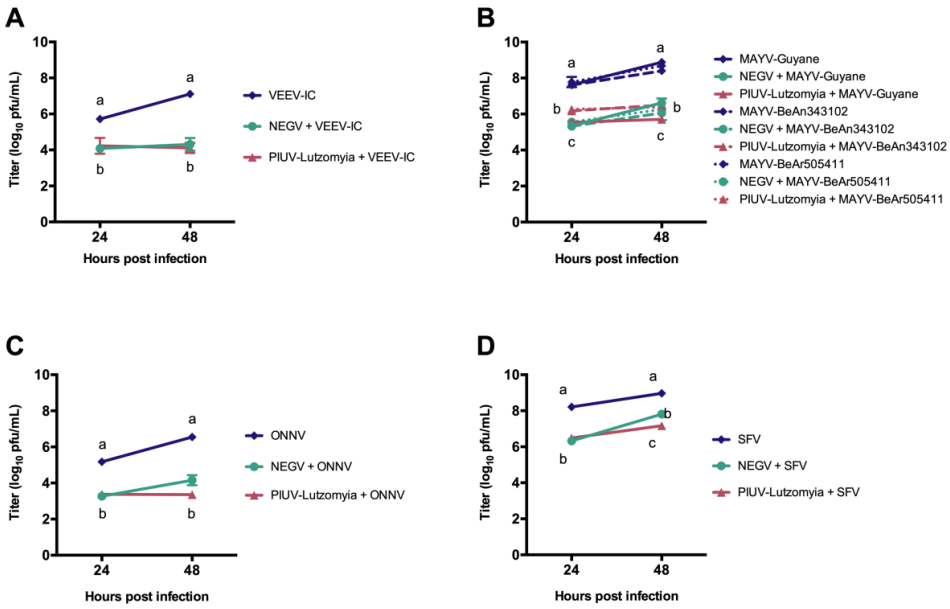

626

627 Figure 4.

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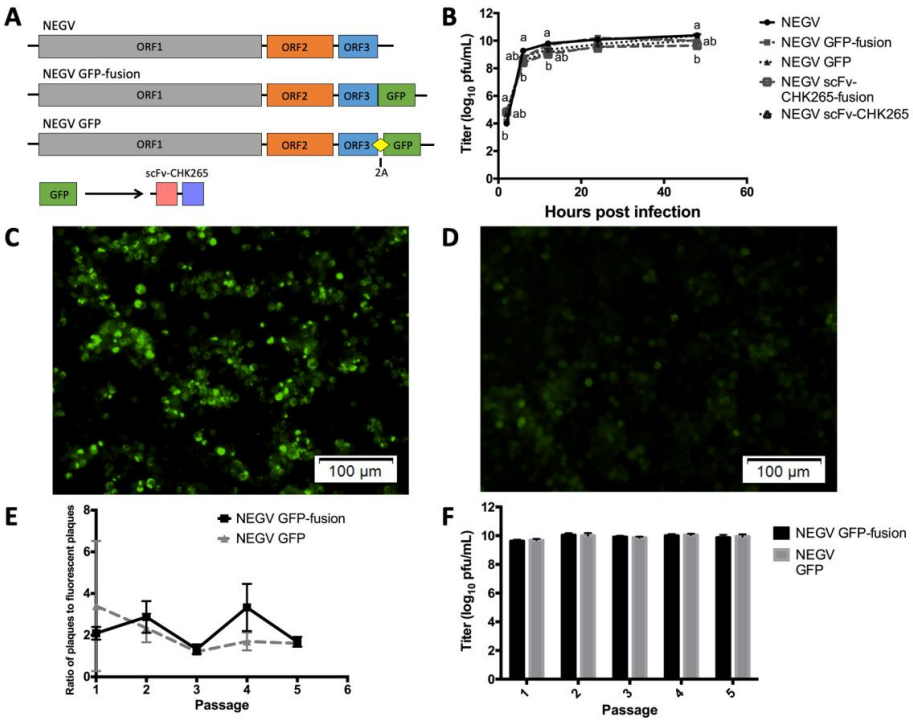

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630 Figure 5.

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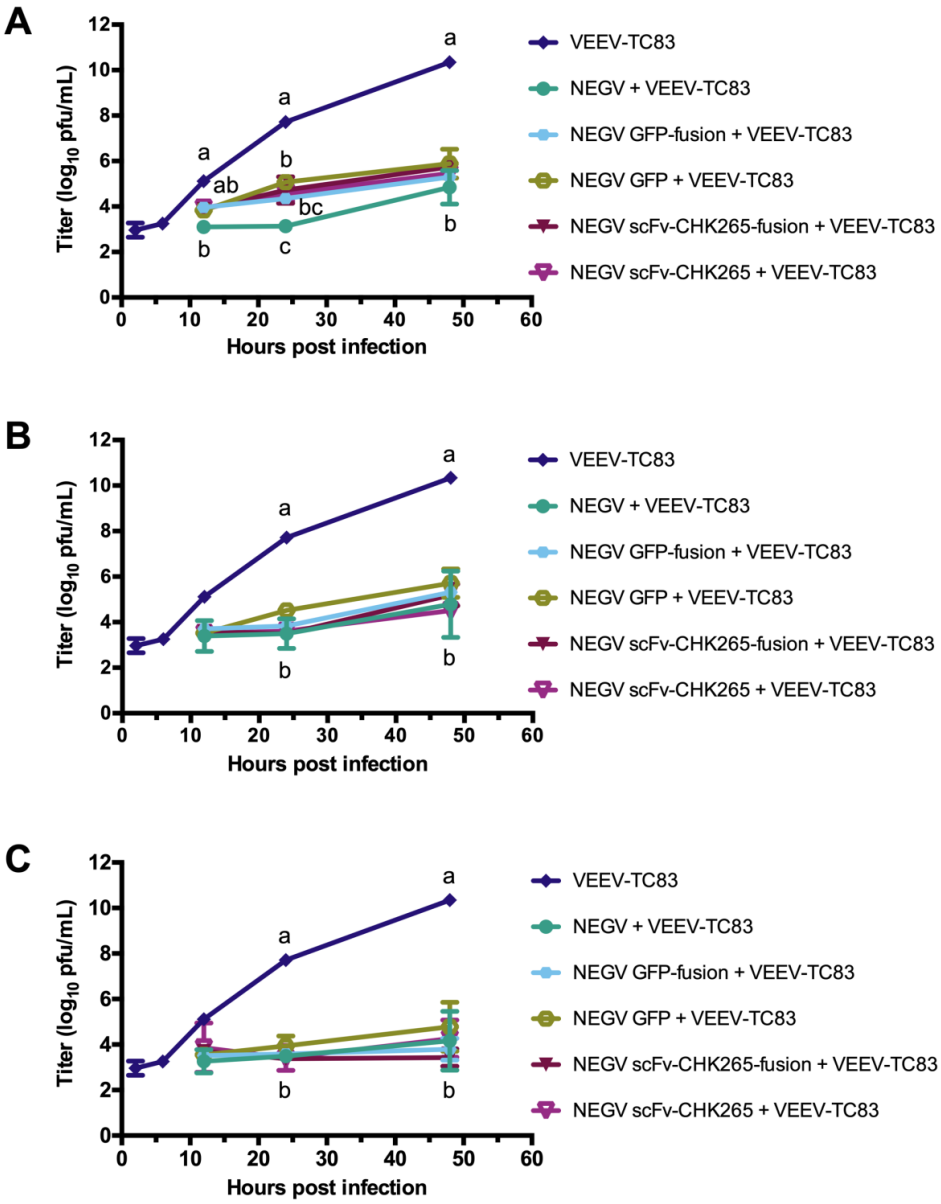

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633 Figure 6.

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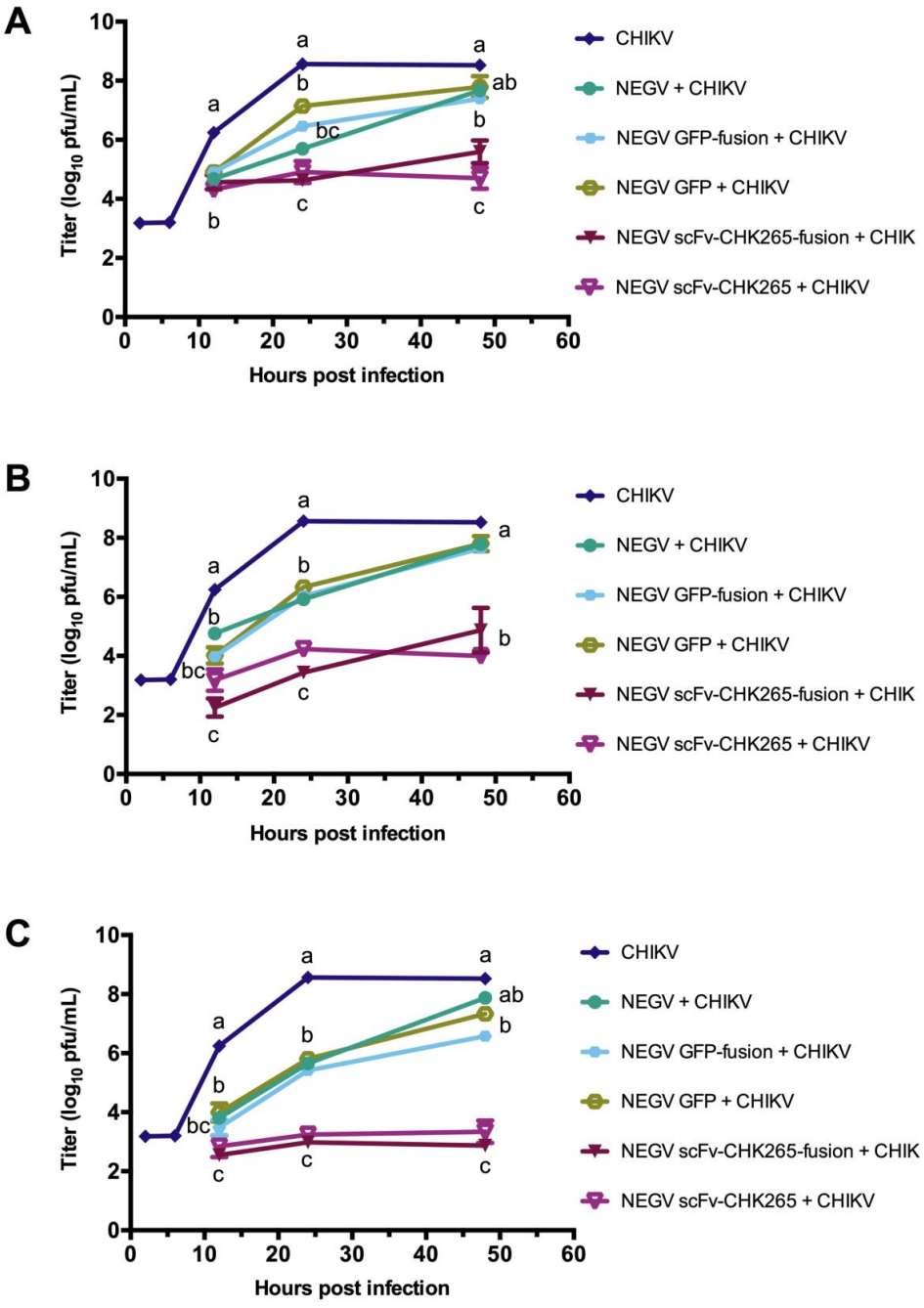

635

636 Figure 7.